Detection of histone deacetylase inhibition by noninvasive magnetic resonance spectroscopy

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Abstract
Histone deacetylase (HDAC) inhibitors are new and promising antineoplastic agents. Current methods for monitoring early response rely on invasive biopsies or indirect blood-derived markers. Our goal was to develop a magnetic resonance spectroscopy (MRS)–based method to detect HDAC inhibition. The fluorinated lysine derivative Boc-Lys-(Tfa)-OH (BLT) was investigated as a 19F MRS molecular marker of HDAC activity together with 31P MRS of endogenous metabolites. In silico modeling of the BLT–HDAC interaction and in vitro MRS studies of BLT cleavage by HDAC confirmed BLT as a HDAC substrate. BLT did not affect cell viability or HDAC activity in PC3 prostate cancer cells. PC3 cells were treated, in the presence of BLT, with the HDAC inhibitor p-fluorosuberylanilide hydroxamic acid (FSAHA) over the range of 0 to 10 μmol/L, and HDAC activity and MRS spectra were monitored. Following FSAHA treatment, HDAC activity dropped, reaching 53% of control at 10 μmol/L FSAHA. In parallel, a steady increase in intracellular BLT from 14 to 32 fmol/cell was observed. BLT levels negatively correlated with HDAC activity consistent with higher levels of uncleaved BLT in cells with inhibited HDAC. Phosphocholine, detected by 31P MRS, increased from 7 to 16 fmol/cell following treatment with FSAHA and also negatively correlated with HDAC activity. Increased phosphocholine is probably due to heat shock protein 90 inhibition as indicated by depletion of client proteins. In summary, 19F MRS of BLT, combined with 31P MRS, can be used to monitor HDAC activity in cells. In principle, this could be applied in vivo to noninvasively monitor HDAC activity. [Mol Cancer Ther 2006;5(5):1325–34]

Introduction
Acetylation and deacetylation of nucleosomal core histones play an important role in the modulation of chromatin structure and the regulation of gene expression. The acetylation status of histones is controlled by the activities of histone acetyltransferases and histone deacetylases (HDAC), which, respectively, catalyze removal or addition of acetyl groups onto the ε-amino of lysine residues in the histone tail. Disruptions in HDACs and histone acetyltransferases have been associated with cancer development (1, 2). Conversely, it has been shown that HDAC inhibitors (HDACI) lead to differentiation, growth arrest, and/or apoptosis in treated cells and tumors (1, 3–6). Consequently, HDACIs are currently in clinical trials and show promising results in several different tumor types (1, 2, 7–12). The exact mechanism of action of HDACIs is not entirely clear. Reactivation of silenced tumor suppressor genes occurs following HDAC inhibition in some cases (13, 14). However, HDACIs can lead not only to gene stimulation but also to gene repression (15, 16). Nonetheless, many of the modulated genes mediate proliferation, cell cycle progression, or apoptosis and include p21/WAF1, caspases, p53, vascular endothelial growth factor, HER-2/neu, and bcr/abl (17–21). In addition, acetylation of nonhistone proteins is likely involved in the activity of HDACIs, and HDAC substrates include pRb, E2F, and heat shock protein (Hsp) 90 (22).

As mentioned, several HDACIs are currently in clinical trials. However, at present, there is no direct noninvasive means to measure drug delivery to the tumor tissue, drug–target interaction, or molecular response. Response to HDACIs in clinical trials is correlated with acetylation of peripheral blood mononuclear cells or acetylation of histones in tumor biopsy specimens (7, 9, 10, 12). Blood tests are well tolerated by patients but provide only an indirect indicator of drug delivery and activity at the tumor site. Biopsies reliably assess drug action but are surgically invasive. A further difficulty is that response in many cases is associated with tumor stasis, rather than shrinkage (7), limiting the use of traditional imaging methods. Determining the appropriate, biologically relevant, drug dose and assessing drug action at the tumor site, in vivo, present a challenge (23, 24). A noninvasive method of assessing drug delivery to, and the effect on, the intended molecular target is therefore needed.

Magnetic resonance spectroscopy (MRS) presents a noninvasive nondestructive method, which can provide longitudinal pharmacokinetic and pharmacodynamic...
Detection of HDAC Inhibition by MRS

Biomarkers of drug delivery and action at defined anatomic locations in individual cancer patients. \(^{31}P\) MRS has been used in studies of fluorinated chemotherapeutic agents in cells, animal models, and patients (25–27) and also provides a tool to assess different physiologic variables, including oxygenation, pH, and gene expression (28–30). In addition, MRS can monitor changes in cellular metabolites that are associated with clinical response to traditional chemotherapy or radiotherapy. An increase in choline-containing metabolites, as detected using either \(^{31}P\) or \(^{1}H\) MRS, is associated with cell transformation, and a drop in those metabolites is typically associated with response to treatment (31–33). More recently, \(^{31}P\) MRS has been used to identify biomarkers of response to novel targeted therapies. We have shown that MRS can detect several metabolic changes associated with inhibition of the mitogen-activated protein kinase pathway (34, 35) and the phosphatidylinositol 3-kinase pathway (36) as well as response to the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17AAG; refs. 37, 38).

Several of the genes and proteins modulated by HDAC inhibition are expected to lead to MRS detectable changes. These include down-regulation of receptor tyrosine kinases and their downstream effector molecules (16, 17, 20, 21, 39), which have been associated with a drop in phosphocholine (34, 36, 40); modulation of p53 (19), which could affect phosphocholine levels (41); and Hsp90 acetylation and inhibition (22, 42, 43), which could lead to increased phosphocholine and glycerophosphocholine (37). Thus, \(^{31}P\) MRS could be used to monitor metabolic changes associated with inhibition of HDAC. However, any metabolic changes observed in the \(^{31}P\) spectrum represent indirect and often nonspecific downstream events. Specific direct indicators of drug activity on the intended molecular target are therefore needed to complement the downstream metabolic changes.

We show here for the first time the use of a fluorinated HDAC substrate, the lysine derivative Boc-Lys-(Tfa)-OH (BLT), as a specific spectroscopic indicator to directly monitor HDAC inhibition. We show that intracellular BLT levels, as determined by \(^{31}P\) MRS, are negatively correlated with HDAC activity. In addition, using \(^{31}P\) MRS, we show that phosphocholine levels also negatively correlate with HDAC activity. Our results indicate that MRS can be used as a method for assessing HDAC inhibition and its downstream signaling and metabolic effects. This methodology could eventually be translated to the clinic where drug delivery and molecular activity would be monitored noninvasively over time leading to optimized drug scheduling and dosing.

Materials and Methods

In silico Modeling of BLT Docking into HDAC

Docking was done using the FlexX 1.13.5 software as provided in Sybyl7.1 (Tripos, Inc., St. Louis, MO) running on a 4-Processor R16000 SGI Tezro. Unless otherwise noted, all defaults were used in the docking experiment. The structure of BLT was drawn into Sybyl using the Sketch module, and types were modified to correspond to the protonated state at pH 7. Charges were not assigned to the molecule, because FlexX uses formal charges that are assigned during the actual run. The crystal structure of HDAC8 complexed with suberoylanilide hydroxamic acid (SAHA) was retrieved from the RCSB (ID code 1T69; ref. 44). The binding site was then defined as 6.5 Å around the SAHA ligand. In the customized setting, the zinc atom was added as a template. For H142 and H143, the histidines were selected to be in the α protonated state. This particular protonation state was found to be necessary for proper docking of the hydroxamic acid–based inhibitors. The CSCORE method (45) was used in the ranking of the 30 requested configurations.

MRS Studies of BLT Cleavage

To confirm that MRS can be used to monitor cleavage of the HDAC substrate, 0.6 mmol/L BLT (Advance Chem-Tech, Louisville, KY) was incubated alone or with 28 units recombinant HDAC-8 (Biomol, Plymouth Meeting, PA) in a total volume of 500 μL HDAC assay buffer (Biomol; composed of 25 mmol/L Tris-HCl, 137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl₂, formulated to maintain HDAC activity). \(^{19}F\) MRS was used to monitor the decrease in BLT and buildup of the cleavage product TFA by acquiring \(^{1}H\)-decoupled spectra at 10-minute intervals on an Avance DPX300 Bruker spectrometer (Bruker Biospin, Rheinstetten, Germany) using a 30° flip angle, 3-second relaxation delay, and 128 scans. A sealed insert containing C₆F₆ served as a quantification and chemical shift reference (−164.9 ppm relative to CFCl₃).

Cell Culture, Cell Proliferation, and HDAC Activity

PC3 human prostate cancer cells were routinely cultured in DMEM/F-12 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (HyClone, Logan, UT) and 10,000 units/mL penicillin, 10,000 μg/mL streptomycin, and 25 μg/mmol amphotericin B (Life Technologies) at 37°C in 5% CO₂.

To assess the effect on cell proliferation of BLT, the colorimetric WST-1 cell proliferation assay (Roche, Indianapolis, IN) was used following the manufacturer’s instructions. Briefly, 20 × 10³ cells per well were seeded in 96-well plates. Cells were then treated for 24 hours with BLT at 5, 10, 20, 50, 100, 200, 500 μmol/L and 1, 2, 5, and 10 mmol/L or with matched DMSO (1:20,000 to 1:10). Subsequently, cells were incubated for 4 hours with the WST-1 cell proliferation reagent and absorbance was read at 440 nm using a Tecan Freedom Eco liquid handler equipped with the SAFIRE monochromator-based microplate reader (Tecan U.S., Research Triangle Park, NC).

The effect on cell proliferation of p-fluoro-SAHA (FSAHA; synthesized in-house based on a previously described method; ref. 46), the fluorinated derivative of the clinically relevant HDACI SAHA, was also determined using the WST-1 assay as described above. Cells were treated with 2, 5, and 10 μmol/L FSAHA either in the presence of 1 mmol/L BLT or in the presence of DMSO (in which BLT had to be dissolved first due to its low
solubility in growth medium). Note that controls were treated with DMSO to clearly identify effects that are due to the compound being investigated rather than its vehicle (DMSO). FSAHA dose was based on previous findings (4).

The effect on HDAC activity of BLT and the HDACIs FSAHA and SAHA (also synthesized in-house based on the previously described method; ref. 46) was determined using the Fluor-de-Lys fluorometric assay (Biomol) following the manufacturer’s instructions. Briefly, 20 × 10^3 cells per well were seeded in 96-well plates and incubated for 24 hours with (a) 1 mmol/L BLT, (b) 2, 5, and 10 μmol/L FSAHA, (c) 2, 5, and 10 μmol/L SAHA, (d) 2, 5, 7, 8, 9, and 10 μmol/L FSAHA in the presence of 1 mmol/L BLT, and (e) 2, 5, 7, 8, 9, and 10 μmol/L FSAHA in the presence of vehicle (DMSO). The Fluor-de-Lys substrate was then added for 1 hour, medium was removed, cells were rinsed with PBS and incubated for 10 minutes with the Fluor-de-Lys developer, and fluorescence was read at 460 nm using the Tecan microplate reader as above. Results were normalized to cell density as determined using the WST-1 assay in the same 96 well plate.

**MRS Studies of HDAC Activity**

For MRS studies, PC3 cells were treated for 24 hours with FSAHA at 2, 5, 7, 8, 9, and 10 μmol/L in the presence of 1 mmol/L BLT or with 1 mmol/L BLT alone. Cells (≈1 × 10^7–1.5 × 10^7) were then extracted using the dual-phase extraction method as described previously (37, 47). Briefly, cells were extensively rinsed with ice-cold saline to remove any residual extracellular BLT and medium. Cells were then fixed in 10 mL ice-cold methanol, scraped off the surface of the culture flask, collected into glass tubes, and vortexed. Ice-cold chloroform (10 mL) was then added followed by 10 mL ice-cold deionized water. Following phase separation and solvent removal, the water-soluble fraction was reconstituted in 250 μL deuterium oxide and 250 μL DMSO for 19F magnetic resonance measurements. To perform the 31P magnetic resonance measurement, 100 μL EDTA and 50 μL methylene diphosphonic acid in deuterium oxide were added to a final concentration of 10 and 0.35 mmol/L, respectively. The number of cells extracted was determined by counting a separate flask of cells. 19F MRS of the water-soluble metabolites were recorded as above. Metabolite concentrations were determined by integration and comparison with the area of the internal methylene diphosphonic acid reference, normalizing to cell number and correcting for saturation effects (correction factors were determined as above by acquiring a fully relaxed quantitative spectrum using a 90° flip angle and a 30-second relaxation delay).

To monitor the fluorinated metabolites, the lipid phase was reconstituted in 500 μL CDCl3. To monitor the fluorinated metabolites in cellular protein, the protein pellet obtained during cell extraction was dissolved in 1 mL of 0.5 mol/L NaOH and heated to 60°C for 1 hour. Samples were then analyzed by 19F MRS as above.

**Analysis of TFA in Extracellular Medium**

To assess buildup of TFA in medium, samples of extracellular medium were collected and analyzed using gas chromatography-mass spectroscopy. To be able to use standard capillary gas chromatography-mass spectroscopy, TFA had to be derivatized. This was done using a modification of the procedure of Scott et al. (48) as follows. Sample (1 mL) or TFA standard in medium was treated with 20 mg NaCl, 60 μL HCl, and 30 μL each of 100 mmol/L 1,3-dicyclohexylcarbodiimide (Sigma-Aldrich Co., St. Louis, MO) and 2,4-difluoroaniline (Sigma-Aldrich) and adjusted to a final volume of 1.320 mL in ethyl acetate. Mixture was vortexed for 60 minutes at room temperature, an additional 50 mg NaCl was added, and sample was briefly vortexed. Following phase separation, the organic layer was removed and stored. The aqueous phase was further extracted twice with 200 μL ethyl acetate. All three ethyl acetate extracts were combined, treated with 50 μL of 3 mol/L HCl and 50 μL saturated anhydrous sodium sulfate, and vortexed and another 20 mg anhydrous sodium sulfate was added and mixed. The organic phase was then evaporated to dryness under a dry nitrogen gas stream. The residue was dissolved in 250 μL toluene and transferred into sample vials for analysis. Sample extract (3 μL) was analyzed using an Agilent (Wilmington, DE) 6890N GC coupled to 5973N MSD in splitless injection mode. TFA was resolved using a Supelco (St. Louis, MO) Omegawax 250 capillary column (30 m × 0.25 mm). Column temperature was 100°C for 1 minute, ramping to 230°C at 25°C/min, and then held at 230°C for 2 minutes. Detection was done in EI-positive mode monitoring the m/z 225 ion.

**Western Blot Analysis of Protein Levels**

PC3 cells were lysed using cell lysis buffer [0.1% NP40, 50 mmol/L HEPES (pH 7.4), 250 mmol/L NaCl, 1 mmol/L DTT, 1 mmol/L EDTA, 1 mmol/L NaF, 10 mmol/L β-glycerophosphate, 0.1 mmol/L sodium orthovanadate, 1 μL/mL protease inhibitor cocktail set III (Calbiochem, La Jolla, CA), 1 mmol/L phenylmethylsulfonyl fluoride]. Lysates were centrifuged at 12,000 rpm for 10 minutes (4°C), the protein supernatant was collected, and total protein concentrations were determined using Bio-Rad DC protein assay reagents (Bio-Rad, Hercules, CA). Proteins were separated by SDS-PAGE using 10% gels and transferred electrochemically to 0.45 μm nitrocellulose membranes. Membranes were blocked in blocking buffer containing 5% nonfat dry milk in TBS (pH 7.6) and 0.1% Tween 20 and incubated overnight at 4°C with primary...
antibodies as follows: c-Raf (1:1,000; Cell Signaling Technology, Danvers, MA), cyclin-dependent kinase 4 (cdk4; 1:2,000; Cell Signaling Technology), Hsp70 (1:2,000; Stressgen, Victoria, British Columbia, Canada), and glyceraldehyde-3-phosphate dehydrogenase (1:5,000; Stressgen). This was followed by 1-hour incubation with horseradish peroxidase–conjugated secondary anti-rabbit (Cell Signaling Technology) and anti-mouse (Cell Signaling Technology) antibodies at dilutions of 1:1,000 and 1:2,000, respectively. Membranes were washed with enhanced chemiluminescence reagents (LumiGLO & Peroxide, Cell Signaling Technology) for 1 minute and exposed to Hyperfilm (Amersham Biosciences, Piscataway, NJ), which was developed on a Konica SRX-101 automatic developer (Konica, Tokyo, Japan).

**Statistical Analysis**

All results represent the average of at least three experiments and are expressed as mean ± SD. Data were analyzed by Wilcoxon-Mann-Whitney rank test and *P* < 0.05 was considered significant. A Spearman’s rank correlation was used to analyze correlations. KaleidaGraph (Synergy Software, Essex Junction, VT) and Statistica (Statsoft, Tulsa, OK) software were used.

**Results**

**BLT Is a HDAC Substrate**

First, it was necessary to test the hypothesis that a fluorinated compound composed of a modified lysine could serve as a MRS-detectable substrate of HDAC and could therefore be used to assess HDAC inhibition. We investigated the commercially available BLT and first performed computer modeling to estimate the BLT-HDAC interaction. The known structure of HDAC8 was used (44). The top-ranking consensus scored configuration of BLT docked with HDAC8 is shown in Fig. 1. The interaction of the carbonyl group with the Y306 is consistent with the proposed model of how acetylated lysine would interact in the catalytic site (49). In the docked structure, there is a hydrogen bond to the backbone carbonyl of G151 and a hydrogen bond between side chain NH and D101. The aliphatic side chain interacts with two phenylalanine groups (F152 and F208) and the interaction is seen in the complex of the SAHA as well (44). The Boc group consistently selected to position nearby F207 and F208 rather than the area occupied in the reported complex with SAHA. This may result from the asymmetry that is present within the BLT and not within SAHA, which forces the choice between optimizing the orientation of the carboxyl group and the Boc group.

Next, 19F MRS was used to confirm that recombinant HDAC8 does indeed cleave BLT *in vitro*. As illustrated in Fig. 2, a drop in BLT levels was detected over time accompanied by an increase in TFA levels, consistent with cleavage of BLT by HDAC8 to form TFA and the 19F magnetic resonance invisible Boc-Lys. No changes in BLT levels or any buildup of TFA could be detected in the control sample, which contained no HDAC8. This confirmed that BLT is indeed a substrate of HDAC8 and that its cleavage by HDAC can be monitored by MRS.

**BLT Does Not Affect Cell Viability or HDAC Activity**

Before using BLT as a marker of HDAC activity in cells, it was necessary to rule out its toxicity. The WST-1 assay was used to investigate the effect on PC3 cells of a range of BLT concentrations from 5 μmol/L to 10 mmol/L compared with matched DMSO controls. BLT did not significantly affect cell proliferation compared with controls up to a...
concentration of 10 mmol/L (P < 0.03 for 10 mmol/L and P > 0.4 for all other concentrations from 5 μmol/L to 5 mmol/L; data not shown). We therefore chose to perform MRS experiments with a BLT concentration of 1 mmol/L, which was expected to lead to a MRS detectable signal. Cell numbers following 24-hour treatment with 1 mmol/L BLT represented 95 ± 4% of controls (P > 0.1).

Next, it was necessary to confirm that 1 mmol/L BLT did not affect HDAC activity in cells. Using the Fluor-de-Lys assay, we determined that incubation of PC3 cells for 24 hours with 1 mmol/L BLT resulted in HDAC activity levels of 102 ± 9% (P > 0.3) relative to DMSO-treated controls, indicating no statistically significant effect of 1 mmol/L BLT on HDAC activity.

Inhibition of HDAC Activity and Cell Proliferation by HDACI Treatment Is Not Affected by the Addition of BLT

Before using MRS of BLT to assess the effect of HDACIs, it was necessary to confirm that the addition of BLT will not modify the biological effects of the HDACI. To this end, we investigated both cell proliferation and HDAC activity. Figure 3A illustrates our findings. Treatment with FSAHA, the fluorinated derivative of the HDACI SAHA, resulted in a significant drop in cell proliferation relative to control at all three doses investigated down to 87 ± 5% at 2 μmol/L, 79 ± 3% at 5 μmol/L, and 66 ± 6% at 10 μmol/L (P < 0.03 for all three doses). Importantly, the presence of BLT did not further affect cell proliferation. In the presence of BLT, cell proliferation dropped to 86 ± 1% at 2 μmol/L, 77 ± 7% at 5 μmol/L, and 63 ± 2% at 10 μmol/L (P < 0.03 relative to controls and P > 0.5 relative to FSAHA). Figure 3B illustrates the effect on HDAC activity of FSAHA and FSAHA in the presence of 1 mmol/L BLT. The lower concentration of FSAHA (2 μmol/L) did not lead to a statistically significant drop in HDAC activity (93 ± 16%), but the higher concentrations of FSAHA resulted in significant inhibition of HDAC activity (58 ± 14% at 5 μmol/L and 41 ± 8% at 10 μmol/L; P < 0.03). Again, the presence of BLT did not significantly alter the effect of FSAHA treatment (down to 88 ± 12%, 69 ± 9%, and 53 ± 5% for 2, 5, and 10 μmol/L, respectively; P > 0.1 relative to FSAHA).

Because SAHA is being investigated in clinical trials, it was necessary to confirm that the biological effects of its fluorinated derivative FSAHA were comparable with those of SAHA. The effects of SAHA and FSAHA on HDAC activity were compared at 2, 5, and 10 μmol/L. The effect of SAHA on HDAC activity was comparable with that observed with FSAHA at 2 and 5 μmol/L (84 ± 10% at 2 μmol/L and 51 ± 3% at 5 μmol/L; P > 0.7 relative to FSAHA). However, treatment with 10 μmol/L SAHA resulted in a greater inhibition of HDAC activity compared with 10 μmol/L FSAHA (29 ± 3%; P < 0.03 relative to FSAHA). Nonetheless, we reasoned that if MRS can detect the effect of FSAHA on HDAC activity, it would also be able to detect the potentially larger effect of SAHA.

MRS of BLT Can Be Used to Assess HDAC Inhibition in Cells

Figure 4A illustrates the 19F MRS spectra recorded from extracts of PC3 cells. Control cells cultured in the presence...
of 1 mmol/L BLT contained 14 ± 4 fmol/cell BLT. This value increased significantly to 32 ± 4 fmol/cell in cells treated with 10 μmol/L FSAHA in the presence of BLT (P < 0.0002), consistent with intracellular uncleaved BLT levels being higher when HDAC is inhibited. No signal could be detected from FSAHA, which was expected to resonate at -121 ppm. In addition, no 19F signal was observed from the lipid phase or the protein pellet of the cells.

Interestingly, TFA levels observed in both treated and control cells remained, within experimental error, unchanged. The average TFA concentration observed in control cells was 1.1 ± 0.6 versus 1 ± 1 fmol/cell in cells treated with 10 μmol/L FSAHA. We therefore speculated that TFA produced inside the cell was removed into the extracellular compartment. To test this hypothesis, gas chromatography-mass spectroscopy was used to determine the levels of TFA present in cellular growth medium. In control cells, the level of TFA was 5 ± 0.4 μg/mL but was only 3.4 ± 0.7 μg/mL in the medium obtained from 10 μmol/L FSAHA-treated cells (P < 0.03).

To confirm that the intracellular BLT levels detected by MRS are indicative of inhibition of cellular HDAC, we next monitored HDAC activity and cellular BLT levels in cells treated with a range of FSAHA concentrations from 2 to 10 μmol/L. HDAC activity dropped significantly for all concentrations greater than 2 μmol/L. An increase in BLT levels was observed for all FSAHA concentrations investigated and reached statistical significance when HDAC activity dropped to 74% relative to controls. Furthermore, a negative correlation was observed between HDAC activity and the level of intracellular BLT (ρ = -0.75; P < 0.05; Fig. 4B).

31P MRS Can Be Used to Assess HDAC Inhibition in Cells

Because HDAC inhibition leads to modulation of several genes that are associated with MRS detectable metabolic changes, we questioned whether response to treatment with HDACIs is also detectable in the 31P MRS of treated cells. 31P MRS was used to investigate the same PC3 samples investigated by 19F MRS. As illustrated in Fig. 5A, treatment with 10 μmol/L FSAHA resulted in a significant increase in phosphocholine levels from 7 ± 1 to 16 ± 2 fmol/cell (P < 0.01). None of the other metabolites observed in the 31P MRS spectrum were altered. As in BLT, phosphocholine levels for cells treated with lower concentrations of FSAHA also showed an increase relative to controls, reaching statistical significance when HDAC activity dropped to 74% relative to controls. Phosphocholine levels also negatively correlated with HDAC activity (ρ = -0.86; P < 0.02; Fig. 5B).

31P MRS Changes Are Consistent with Depletion of Hsp90 Client Proteins cdk4 and c-Raf-1

The increase in phosphocholine observed in our spectra following response to HDACI treatment is an unusual observation and only previously reported following treatment with the Hsp90 inhibitor 17AAG (37). Hsp90 inhibition has also been reported following HDACI treatment. To test the hypothesis that the 31P MRS changes observed here were associated with inhibition of Hsp90, we monitored the levels of the Hsp90 client proteins c-Raf-1 and cdk4. Figure 6 indicates depletion of these two Hsp90 client proteins following treatment with FSAHA. However, induction of Hsp70, which has also been reported following inhibition of 17AAG, was not observed in our cells (Fig. 6).
Discussion

The recent shift to development of antineoplastic therapies that target specific alterations in cancerous cells has been fueled by the clinical successes of tyrosine kinase inhibitors, such as imatinib, gefitinib, and trastuzumab (50, 51). HDACIs are a novel class of anticancer drugs that target HDACs. Modulations in gene expression resulting from the activities of HDACs and their counterpart histone acetyltransferases have been associated with cell transformation (52, 53). Conversely, response to HDACIs has been associated with alterations in expression of several oncoproteins and results in induction of differentiation, growth arrest, or apoptosis (1–3, 16, 54). Response to HDACIs in clinical trials has been promising (1, 2, 7, 11, 12). Unfortunately, current methods to monitor response in vivo rely on either surgically invasive biopsies or blood-derived markers (7, 12). There is therefore a need for a direct, reliable, and noninvasive method to monitor drug delivery and response to HDACIs at the tumor site.

Here, we have investigated the fluorinated lysine derivative BLT as a MRS marker of HDAC activity. BLT is detectable by $^{19}$F MRS and its cleavage by HDAC was expected to produce TFA and Boc-Lys. In silico and in vitro studies confirmed that BLT is a substrate of HDAC8 and therefore most likely a substrate of other class I and II HDACs. BLT did not affect cell viability or HDAC activity. Most importantly, we show here that the intracellular levels of BLT, as measured by $^{19}$F MRS, are correlated with cellular HDAC activity.

Fluorine in the body is in the form of solid fluorides with very short $T_2$ relaxation times, producing wide and virtually nondetectable MRS peaks. In vivo $^{19}$F MRS therefore presents the advantage that there are no naturally observable fluorinated molecules. Consequently, exogenously administered fluorine-containing compounds are observed without interference (28). By introducing a fluorinated HDAC substrate, it is therefore straightforward to monitor its fate and thus assess HDAC activity directly in the target tissue.

We have shown previously that $^{31}$P MRS provides a noninvasive method for the detection of metabolic biomarkers associated with response to targeted therapies (34, 37). Here, we have applied this methodology to monitor the downstream metabolic effects correlated with HDAC inhibition, complementing the use of $^{19}$F MRS to monitor drug activity. We show that phosphocholine levels increase following HDAC inhibition and are correlated with the level of this inhibition. This provides a downstream metabolic biomarker of tumor response to HDACI treatment, further confirming activity of the drug on its target.

MRS is a noninvasive method that can be readily translated to the clinic. Our investigations therefore concentrated on a derivative of the clinically relevant HDACI SAHA (10). We chose to concentrate our studies on FSAHA, rather than SAHA, because we reasoned that if a significant level of FSAHA accumulates intracellularly it would be possible to simultaneously monitor both the delivery of FSAHA and its effect on HDAC activity by $^{19}$F MRS. FSAHA could not be detected in any of our spectra. Therefore, we conclude that the intracellular level of FSAHA is below MRS detection level ($< 0.1$ fmol/cell).

Current phase I trials (10) found that the mean plasma concentration of SAHA in vivo was $< 600$ mg/mL, which is also expected to be below detection level.

The inhibitory effect of FSAHA was slightly lower at 10 $\mu$mol/L than that of SAHA. We have not investigated the reasons for this difference. Nonetheless, this observation does not affect the value of our MRS findings. Because we

![Figure 5](https://example.com/figure5.png)

**Figure 5.** A, representative $^1$H-decoupled $^{31}$P MR spectra of PC3 cell extracts. Cells were treated for 24 h with 10 $\mu$mol/L FSAHA in the presence of 1 mmol/L BLT (top) or with 1 mmol/L BLT alone (bottom). Average phosphocholine (PC) content in the FSAHA-treated cells was 15 fmol/cell compared with 7 fmol/cell in controls. Spectra are the result of 3,000 scans acquired using a 30° flip angle and a 3-second relaxation delay. Reference was methylene diphosphonic acid. B, phosphocholine levels as a function of HDAC inhibition. Phosphocholine levels were determined by $^{31}$P MRS as in A. HDAC inhibition was determined using the Fluor-de-Lys assay. Bars, SD. Line, best linear fit. Spearman’s rank correlation indicates that phosphocholine levels negatively correlated with HDAC activity ($\rho = -0.86; P = 0.02$).
are able to detect the effects of the less potent fluorinated inhibitor, we believe we would also be able to detect the effects of the parent inhibitor SAHA or other HDACIs of equal or greater efficacy.

Surprisingly, TFA, the cleavage product of BLT, was constant in the intracellular compartment of control and HDACI-treated cells. However, an analysis of the extracellular medium showed that TFA was lower by an average 1.6 µg/mL in the medium of 10 µmol/L SAHA-treated cells compared with controls. On average, intracellular BLT increased by 18 fmol/cell in those cells. If any TFA produced through cleavage of BLT by HDACs is removed into the extracellular medium, this would lead to TFA levels lower by 1.3 µg/mL in the medium of HDAC-inhibited cells. This number is consistent with the TFA levels determined experimentally in our extracellular medium. We conclude that TFA produced by cleavage of BLT is removed from the intracellular compartment into the medium, in line with previous findings (55).

In vivo, depending on the rate of TFA clearance, it is possible that both BLT and TFA will be present in the tumor region. However, due to the small chemical shift difference between BLT and TFA, monitoring TFA is expected to be difficult.

In addition to directly assessing HDAC activity by 19F MRS of BLT, a unique metabolic biomarker of response was afforded by using 31P MRS to monitor the intrinsic cellular metabolites. Inhibition of cell growth following chemotherapeutic treatment as well as signaling inhibition are typically associated with a magnetic resonance visible drop in phosphocholine levels (31, 34–36, 56). The increase in phosphocholine observed here is therefore unusual and has been observed previously only following response to treatment with 17AAG (37, 38). 17AAG causes inhibition of Hsp90, which results in depletion of its client proteins, including cdk4 and c-Raf-1, as well as up-regulation of Hsp70 (57, 58). Interestingly, HDACI treatment results in increased Hsp90 acetylation also leading to inhibition of its activity and depletion of client proteins (39, 43, 59). In the specific case of SAHA, a drop in both cdk4 and c-Raf-1 has been observed in some cases (16) but not in others (60). Our Western blot analysis indicates depletion of both cdk4 and c-Raf-1 in treated cells. However, we did not observe any up-regulation of Hsp70. Thus, it is not entirely clear if the depletion of cdk4 and c-Raf-1 is a direct result of HDAC inhibition or occurs subsequent to Hsp90 acetylation following HDAC inhibition. Nonetheless, we believe that the increase in phosphocholine observed by MRS is associated with the depletion of cdk4 and c-Raf-1. The mechanism linking cdk4 and c-Raf-1 with modulation of phosphocholine remains to be elucidated, but the results described here following HDACI treatment are entirely consistent with our earlier observations (37). It should be noted that we have also previously observed an increase in glycerophosphocholine following response to 17AAG. In this study, this metabolite remained below detection level; thus, it is not clear if its levels are altered by HDACI treatment.

In future, these methods could be applied in vivo. Full toxicity studies of BLT still need to be done. However, preliminary experiments in our laboratory indicate that an i.p. injection of 100 mg/kg BLT once weekly on three consecutive weeks (a schedule consistent with monitoring response to HDACIs in vivo) results in no detectable toxicity to the animal. Importantly, this dose was sufficient to produce a MRS visible BLT signal in s.c. PC3 tumors with a temporal resolution of 5 minutes at 4.7 T. The BLT signal remained detectable within the tumor region for over 2 hours. As expected, the TFA peak could not be easily resolved in our preliminary in vivo studies. However, based on the data presented here, it is expected that intratumoral BLT levels will be higher in HDACI-treated tumors compared with controls; therefore, tumoral BLT levels could be used to assess HDAC activity in vivo. Future work in our laboratory is aimed at confirming this point. Downstream metabolic biomarkers could also be assessed in our preliminary studies. We were able to acquire a 31P spectrum from PC3 s.c. tumors in 30 minutes, providing a means for monitoring phosphocholine levels. This is consistent with previous studies (37) in which an increase in phosphocholine could be monitored as an indicator of response to 17AAG treatment. 1H MRS, with its greater sensitivity compared with 31P, could also be used to monitor the total choline signal as a downstream metabolic marker of response to HDAC inhibition.

In summary, we present here a dual method for noninvasively monitoring response to HDACIs. 19F MRS of the targeted molecular imaging agent BLT can be used to monitor delivery and activity of HDACIs at the tumor site, whereas 31P MRS can be used to monitor the downstream metabolic consequences of HDAC inhibition. Together, these two MRS methods provide both a direct marker of HDAC inhibition and a downstream biomarker of cellular response to the inhibition. We believe that combining both indicators provides a more powerful tool than a single

![Figure 6](https://example.com/image.png)
marker alone, particularly at lower levels of HDAC inhibition when the changes observed in either marker alone are relatively small. The combination of $^{19}$F and $^{31}$P (or $^2$H) MRS could thus serve as a reliable noninvasive modality to assess HDAC inhibition.

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