Histone Deacetylase Inhibition Increases Levels of Choline Kinase α and Phosphocholine Facilitating Noninvasive Imaging in Human Cancers

Mounia Beloueche-Babari1, Vaitha Arunan1, Helen Troy1, Robert H. te Poele3, Anne-Christine Wong Te Fong1, L. Elizabeth Jackson1, Geoffrey S. Payne1, John R. Griffiths2, Ian R. Judson3, Paul Workman3, Martin O. Leach1, and Yuen-Li Chung1

Abstract
Histone deacetylase (HDAC) inhibitors are currently approved for cutaneous T-cell lymphoma and are in midstage trials for other cancers. The HDAC inhibitors LAQ824 and SAHA increase phosphocholine (PC) levels in human colon cancer cells and tumor xenografts as observed by magnetic resonance spectroscopy (MRS). In this study, we show that belinostat, an HDAC inhibitor with an alternative chemical scaffold, also caused a rise in cellular PC content that was detectable by 1H and 31P MRS in prostate and colon carcinoma cells. In addition, 1H MRS showed an increase in branched chain amino acid and alanine concentrations. 13C-choline labeling indicated that the rise in PC resulted from increased de novo synthesis and correlated with an induction of choline kinase α expression. Furthermore, metabolic labeling experiments with 13C-glucose showed that differential glucose routing favored alanine formation at the expense of lactate production. Additional analysis revealed increases in the choline/water and phosphomonoester (including PC)/total phosphate ratios in vivo. Together, our findings provide mechanistic insights into the impact of HDAC inhibition on cancer cell metabolism and highlight PC as a candidate noninvasive imaging biomarker for monitoring the action of HDAC inhibitors. Cancer Res; 72(4); 990–1000. ©2011 AACR.

Introduction
Histone acetylation is a key regulator of eukaryotic gene expression which controls DNA accessibility to transcription factors and mRNA transcription. The histone acetylation/deacetylation balance is maintained by the opposing activities of histone acetyl transferases and histone deacetylases (HDACs) resulting in cell-specific gene expression patterns (1). Deregulation of histone acetylation results in abnormal gene expression profiles involved in controlling cell proliferation, differentiation and apoptosis, and is associated with malignancy (2–5).

HDACs also act on other nonhistone proteins that are subject to regulation by acetylation including some transcription factors (e.g., E2F) and the heat shock protein 90 (HSP90) molecular chaperone, which maintains the conformational stability of several oncogenic proteins (e.g., ErbB2; ref. 6). HDAC inhibition is a promising antitumor approach for simultaneously targeting multiple oncogenic players and pathways. Several HDAC inhibitors have been described that induce potent antitumor effects in cells and tumor xenografts (6–8). The HDAC inhibitors SAHA (vorinostat) and depsipeptide FK228 (romidepsin) have gained U.S. Food and Drug Administration approval for cutaneous T-cell lymphoma treatment and many more are currently under clinical evaluation (6, 8–11). One example is belinostat which has shown promising activity in preclinical cancer models and in patients (8, 12).

The development and evaluation of novel HDAC inhibitors require the identification and validation of pharmacodynamic (PD) biomarkers of drug activity. These are important because they inform on the inhibition of the intended biochemical target, help assess response dynamics, aid treatment schedule and dose planning, and subsequently allow therapeutic efficacy assessment (13–15). In contemporary drug development, noninvasive endpoints of target modulation are highly desirable as they do not involve surgical intervention and allow longitudinal studies in the same patient to be carried out (15–17).

Noninvasive imaging of cancer metabolism is a valuable approach for PD biomarker discovery that exploits the altered metabolic features of tumors relative to normal tissues,
including increased lipid synthesis and aerobic glycolysis (18, 19). These metabolic changes are increasingly being investigated as diagnostic as well as treatment response biomarkers, with techniques such as magnetic resonance spectroscopy (MRS) being of particular value for translating findings from preclinical models to humans (16, 17, 20–22).

MRS allows the detection of many metabolites (e.g., those related to glucose, protein, and lipid metabolism) and in preclinical studies has shown that response to molecularly targeted therapeutics is often associated with altered metabolism (17, 20). For example, inhibitors of HSP90 (23, 24), phospholipase Cγ1 (25), mitogen-activated protein kinase (26), or phosphoinositide 3-kinase (27, 28) have all been shown to alter choline phospholipid metabolism in human cancer cells. In the case of the HDAC inhibitor LAQ824, both in vitro and in vivo MRS showed increased phosphorycholine (PC) levels in both human colon cancer cells and tumors posttreatment (29). A similar effect was also observed in human colon and prostate cancer cells treated with the HDAC inhibitor SAHA (29) or its fluoron analogue (30), respectively. Furthermore, LAQ824 treatment caused a substantial reduction in tumor bioenergy-related metabolites [e.g., nucleotide triphosphate (NTP) and glucose] that was observed in vivo but not in vitro (29). This effect was attributed to the antiangiogenic action of LAQ824, whereas the rise in PC was likely to relate to the effect of HDAC inhibition on tumor cell metabolism (29) although the molecular and biochemical mechanisms behind this change remain unclear.

Here, we assess (i) whether similar metabolic effects would be observed with the alternative chemotype (chemical scaffold) HDAC inhibitor and probe compound belinostat and (ii) the molecular and biochemical processes underlying the observed metabolic alterations.

We show that HDAC inhibition with belinostat in human cancer cells leads to increased alanine and branched-chain amino acid (BCAA) content that was associated with altered glucose utilization. Belinostat also increased PC levels, thus confirming our previous finding with the alternative chemotype agent LAQ824. Importantly, we show for the first time that this effect is associated with induction of choline kinase α (ChoKα) gene and protein expression. The increase in PC is also observed in belinostat-treated tumors in vivo, thus supporting the role of PC as a potential noninvasive metabolic imaging biomarker of HDAC inhibition.

Materials and Methods

Cell culture

Human HT29 colon and PC3 prostate carcinoma cells (American Type Culture Collection; ATCC) were grown in Dulbecco’s Modified Eagle’s Medium or RPMI, respectively, containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a 37°C humidified 5% CO2 atmosphere. Cells were preserved and propagated according to ATCC’s protocols, screened monthly for mycoplasma and passed for no longer than 3 months. All cell culture materials were from Life Technologies.

Western blot

Analysis of the molecular effects of HDAC inhibition was conducted by Western blotting as previously described (29). The primary antibodies rabbit antiacetyl histone-3 (Millipore), rabbit anti-ChoKα (Sigma-Aldrich), mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Chemicon), and rabbit α-tubulin (Cell Signaling Technology) were used. The secondary anti-rabbit and anti-mouse antibodies were from GE Healthcare Life Sciences (Buckinghamshire).

Growth inhibition and cell-cycle analysis

Cell counts were carried out on a Beckman Coulter Vi-Cell Cell Viability Analyzer. The impact of belinostat on cell proliferation and cell-cycle distributions was assessed with the sulforhodamine B (SRB) assay and flow cytometry, respectively, as previously described (27).

Cell treatment for in vitro MRS

PC3 and HT29 cells were treated for 24 hours with belinostat (0.9 and 2 μmol/L, respectively), to obtain a 30% to 50% reduction in cell counts and induction of histone-3 acetylation as a characteristic molecular biomarker of HDAC inhibition (9). HT29 cells were further treated with 2 μmol/L belinostat for 4 and 16 hours to assess response time dynamics. Control cells were treated with 0.01% dimethyl sulfoxide (DMSO).

For 13C-tracer experiments, HT29 cells were treated as above for 16 hours followed by a further 3-hour incubation in fresh medium containing DMSO or 2 μmol/L belinostat and 28 μmol/L [1,2-13C]-choline (Cambridge Isotope Laboratories; final concentration 56 μmol/L) or 5 mmol/L [1–13C]-glucose (Sigma-Aldrich; final concentration 25 mmol/L).

At the end of treatment, cells were extracted with a dual-phase method (26) and samples lyophilized for MRS analysis.

Quantitative real-time PCR

Total RNA was extracted with the RNAeasy Kit (QIAGEN), and 500 ng was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Samples were diluted 1:5, and 1 μL used in the Taqman assay, using Taqman universal master mix, and the Hs03682798_m1 assay for the ChoKα gene CHKA (CHKA specific primers and FAM-labeled probe) multiplexed with the 4326314E assay for large ribosomal protein 0 gene LR0 (LRP0-specific primers and VIC-labeled probe; Applied Biosystems). mRNA levels of CHKA and LRP0 were determined for each sample in the same well on the ABI 7900HT. CHKA mRNA levels were expressed relative to those of LRP0.

HT29 human colon tumor xenograft model

MF-1 male nude mice were injected subcutaneously in the flank with 5 × 106 HT29 human colon carcinoma cells. Tumor volume was calculated by measuring the length, width, and depth using calipers and the formula L × W × D × (π/6). Once an appropriate tumor volume (~350 mm3) was established (3–4 weeks later), mice were randomized into 2 groups; one group was treated with belinostat in vehicle (10% DMSO in water) at 60 mg/kg p.o. once a day for 3 days (days 1, 2, and 3; n = 13), and
one group was treated with vehicle alone (n = 12). A cohort of animals (6 vehicle- and 6 belinostat treated) was used in the MRS study, and tumors were excised on day 3 for Western blotting or in vitro MRS. Another animal cohort (6 vehicle- and 7 belinostat treated) was used to study tumor growth delay where animals were treated as above and tumor volumes monitored for a further 6 days after the last dose. Animals were treated in accordance with local and national ethical requirements and with the UK National Cancer Research Institute (NCRI) Guidelines for the Welfare and Use of Animals in Cancer Research (31).

**In vivo MRS of HT29 tumors**

Mice were anesthetized as previously described (29) and scanned in a 7T Bruker Magnetic Resonance System spectrometer with tumors positioned in the center of a 15-mm 2-turn $^1$H/$^31$P surface coil. In vivo localized PRESS $^1$H MRS (echo time (TE) = 136 ms and repetition time (TR) = 4 s) and image-selected spectroscopy $^{31}$P MRS (TR = 2 s) of the tumors were carried out before treatment (day 0) and the last day of treatment (day 3). $^1$H and $^{31}$P MR spectra were quantified with jMRUI as previously described (24). After the final scan, tumors were excised and stored at −80°C for subsequent in vitro MRS or Western blotting.

The surface coils used to obtain the $^{31}$P MRS signal from subcutaneous tumors in vivo have a nonuniform spatial sensitivity making it difficult to normalize to an external standard. Therefore, the signal intensities observed by in vivo $^{31}$P MRS are expressed as metabolite ratios.

**In vitro MRS of tumor extracts**

Freeze-clamped HT29 tumors (~200 mg) were extracted in 6% ice-cold perchloric acid (PCA) as previously described (29). Neutralized extracts were freeze dried and reconstituted in 1 mL of D$_2$O and 0.5 mL was then analyzed. Sodium 3-trimetilsilyl-2,2,3,3-tetradeteropropionate (TSP; 50 μL, 5 mmol/L) was added as an internal chemical shift and quantification reference. The pH of the samples was reneutralized with PCA or KOH followed by acquisition of water-suppressed $^1$H MRS spectra. For $^{31}$P MRS, EDTA (50 μL, 60 mmol/L) was added to chelate metals ions, and methylene-diphosphonic acid (internal reference; 50 μL, 5 mmol/L) was added.

**In vitro MRS of cell extracts and media samples**

Lyophilized samples of the aqueous fraction of cell extracts were processed as above. The lipid phase of cell extracts was reconstituted in CDCl$_3$ containing 0.56 mmol/L trimethyl silane (internal standard). Media samples from the [1–13C]glucose experiments were prepared by adding 50 μL D$_2$O and 50 μL TSP (5 mmol/L, internal reference) to 0.45 mL of medium.

$^1$H and $^{31}$P spectra were acquired as previously described (23). $^{13}$C MR spectra were acquired using power gated composite pulse $^1$H decoupling, a 30 degrees flip angle, a 2 seconds repetition delay, a spectral width of 220 ppm and 32 K data points. Spectral processing and metabolite quantitation were carried out as previously described (23).

**Statistical analysis**

Statistical significance was assessed with Student t tests with $P \leq 0.05$ considered to be significant. Pearson correlation analysis was conducted with GraphPad Prism (version 5.01). Data represent the mean ± SE.

**Results**

**Belinostat treatment alters cellular metabolism in human HT29 and PC3 carcinoma cells**

Belinostat inhibited proliferation in HT29 colon and PC3 prostate cancer cells as shown by the sulforhodamine B (SRB) assay (Supplementary Table S1). The respective exposure of HT29 and PC3 cells to 2 μmol/L (5 × GI$_{50}$) and 0.9 μmol/L (3 × GI$_{50}$) belinostat for 24 hours led to a substantial reduction in cell counts to 57% ± 3% and 77% ± 2% of controls, respectively ($P \leq 0.001$). Western blotting showed the induction of histone-3 acetylation posttreatment with belinostat consistent with HDAC inhibition in both cell lines (Fig. 1A). Significant alterations in cell-cycle profiles were observed after belinostat treatment characterized by a build up in the G$_1$ and G$_2$–M cell populations concomitant with a reduction in the S phase fraction in HT29 cells (Supplementary Fig. S1).

To evaluate the metabolic effects of HDAC inhibition, we analyzed the $^1$H and $^{31}$P MR spectra from the aqueous fractions of HT29 and PC3 cell extracts posttreatment with belinostat.

Relative to controls, $^1$H MRS revealed time-dependent increases in PC levels in HT29 cell extracts which were also present in PC3 cell extracts following a 24-hour exposure to belinostat (Fig. 1B and C). Decreased glycerophosphocholine (GPC) levels were also recorded in HT29 cells at 16 and 24 hours, and PC3 cells at 24 hours; nevertheless, the total choline (tCho) signal comprising PC + GPC remained higher, relative to controls, following belinostat treatment in both cell lines (Fig. 1C).

In addition, we also observed time-dependent increases in levels of the BCAAs (comprising valine, leucine, and isoleucine), alanine, and threonine in belinostat-treated HT29 cells compared with controls (Fig. 1B and C). Similar effects on BCAA and alanine were also observed in PC3 cells at 24 hours following belinostat treatment (Fig. 1C).

$^{31}$P MRS analysis confirmed the $^1$H MRS observed changes showing that, relative to controls, PC levels were not significantly altered at 4 hours (19 ± 3 vs. 19 ± 2 fmol per cell; n = 3) but increased significantly at 16 hours (17 ± 2 vs. 25 ± 2 fmol per cell; n = 5, $P = 0.02$) and 24 hours (18 ± 1 vs. 27 ± 2 fmol per cell; n = 6, $P = 0.0003$). GPC levels decreased significantly at 16 hours (7 ± 1 vs. 4 ± 1 fmol per cell; $P = 0.02$) and 24 hours (8 ± 1 vs. 6 ± 1 fmol per cell; $P = 0.03$) posttreatment. Data from the 24-hour time point are further summarized in Table 1, which also shows a similar increase in PC levels in PC3 cells treated with belinostat.

$^1$H MRS analysis of the lipid fraction of HT29 cell extracts indicated that phosphatidylcholine (PtdCho) levels, determined by integrating the N-trimethyl resonance at 3.2 ppm, increased from 204 ± 18 arbitrary units per cell to 290 ± 28 arbitrary units per cell following belinostat treatment (n = 4, $P = 0.04$).
HDAC inhibition with belinostat alters glucose utilization

To investigate the basis for the rise in amino acid (AA) levels, HT29 cells were cultured in the presence of [1–13C]-glucose for 3 hours to monitor tracer uptake and incorporation into glycolytic intermediates. 13C MRS of cell extracts indicated that, following belinostat treatment, no significant effects were observed on intracellular

Table 1. The effect of the HDAC inhibitor belinostat on the 31P-containing aqueous metabolites in human HT29 colorectal and PC3 prostate carcinoma cells following a 24-hour exposure to 2 and 0.9 μmol/L, respectively

<table>
<thead>
<tr>
<th>Metabolites (fmol per cell)</th>
<th>HT29 (n = 6)</th>
<th></th>
<th></th>
<th></th>
<th>PC3 (n = 4)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Belinostat</td>
<td>P*</td>
<td>Control</td>
<td>Belinostat</td>
<td>P*</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>18 ± 1</td>
<td>27 ± 1</td>
<td>0.0003</td>
<td>17 ± 1</td>
<td>24 ± 1</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
<td>7 ± 1</td>
<td>8 ± 1</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>GPC</td>
<td>8 ± 1</td>
<td>6 ± 1</td>
<td>0.03</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>GPE</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
<td>0.37</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>NTP</td>
<td>8 ± 1</td>
<td>8 ± 1</td>
<td>0.86</td>
<td>10 ± 1</td>
<td>11 ± 2</td>
<td>0.69</td>
<td></td>
</tr>
</tbody>
</table>

*2-Tailed unpaired t test comparing changes between control and belinostat-treated cells. Abbreviations: ND, not detectable; PE, phosphoethanolamine; GPE, glycerophosphoethanolamine.
The effect of belinostat treatment (16 + 3 hours) on \(^{13}\)C-glucose utilization in HT29 human colon cancer cells. A, \(^{13}\)C spectra of HT29 cells incubated in \([1-^{13}\text{C}]\)-glucose for 3 hours showing the effect of belinostat treatment (2 \(\mu\text{mol/L}\) on \([4-^{13}\text{C}]\)-glutamate (Glut), \([3-^{13}\text{C}]\)-lactate (Lac), and \([3-^{13}\text{C}]\)-alanine (Ala)). B, quantitation of \(^{13}\)C-labelled glucose, lactate (intra- and extracellular), alanine, and glutamate in control and belinostat-treated HT29 cells following a 3-hour incubation in \([1-^{13}\text{C}]\)-glucose showing increased alanine formation and decreased lactate production.

\(^{13}\)C-glucose levels; however, extracellular \([3-^{13}\text{C}]\)-lactate (measured by \(^{13}\)C MRS of cell culture media) decreased markedly to 25% ± 11% relative to controls \((n = 4, P = 0.007)\). Interestingly, the reduction in \(^{13}\)C-lactate production was paralleled with a mate/\([3-^{13}\text{C}]\)incorporation into \([3-^{13}\text{C}]\)PC post-vehicle or belinostat treatment.

To characterize the molecular drivers of this effect, we assessed the expression of ChoK, the enzyme that catalyses the formation of PC from its precursor choline. Quantitative real-time (qRT)-PCR analysis indicated that following belinostat treatment the levels of ChoK mRNA were induced by 2.3-fold at 4 hours, 3.5-fold at 16 hours, and 3.2-fold at 24 hours relative to controls (Fig. 3A), and this effect correlated strongly with the increase in PC levels observed by MRS \((r = 0.99, P = 0.02)\). ChoK mRNA was also induced to 148% ± 8% relative to controls \((n = 9, P = 0.002)\) in PC3 cells following a 24-hour treatment with belinostat. Western blotting of the HT29 cell samples from the \(^{13}\)C-choline labeling experiment showed induction of ChoK protein expression up to 260% ± 45% \((n = 3, P = 0.018)\) of controls following exposure to belinostat (Fig. 3D).

**HDAC inhibition with belinostat increases levels of choline-containing metabolites of HT29 xenografts in vivo**

To assess if any of the metabolic alterations observed in cells would be translatable to in vivo tumor models, we evaluated the metabolite profiles of HT29 tumor xenografts following treatment with belinostat.

Following 3 days of treatment, tumor volumes increased by 20% ± 3% in the vehicle-treated group and 3% ± 2% in the belinostat-treated group \((P < 0.0001\); Fig. 4A). After therapy cessation, tumor growth inhibition in the belinostat-treated group became statistically insignificant relative to controls by day 7 \((P = 0.9)\). Western blots of the excised tumors following 3 days of treatment showed increased histone-3 acetylation in the belinostat-treated group (Fig. 4B), thus confirming the expected inhibitory effect of belinostat on HDAC in HT29 xenografts.

In vivo \(^{31}\)P MR spectra from a HT29 tumor pre- and post-belostatin treatment are shown in Fig. 4C (left), in which resonances from tCho and lipids can be observed. A significant increase \((169\% ± 25\%\) of post-/pretreatment values, \(P = 0.02)\) was observed in the belinostat-treated group \((P < 0.0001\); Fig. 4A). After therapy cessation, tumor growth inhibition in the belinostat-treated group became statistically insignificant relative to controls by day 7 \((P = 0.9)\). Western blots of the excised tumors following 3 days of treatment showed increased histone-3 acetylation in the belinostat-treated group (Fig. 4B), thus confirming the expected inhibitory effect of belinostat on HDAC in HT29 xenografts.

In vivo \(^{31}\)P MR spectra from a HT29 tumor pre- and post-belostatin treatment are shown in Fig. 4C (right), in which resonances from phosphomonoesters (PME), phosphodiester (PDE), inorganic phosphate (Pi), α-, β-, γ-NTP and phosphocreatine (PCr) can be observed. Significant increases in PME/TotP \((P = 0.01)\) and Pi/TotP ratios \((P = 0.03)\) were observed post-belinostat treatment (Table 2). No significant change in metabolite ratios was observed in the vehicle-treated group (Table 3).
in vivo high resolution MRS analyses of HT29 tumor extracts showed elevated PC \( (P = 0.04) \), GPC \( (P = 0.04) \), and free choline \( (P = 0.01) \) levels and reduced glucose \( (P = 0.01) \) and formate levels in belinostat-treated HT29 tumor extracts relative to vehicle-treated tumors (Table 4) as detected by \(^{1}H\) MRS. \(^{31}P\) MRS analysis showed increased PC \( (P = 0.03) \) and GPC \( (P = 0.05) \) levels in belinostat-treated tumors (Table 5) relative to controls, consistent with the in vivo \(^{1}H\) and \(^{31}P\) MRS changes.

**Discussion**

HDAC inhibitors are targeted anticancer agents currently approved for cutaneous T-cell lymphoma and in mid-late stage trials for other cancers (1, 10, 11). The development of such agents requires the discovery and validation of biomarkers, and particularly those that are noninvasive, to monitor target modulation and aid treatment planning and evaluation.

Imaging of tumor metabolism is a promising approach for biomarker discovery as it exploits the distinct metabolic characteristics of the tumor to inform on its behavior following therapy (17, 20–22). Moreover, studying tumor metabolism informs on the metabolic pathways modulated by targeted agents thereby providing a means for investigating potential mechanisms of action.

Using MRS, we have previously shown that HDAC inhibition with LAQ824 leads to increased PC levels in HT29 human colon carcinoma cells in vitro and tumors in vivo (29). The aims of this study were to (i) confirm this observation with belinostat in HT29 cells in vitro and in vivo in addition to PC3 human prostate cancer cells in vitro and (ii) investigate the mechanism(s) underlying this effect.

The clinical development of belinostat is now primarily directed toward the use of this drug in combination with chemotherapy. In this study, belinostat served as an alternative chemotype probe to the agents currently in the clinic. Probe compounds have considerable value for interrogating the molecular function of target proteins and the downstream biological processes they mediate, and for biomarker discovery and validation (32).

HT29 and PC3 cells were treated in vitro with belinostat at a concentration and duration that led to target modulation (shown by histone-3 hyperacetylation) and inhibition of cell proliferation (shown by SRB assays and cell counts). \(^{31}P\) MRS metabolic analysis under these conditions indicated that the most significant effect observed in both HT29 and PC3 cells after 24 hours of treatment was a rise in PC content, which was time-dependent in HT29 cells. When detectable (in HT29 cells), GPC levels decreased post-belinostat treatment. The changes in PC and GPC were confirmed by \(^{1}H\) MRS which indicated that the tCho signal was also significantly higher following belinostat treatment in both cell lines. The rise in PC is in line with our previous findings with LAQ824 and SAHA in HT29 cells (29) indicating that it is not inhibitor chemotype-specific or cell line/tissue type-dependent.

Furthermore, belinostat treatment resulted in increased BCAAs, alanine, and threonine levels in both HT29 and PC3 cells as shown by \(^{1}H\) MRS. These changes were also time-dependent in HT29 cells.
Next, we set out to investigate the metabolic processes underlying the increases in AA and PC levels following HDAC inhibition. An increase in AAs could reflect (i) elevated uptake, (ii) increased de novo formation from glycolytic intermediates or other AAs, (iii) increased proteolysis, (iv) decreased utilization (e.g., in protein synthesis), or a combination of the above.

Measurement of HT29 cell lysates by the BIO-RAD method revealed a 10% rise in protein content of belinostat-treated cells relative to controls (data not shown). Therefore, it is unlikely that the rise in AAs observed here could reflect increased proteolysis or decreased protein synthesis as this would be expected to reduce the overall cellular protein mass.

To assess whether the AA rise was due to increased synthesis from glycolytic intermediates, we analyzed the 13C MRS profiles of belinostat-treated cells incubated in [1-13C]-glucose for 3 hours. This short incubation time ensured that the 13C-labelled metabolites detected were formed directly from the 13C-glucose precursor rather than other prelabeled metabolic intermediates.

This experiment revealed increases in 13C-glucose–derived 13C-alanine (up to ~3-fold) which coincided with decreased 13C-lactate production by up to 75% of controls. Moreover, there was a trend toward an increase in cellular 13C-glutamate and 13C-glucose although neither of these effects was statistically significant.
Taken together, these observations suggest that belinostat treatment led to differential metabolic routing that favors the conversion of pyruvate to alanine at the expense of lactate synthesis.

A previous report showed inhibition of glucose transport concomitant with reduced hexokinase activity following HDAC inhibition (33). Here, we did not observe a reduction in $^{13}$C-glucose accumulating in the cells, and the combined level of downstream $^{13}$C-intermediates (lactate + alanine + glutamate) formed from $^{13}$C-glucose seemed to be comparable between control and treated cells (Fig. 2A and B). These observations suggest that glucose uptake was probably unaltered under our experimental conditions although more work is required to test this hypothesis.

Furthermore, and although neither of the changes in individual content of intracellular $^{13}$C-lactate or $^{13}$C-glutamate following belinostat treatment were statistically significant, the $^{[4,13]}$C-glutamate/$^{[3,13]}$C-lactate ratio increased by approximately 3-fold. This effect could suggest an altered balance between glycolysis and Krebs cycle metabolism as previously reported (34).

Next, we investigated the basis for the PC rise following belinostat treatment. PC is formed via 2 main routes: (i) de novo synthesis through ChoK-catalyzed phosphorylation of its precursor choline and (ii) release from membrane PtdCho via PtdCho-specific phospholipase C or release of choline from PtdCho via phospholipase D followed by ChoK-mediated phosphorylation (35).

To distinguish these 2 processes, we assessed levels of $^{13}$C-PC in cells incubated in $^{[1,2,13]}$-choline for 3 hours. This short duration ensured that the $^{13}$C-PC observed was formed de novo from exogenous $^{13}$C-choline, thus excluding contributions from membrane PtdCho-derived PC (because the $^{13}$C-choline would take much longer than 3 hours to be incorporated into PtdCho and then be released again (36)).

$^{13}$C MRS indicated that the levels of $^{13}$C-PC formed were approximately 1.5-fold higher in belinostat-treated cells relative to controls. The amplitude of this increase is similar to that observed by $^1$H and $^{31}$P MRS indicating that the elevation in steady-state PC is driven primarily by increased de novo synthesis.

To further delineate the molecular processes driving this effect, we assessed the expression of ChoK$^\alpha$. qRT-PCR analysis revealed an induction in ChoK$^\alpha$ gene expression in HT29 cells which correlated strongly with the rise in PC levels measured by MRS and which was also confirmed by ChoK$^\alpha$ protein expression in these cells. A similar increase in ChoK$^\alpha$ expression was also observed in PC3 cells, albeit to a lesser extent. These findings point to the induction of ChoK$^\alpha$ expression as a key driver of the rise in PC observed by MRS following belinostat treatment in both HT29 and PC3 cells.

PtdCho levels increased by approximately 1.4-fold in belinostat-treated HT29 cells relative to controls, as shown by $^1$H MRS, indicating that the de novo formed PC was used to supply PtdCho synthesis. This finding concurs with the previously reported role of HDAC inhibition in inducing the expression of CTP-PC cytidylyltransferase, the rate-limiting enzyme in PtdCho biosynthesis (37). Interestingly, levels of GPC, which is a PtdCho breakdown product, fell post-belinostat treatment in cells (but not tumors), suggesting that HDAC inhibition probably also inhibited PtdCho degradation, which together with activation of the synthetic pathway led to net augmentation in this membrane phospholipid. However, this effect did

<table>
<thead>
<tr>
<th>Metabolite ratios</th>
<th>Pre-belinostat</th>
<th>Post-belinostat</th>
<th>$P^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PME/TotP</td>
<td>0.13 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>$\beta$-NTP/TotP</td>
<td>0.22 ± 0.02</td>
<td>0.21 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>$\beta$-NTP/PI</td>
<td>5.28 ± 1.35</td>
<td>3.01 ± 0.93</td>
<td>0.02</td>
</tr>
<tr>
<td>P/TotP</td>
<td>0.05 ± 0.02</td>
<td>0.10 ± 0.01</td>
<td>0.03</td>
</tr>
</tbody>
</table>

NOTE: NS, $P > 0.1$.

$^a$2-Tailed paired t test comparing changes pre- and post-belinostat treatment within the same group of animals.

<table>
<thead>
<tr>
<th>Metabolite ratios</th>
<th>Pre-vehicle</th>
<th>Post-vehicle</th>
<th>$P^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PME/TotP</td>
<td>0.14 ± 0.02</td>
<td>0.12 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>$\beta$-NTP/TotP</td>
<td>0.21 ± 0.02</td>
<td>0.23 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>$\beta$-NTP/PI</td>
<td>4.10 ± 1.86</td>
<td>6.16 ± 1.43</td>
<td>NS</td>
</tr>
<tr>
<td>P/TotP</td>
<td>0.09 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>NS</td>
</tr>
</tbody>
</table>

NOTE: NS, $P > 0.1$.

$^a$2-Tailed paired t test comparing changes pre- and post-vehicle treatment within the same group of animals.
Indeed, ChoKdoxically, more commonly associated with malignancy (38). The induction in ChoK observed following HDAC inhibition and the ensuing increase in PC are unusual and, paradoxically, more commonly associated with malignancy (38). Indeed ChoK expression is linked to oncogene activation (39) and correlates with poor patient prognosis (40). Further studies are required to elucidate the significance of the choline metabolism effects observed here in relation to HDAC inhibitor–induced antitumor activity.

Finally, and to assess whether any of the changes observed in cancer cells could serve as potential noninvasive biomarkers of HDAC inhibition, we investigated the effect of belinostat in tumor xenografts derived from the same HT29 cells used in vitro. In vivo 1H MRS revealed increased tCho/water ratio following belinostat treatment. Although therapy-induced changes in water content can contribute to a rise in tCho/water, increased PME/TotP was also observed by in vivo 31P MRS following treatment with belinostat. Furthermore, ex vivo MRS revealed increased PC and GPC levels in belinostat-treated tumors relative to controls. This indicates that the in vivo MRS-detectable changes are primarily due to increased PC and GPC content in the drug-treated group.

The rise in PC concurs with our in vitro cell observations and with our previous findings with LAQ824 and SAHA (29), indicating that this effect is likely to be associated with the mechanism of action of HDAC inhibitors. We also found an increase in tumor GPC following belinostat treatment, but a decrease in GPC was observed in belinostat-treated cells and previously in LAQ824-treated tumor extracts (29).

Interestingly, although HDAC inhibition led to significant increases in cellular AA levels and previously in HT29 tumors in vivo post-LAQ824 treatment (29), no significant effects on AA content were recorded in the belinostat-treated tumors. The basis for this difference is unclear and may relate to drug-induced physiologic effects that may vary under the different treatment conditions.

In line with our previous findings with LAQ824 (29), glucose concentration fell in the belinostat-treated tumors compared with controls albeit to a lesser extent compared with LAQ824 (35% vs. 61%). This effect did not correlate with major changes in tumor bioenergy metabolites as seen with LAQ824 (29). The differences in bioenergetic effects between the 2 HDAC inhibitors may be due to the additional antivascular effects of LAQ824 (41).

In summary, we show that the most consistent and reproducible metabolic signature observed following HADC inhibition in cells and in vivo tumors is increased PC levels. Importantly, we show for the first time that this effect is driven by

### Table 4. In vitro MRS of HT29 tumors: 1H MRS

<table>
<thead>
<tr>
<th>Metabolites (µmol/g w.wt)</th>
<th>Control (n = 6)</th>
<th>Belinostat (n = 6)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>2.57 ± 0.25</td>
<td>3.33 ± 0.21</td>
<td>0.04</td>
</tr>
<tr>
<td>GPC</td>
<td>2.29 ± 0.17</td>
<td>3.02 ± 0.26</td>
<td>0.04</td>
</tr>
<tr>
<td>Free choline</td>
<td>0.04 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.79 ± 0.07</td>
<td>0.51 ± 0.07</td>
<td>0.01</td>
</tr>
<tr>
<td>Formate</td>
<td>0.24 ± 0.05</td>
<td>0.13 ± 0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>Lactate</td>
<td>8.35 ± 1.01</td>
<td>7.20 ± 1.00</td>
<td>NS</td>
</tr>
</tbody>
</table>

NOTE: NS, P > 0.1.

*2-Tailed unpaired t test comparing changes between vehicle- and belinostat-treated groups.

### Table 5. In vitro MRS of HT29 tumors: 31P MRS

<table>
<thead>
<tr>
<th>Metabolites (µmol/g w.wt)</th>
<th>Control (n = 5)</th>
<th>Belinostat (n = 5)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>1.48 ± 0.06</td>
<td>1.96 ± 0.27</td>
<td>0.09</td>
</tr>
<tr>
<td>PC</td>
<td>1.85 ± 0.12</td>
<td>2.59 ± 0.25</td>
<td>0.03</td>
</tr>
<tr>
<td>GPE</td>
<td>0.99 ± 0.10</td>
<td>1.17 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>GPC</td>
<td>1.78 ± 0.22</td>
<td>2.39 ± 0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>Pi</td>
<td>3.86 ± 0.29</td>
<td>4.06 ± 0.23</td>
<td>NS</td>
</tr>
</tbody>
</table>

NOTE: NS, P > 0.1.

*2-Tailed unpaired t test comparing changes between vehicle- and belinostat-treated groups.
increased de novo formation involving upregulation of ChoKα mRNA and protein expression.

Further studies are required to delineate the precise molecular links between HDAC and ChoKα expression and to unravel the significance of the choline metabolic effects in relation to drug-induced anticancer activity. Our findings also support the role of PC as a potentially useful noninvasive metabolic imaging biomarker for monitoring the action of HDAC-targeted therapeutics.

Disclosure of Potential Conflicts of Interest

M. Beloueche-Babari, V. Arunan, R.H. te Poel, A.-C.W. Te Fong, L.E. Jackson, L.R. Judson, P. Workman, M.O. Leach, and Y.-L. Chung are employees of The Institute of Cancer Research, which has a commercial interest in HDAC inhibitors and which operates a “rewards to inventors” scheme. P. Workman was a founder of Chrona Therapeutics and is Chairman of its Scientific Advisory Board. P. Workman also a Cancer Research UK Life Fellow.

References

24. Al-Saffar NM, Jackson LE, Raynaud FI, Clarke PA, Ramirez de MA, Lacaq JC, et al. The phosphoinositide 3-kinase inhibitor PI-103 down-regulates choline kinase alpha leading to phosphocholine and total

Acknowledgments

The authors thank Dr S.P. Robinson for help with setting up the in vivo MRS facility. Belinostat was generously provided by TopoTarget and the National Cancer Institute, NIH.

Grant Support

This work was supported by the Cancer Research UK and EPSRC Cancer Imaging Centre in association with the MRC and Department of Health (England) grant C3060/A10334 and by Cancer Research UK project grant C3060/6916 and programme grant C309/A8274, and NHS funding to the NIHR Biomedical Research Centre.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 12, 2011; revised November 30, 2011; accepted December 16, 2011; published OnlineFirst December 22, 2011.
choline decrease detected by magnetic resonance spectroscopy. Cancer Res 2010;70:5507–17.


Histone Deacetylase Inhibition Increases Levels of Choline Kinase α and Phosphocholine Facilitating Noninvasive Imaging in Human Cancers

Mounia Beloueche-Babari, Vaitha Arunan, Helen Troy, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-2688

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/12/22/0008-5472.CAN-11-2688.DC1

Cited articles
This article cites 41 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/72/4/990.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/72/4/990.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.