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The Synthesis and Evaluation of N¹-(4-(2-[¹⁸F]-fluoroethyl)phenyl)-N⁸-hydroxyoctanediamide ([¹⁸F]-FESAHA), A PET Radiotracer Designed for the Delineation of Histone Deacetylase Expression in Cancer

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Abstract

Introduction—Given the significant utility of suberoylanilide hydroxamic acid (SAHA) in chemotherapeutic protocols, a PET tracer that mimics the histone deacetylase (HDAC) inhibition of SAHA could be a valuable tool in the diagnosis, treatment planning, and treatment monitoring of cancer. Here, we describe the synthesis, characterization, and evaluation of N¹-(4-(2-[¹⁸F]-fluoroethyl)phenyl)-N⁸-hydroxyoctanediamide ([¹⁸F]-FESAHA), a PET tracer designed for the delineation of HDAC expression in cancer.

Methods—FESAHA was synthesized and biologically characterized *in vivo* and *in vitro*. [¹⁸F]-FESAHA was then synthesized in high radiochemical purity, and the logP and serum stability of the radiotracer were determined. *In vitro* cellular uptake experiments and acute biodistribution and small animal PET studies were performed with [¹⁸F]-FESAHA in mice bearing LNCaP xenografts.

Results—[¹⁸F]-FESAHA was synthesized in high radiochemical purity via an innovative one-pot procedure. Enzymatic inhibition assays illustrated that FESAHA is a potent HDAC inhibitor, with IC₅₀ values from 3 nM to 1.7 μM against the eleven HDAC subtypes. Cell proliferation experiments revealed that the cytostatic properties of FESAHA very closely resemble those of SAHA in both LNCaP cells and PC-3 cells. Acute biodistribution and PET imaging experiments revealed tumor uptake of [¹⁸F]-FESAHA and substantially higher values in the small intestine, kidneys, liver, and bone.

Conclusion—The significant non-tumor background uptake of [¹⁸F]-FESAHA presents a substantial obstacle to the use of the radiotracer as an HDAC expression imaging agent. The study at hand, however, does present a number of lessons critical to both the synthesis of hydroxamic acid containing PET radiotracers and imaging agents aimed at delineating HDAC expression.

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Keywords

PET; Histone deacetylase; Hydroxamic acid; SAHA; [^{18}F]-FAHA; [^{18}F]-FESAHA

1. Introduction

1.1 Histone Deacetylases and Cancer

The transient and reversible covalent modification of histones plays an important role in the epigenetic regulation of eukaryotic transcription and gene expression.[1,2] Histone acetylation provides a prime example: the acetylation of exposed lysine residues by histone acetylase (HAC) neutralizes the surface of the histone, thereby weakening the interactions between DNA and the nucleosome proteins. In contrast, the deacetylation of the same lysines by histone deacetylase (HDAC) exposes the negative charge on the surface of the histone and enhances the interaction between DNA and the nucleosome particles. Both alterations to the surface structure of the nucleosome significantly modulate transcriptional activity and thus protein expression.[3–7]

This regulation mechanism clearly demands that cells strike a delicate balance between the two modification processes, for small changes in acetylation and de-acetylation patterns may have dramatic effects on DNA transcription levels and, in turn, dire consequences for cellular health.[8,9] Indeed, a wide variety of cancers have been shown to over-express histone deacetylase enzymes.[10–16] Admittedly, however, the exact mechanism by which the over-expression of HDACs leads to cancer has remained a complicated and controversial topic.[17,18] Still, the overall relationship between altered HDAC activity and cancer remains clear, making the enzymes particularly attractive targets for the development of chemotherapeutic agents.[19–21] In humans, 18 different HDAC isoforms have been identified, with all falling into two basic groups: Zn^{2+} -dependent proteases and NAD^{+} -dependent proteases.[22,23] The Zn^{2+} -dependent proteases are the relevant chemotherapeutic targets and are further sub-divided into two classes, class I and class II (the NAD^{+} -dependent proteases are dubbed class III). Importantly, both classes share a homologous ~390-amino-acid deacetylase core and, significantly, a catalytically essential Zn^{2+} ion (a third, less common sub-class, dubbed IIa, actually has two catalytic Zn^{2+} sites).

1.2 SAHA

Over a decade ago, Dr. Paul Marks at Memorial Sloan-Kettering Hospital and Professor Ronald Breslow of Columbia University pioneered the study and chemotherapeutic application of a potent HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA, Figure 1). [24–26] SAHA is capable of inhibiting both class I and class II HDACs at approximately 50 nM and arrests cell proliferation in a wide variety of cells at single micromolar concentrations.[18,21,27,28] The precise mechanisms of the anticancer effects of SAHA are still not completely understood, though both the over-expression of HDACs in many forms of cancer and an abundance of biochemical evidence — including the accumulation or acetylated histones in cells treated with SAHA — suggest that the drug acts through its inhibition of HDAC.[29] Interestingly, additional modes of action for SAHA and similar compounds may also be possible. HDACs have been shown to be responsible for the deacetylation of a variety of other cellular proteins such as Bcl-2 and Hsp90 in addition to their normal histone substrates, and the inhibition of HDACs by SAHA would also prevent the deacetylation of these important proteins.[30,31]

While the precise pathways of SAHA cytotoxicity and specificity remain somewhat elusive, the molecular mechanisms by which the drug inhibits HDACs are far more clear. Structural

and crystallographic studies of SAHA bound to histone deacetylase-like protein (HDLP) reveal that the molecule's hydroxamic acid moiety disables the enzyme by forming a bidentate chelate with the enzyme's catalytically-active Zn^{2+} ion.[32] Further, the aliphatic chain of SAHA makes a number of important van der Waals interactions with the substrate channel of the enzyme, no doubt enhancing the binding strength and potency of the inhibitor. Overall, the crystal structure suggests that aniline ring of the inhibitor, while undoubtedly involved in interactions with the hydrophobic shell of the protein, seems to play a less significant role in the molecular recognition of SAHA than the other two structural moieties, an assertion supported by structure-activity relationships delineated by other researchers.[33–36]

Cellular and molecular mechanisms aside, SAHA has been clinically proven to be an extremely selective, potent, and effective chemotherapeutic in a broad ranges of cancers. SAHA, marketed by Merck as Zolinza™, was first approved in 2006 by the FDA for the treatment of cutaneous T cell lymphoma. In addition, SAHA is currently involved in over 100 clinical trials as either a monotherapy or in combination with other chemotherapeutics for cancers including non-Hodgkin lymphoma, acute myeloid leukemia, Her2-positive breast cancer, and pancreatic cancer.[37] Currently, wide-spread efforts exist to develop more effective HDAC inhibitors, both for a broad spectrum of the enzymes and targeted at specific subtypes.[29,38–41]

1.3 A PET-Radiotracer for HDAC Activity

Given the widespread over-expression of HDACs in cancer and the burgeoning role of HDAC inhibitors as chemotherapeutics, it has become clear that a molecular imaging method for delineating HDAC in tumors would be a valuable tool both in diagnosis and in monitoring the treatment of cancers being treated with SAHA or other HDAC inhibitors. Almost five years ago, the first PET radiotracer targeted at HDACs was synthesized and characterized by Mukhopadhyay, Tong, Gelovani, and Alauddin at M.D. Anderson Cancer Center (Figure 1).[42] Structurally, the radiopharmaceutical, dubbed [^{18}F]-FAHA, resembles SAHA, with an aniline cap and an aliphatic linker group. However, two critical differences exist: the aliphatic linker group is chemically different from that of SAHA and, more importantly, [^{18}F]-FAHA contains a [^{18}F]-fluoroacetamide group in place of SAHA's hydroxamic acid moiety. These changes mandate that [^{18}F]-FAHA acts not as an HDAC inhibitor but rather as an HDAC substrate. Thus, its role is in delineating HDAC activity, not necessarily expression. The idea is both elegant and simple. Upon encountering HDAC, [^{18}F]-FAHA will be deacetylated, producing [^{18}F]-fluoroacetate that is unable to diffuse out of the cell in which it was created. Thus, cells with higher HDAC activity will accumulate more [^{18}F]-fluoroacetate, and thus more activity, than cells with lower HDAC activity. Since the initial report on the promising compound, the radiotracer has been employed to delineate HDAC activity in baboon brain, though with mixed results primarily a result of the extremely rapid metabolism of [^{18}F]-FAHA upon injection.[43] Additional reports of [^{18}F]-FAHA evaluation studies in rodent have also been performed, though to date these results have yet to appear in publication.[44,45]

1.4 The Design of [^{18}F]-FESAHA

Herein, we report the synthesis, characterization, and evaluation of N^1 -(4-(2-[^{18}F]fluoroethyl)phenyl)- N^8 -hydroxyoctanediamide ([^{18}F]-FESAHA), a PET radiotracer designed to delineate histone deacetylase expression in tumors (Figure 1). A PET radiotracer capable of imaging HDAC expression could prove extremely valuable in cancer diagnosis, treatment planning, and treatment monitoring. Interestingly, it is also possible that an HDAC expression PET tracer could even be used in conjunction with tracers for HDAC activity ([^{18}F]-FAHA) to explore the relationship between HDAC activity and expression *in vivo*.

To facilitate the delineation of histone deacetylase expression, [^{18}F]-FESAHA was designed to mimic the structure and, more importantly, HDAC inhibition activity of its parent compound, SAHA. Naturally, one of the most fundamental exigencies of creating an [^{18}F]-PET tracer is the incorporation into the molecule of the radiolabel itself. SAHA does not contain a fluorine atom, so it follows that some structural changes in creating [^{18}F]-FESAHA were mandated. Based on both the crystal structure of SAHA bound to HDLP and published structure-activity relationships of SAHA analog HDAC inhibitors, it was hypothesized that a fluoroethyl modification at the *para* position of the aniline ring would least disturb the binding of the molecule to the active site of the enzyme. This supposition was confirmed both by modeling [^{18}F]-FESAHA in the HDLP active site (Figure 2) and upon learning that the positioning of an ethyl group at the *para* position of the same ring results in very little, if any, attenuation of the molecule's inhibitory effect.[46] Consequently, a *para*-positioned [^{18}F]-fluoroethyl moiety was chosen for [^{18}F]-FESAHA, and a relatively straightforward synthesis of the molecule from suberate and 2-(4-aminophenyl)ethanol was designed.

Though [^{18}F]-FESAHA is one of the first PET radiotracers to incorporate a hydroxamic acid functionality, a couple of previous examples of imaging agents that employ hydroxamic agents, particularly in MMP inhibitors, do exist in the literature.[47–50] However, [^{18}F]-FESAHA is — to the best of the authors' knowledge given extensive literature searches — the first radiotracer (1) to employ this particular synthetic route to a hydroxamic acid and (2) incorporate the hydroxamic acid *after* the ^{18}F radionuclide in a simple one-pot synthesis.

2. Experimental Procedures

2.1 Reagents and Instrumentation

All chemicals, unless otherwise noted, were acquired from Sigma-Aldrich (St. Louis, MO) and were used as received without further purification. All water employed was ultra-pure ($>18.2\text{ M}\Omega\text{cm}$ at $25\text{ }^{\circ}\text{C}$, Milli-Q, Millipore, Billerica, MA), and all other solvents were of the highest grade commercially available. All instruments were calibrated and maintained in accordance with previously reported quality-control procedures. Activity measurements were made using a Capintec CRC-15R Dose Calibrator (Capintec, Ramsay, NJ). For accurate quantification of activities, experimental samples were counted for 1 min on a calibrated Perkin Elmer (Waltham, MA) Automatic Wizard² Gamma Counter using a 400 to 600 keV energy window and decay correction. HPLC was performed using a Shimadzu HPLC equipped with a C-18 reversed-phase column (Phenomenex Luna analytic 4.6 x 250 nm or SemiPrep 21.2 x 100 mm, 5 μm , 1.0 or 8.0 mL/min), 2 LC-10AT pumps, an SPD-M10AVP photodiode array detector, and a BioScan Flow Count *PIN* diode radiodetector. HPLC solvents (H_2O with 0.1% v/v TFA and MeCN with 0.1% v/v TFA) were filtered before use.

No-carrier-added ^{18}F -fluoride ion was produced by the $^{18}\text{O}(p,n)^{18}\text{F}$ nuclear reaction by bombardment of an enriched $^{18}\text{O}\text{-H}_2\text{O}$ target with 11-MeV protons using an EBCO TR-19/9 cyclotron.

2.2 Chemical Synthesis

2.2.1. Synthesis of methyl 8-((4-(2-hydroxyethyl)phenyl)amino)-8-oxooctanoate (1a)—8-methoxy-8-oxooctanoic acid (2 g, 10.6 mmol), HOBt (1.63 g, 10.6 mmol), and DCC (2.2 g, 10.6 mmol) were dissolved in 50 mL DMF and stirred at room temperature. After 15 min, 2-(4-aminophenyl)ethanol (1.23 g, 9 mmol) was added to the reaction mixture. The reaction mixture was stirred at room temperature for 12 h. After 12 h, the DMF was removed by rotary evaporation, and the resultant residue was taken up

in CH_2Cl_2 and washed three times with $\text{NaHCO}_3(\text{aq})$. The organic fractions were then dried with MgSO_4 and concentrated via rotary evaporation. The resultant product was purified with flash chromatography (ISCO Combiflash, gradient 10–80% EtOAc in hexanes over 20 minutes) and concentrated *in vacuo* to yield a white crystalline solid (2.4 g, 83% yield). ESI-MS: 307 $[\text{M}+\text{H}]^+$, 330 $[\text{M}+\text{Na}]^+$. $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ 7.45 ppm (d, 2H), δ 7.17 ppm (d, 2H), δ 3.84 ppm (d, 2H), δ 3.66 ppm (3, 3H), δ 2.83 ppm (d, 2H), δ 2.35–2.25 ppm (m, 4H), δ 1.7–1.5 ppm (m, 4H), δ 1.4–1.3 ppm (m, 4H). $^{13}\text{C-NMR}$ (CDCl_3 , 400 MHz): δ 174.3, 171.4, 136.3, 134.4, 129.5, 120.2, 63.6, 51.5, 38.6, 37.5, 33.9, 28.7, 28.6, 25.3, 24.7.

2.2.2. Synthesis of ethyl 8-((4-(2-hydroxyethyl)phenyl)amino)-8-oxooctanoate

(1b)—8-ethoxy-8-oxooctanoic acid (2.1 g, 10.6 mmol), HOBt (1.63 g, 10.6 mmol), and DCC (2.2 g, 10.6 mmol) were dissolved in 50 mL DMF and stirred at room temperature. After 15 min, 2-(4-aminophenyl)ethanol (1.23 g, 9 mmol) was added to the reaction mixture. The reaction mixture was stirred at room temperature for 12 h. After 12 h, the DMF was removed by rotary evaporation, and the resultant residue was taken up in CH_2Cl_2 and washed three times with $\text{NaHCO}_3(\text{aq})$. The organic fractions were then dried with MgSO_4 and concentrated via rotary evaporation. The resultant product was purified with flash chromatography (ISCO Combiflash, gradient 10–80% EtOAc in Hexanes over 20 minutes) and concentrated *in vacuo* to yield a white crystalline solid (2.6 g, 86% yield). ESI-MS: 322 $[\text{M}+\text{H}]^+$, 344 $[\text{M}+\text{Na}]^+$, 360 $[\text{M}+\text{K}]^+$. $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ 7.45 ppm (d, 2H), δ 7.17 ppm (d, 2H), δ 4.11 ppm (q, 2H), δ 3.83 ppm (d, 2H), δ 2.85 ppm (d, 2H), δ 2.4–2.3 ppm (m, 4H), δ 1.7–1.5 ppm (m, 4H), δ 1.4–1.3 ppm (m, 4H). $^{13}\text{C-NMR}$ (CDCl_3 , 400 MHz): δ 174.4, 170.9, 135.3, 134.9, 127.5, 119.8, 70.6, 60.3, 38.6, 37.5, 33.9, 28.7, 28.6, 25.3, 24.7, 14.2.

2.2.3. Synthesis of methyl 8-((4-(2-fluoroethyl)phenyl)amino)-8-oxooctanoate

(2a)—Compound **1a** (100 mg, 0.32 mmol) was dissolved in 10 mL CH_2Cl_2 and cooled with stirring to 0° C in an ice bath. At 0° C, added dropwise (diethylamino)sulfur trifluoride (DAST, 250 mg, 1.5 mmol, 200 μL) and allowed the reaction mixture to warm to RT over the course of 3 h. After 3 h, 50 mL of CH_2Cl_2 were added to the reaction solution, and the reaction mixture was extracted solution three times with 50 mL $\text{NaHCO}_3(\text{aq})$. The combined organic fractions were dried with MgSO_4 , and the solvent was removed solvent *in vacuo*. The resultant product was purified with flash chromatography chromatography (ISCO Combiflash, gradient 10–80% EtOAc in Hexanes over 20 minutes) and concentrated *in vacuo* to yield a light yellow powdery solid (80 mg, 84% yield). ESI-MS: 310 $[\text{M}+\text{H}]^+$, 333 $[\text{M}+\text{Na}]^+$. $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ 7.46 ppm (d, 2H), δ 7.17 ppm (d, 2H), δ 4.60 ppm (d of t, 2H), δ 3.68 ppm (s, 3H), δ 2.99 ppm (d of t, 2H), δ 2.4–2.3 ppm (m, 4H), δ 1.8–1.6 ppm (m, 4H), δ 1.4–1.3 ppm (m, 4H). $^{13}\text{C-NMR}$ (CDCl_3 , 400 MHz): δ 174.2, 171.2, 136.6, 132.5, 129.5, 119.9, 51.5, 37.5, 36.4, 36.2, 33.9, 28.8, 28.7, 25.3, 24.6.

2.2.4. Synthesis of ethyl 8-((4-(2-fluoroethyl)phenyl)amino)-8-oxooctanoate

(2a)—Compound **1b** (100 mg, 0.32 mmol) was dissolved in 10 mL CH_2Cl_2 and cooled with stirring to 0° C in an ice bath. At 0° C, added dropwise (diethylamino)sulfur trifluoride (DAST, 250 mg, 1.5 mmol, 200 μL) and allowed the reaction mixture to warm to RT over the course of 3 h. After 3 h, 50 mL of CH_2Cl_2 were added to the reaction solution, and the reaction mixture was extracted solution three times with 50 mL $\text{NaHCO}_3(\text{aq})$. The combined organic fractions were dried with MgSO_4 , and the solvent was removed solvent *in vacuo*. The resultant product was purified with flash chromatography chromatography (ISCO Combiflash, gradient 10–80% EtOAc in Hexanes over 20 minutes) and concentrated *in vacuo* to yield a light yellow powdery solid (70 mg, 70% yield). ESI-MS: 324 $[\text{M}+\text{H}]^+$, 346 $[\text{M}+\text{Na}]^+$. $^1\text{H-NMR}$ (CDCl_3 , 3400 MHz): δ 7.46 ppm (d, 2H), δ 7.17 ppm (d, 2H), δ 4.60 ppm (d of t, 2H), δ 4.11 ppm (q, 2H), δ 2.99 ppm (d of t, 2H), δ 2.4–2.3 ppm (m, 4H), δ 1.7–

1.6 ppm (m, 4H), δ 1.4–1.3 ppm (m, 4H), δ 1.25 ppm (t, 3H). ^{13}C -NMR (CDCl_3 , 400 MHz): δ 173.8, 171.2, 136.1, 132.9, 129.5, 119.9, 60.3, 37.5, 36.4, 36.2, 34.2, 28.7, 28.6, 25.3, 24.6, 14.2.

2.2.5. Synthesis of N^1 -(4-(2-fluoroethyl)phenyl)- N^8 -hydroxyoctanediamide (Fluoroethyl-SAHA, FESAHA, 3)

A fresh solution of hydroxylamine was first prepared according to the procedure described in Gediya, *et al.* Compound **2a** (100 mg, 0.32 mmol) was dissolved in 2 mL MeOH and cooled to 0° C in an ice bath. 20 mL of chilled hydroxylamine solution were then added to the reaction mixture, and the reaction mixture was allowed to warm to room temperature while stirring over 2 h. After 2 h, glacial acetic acid was added to neutralize the pH of the reaction solution, and the solvent was removed from *in vacuo*. The crude product was purified via preparative RP-HPLC (C18 column; gradient 0:100 H₂O (0.1% TFA): MeCN (0.1% TFA) to 100:0 in 15 min; retention time 8.2 min) and lyophilized to yield the purified product as a white powdery solid (70 mg, 72% yield). The same procedure can be followed using compound **2b** as the starting material; this route yielded 65 mg pure product as a white solid (67% yield). ESI-MS: 309 [M-H]⁻. ^1H -NMR (d_6 -DMSO, 400 MHz): δ 10.32 ppm (s, 1H), δ 9.81 ppm (s, 1H), δ 8.62 ppm (s, 1H), δ 7.51 ppm (d, 2H), δ 7.17 ppm (d, 2H), δ 4.60 ppm (d of t, 2H), δ 2.90 ppm (d of t, 2H), δ 7.17 ppm (d, 2H), δ 2.28 ppm (t, 2H), δ 1.95 ppm (t, 2H), δ 1.60–1.45 ppm (m, 4H), δ 1.3–1.2 ppm (m, 4H).

2.2.6. Synthesis of ethyl 8-oxo-8-((4-(2-(tosyloxy)ethyl)phenyl)amino)octanoate (4)

Compound **1b** (500 mg, 1.6 mmol) was dissolved with TosCl (611 mg, 3.2 mmol) in dry pyridine (1 mL) and allowed to stir at room temperature for 12 h. After 12 h, 50 mL CH₂Cl₂ were added to the reaction mixture, and the resultant organic fraction was washed three times with NaHCO₃(aq). The organic fractions were then dried with MgSO₄ and concentrated via rotary evaporation. The resultant product was purified with flash chromatography (ISCO Combiflash, gradient 10–80% EtOAc in Hexanes over 20 minutes) and concentrated *in vacuo* to yield a white crystalline solid (650 mg, 92% yield). ESI-MS: 476 [M+H]⁺, 498 [M+Na]⁺. ^1H -NMR (CDCl_3 , 400 MHz): δ 7.69 ppm (d, 2H), δ 7.41 ppm (d, 2H), δ 7.26 ppm (d, 2H), δ 7.05 ppm (d, 2H), δ 4.2–4.1 ppm (m, 4H), δ 2.91 ppm (t, 2H), δ 2.43 ppm (s, 3H), δ 2.4–2.3 ppm (m, 4H), δ 1.8–1.6 ppm (m, 4H), δ 1.4–1.3 ppm (m, 4H), δ 1.25 ppm (t, 3H). ^{13}C -NMR (CDCl_3 , 400 MHz): δ 173.8, 171.3, 144.7, 136.9, 132.8, 131.8, 129.8, 129.3, 137.8, 119.9, 70.6, 60.2, 37.5, 34.7, 34.2, 28.8, 28.7, 25.3, 24.7, 21.6, 14.2.

2.3. Radiosynthesis of N^1 -(4-(2-[^{18}F]-fluoroethyl)phenyl)- N^8 -hydroxyoctanediamide ([^{18}F]-Fluoroethyl-SAHA, [^{18}F]-FESAHA, 6)

[^{18}F]-Fluoride ion (2.8 GBq = 75 mCi) in water, was added to a vial containing 120 μL of 4% tetrabutylammonium bicarbonate and 1 mL acetonitrile. Water was removed azeotropically with acetonitrile (3 $\times$ 1 mL) at 90 °C under a slow stream of argon. [^{18}F]-NBu₄F was dissolved in anhydrous acetonitrile (400 μL) and the solution was transferred to vial containing 3 mg of 8-oxo-8-((4-(2-(tosyloxy)ethyl)phenyl)amino)octanoate (**4**) and the resulting solution was heated at 90 °C for 20 minutes to give [^{18}F]-fluorinated intermediate, ethyl 8-((4-(2-fluoroethyl)phenyl)amino)-8-oxooctanoate (**5**)

The solvent was removed by heating at 90 °C under a slow stream of argon. The vial was cooled to 0 °C using a ice bath and to it 400 μL of hydroxylamine solution in methanol was added and allowed to react for 30 minutes. The product was diluted with 600 μL of water and neutralized with acetic acid just before purification step. Purification was carried out on semi-preparative Gemini NX C18 column (5 μ , 100 Å, 21.2 x 100 mm) using 0.1% trifluoroacetic acid (A) and acetonitrile (B) under gradient conditions given below with a

flow rate of 8 mL/min. The gradient conditions used are 30% B for 0–2 min; 30–95% B from 2–15 min; 95% B for 15–20 min; 95–30% B from 20–25 min. Under these conditions the product eluted with retention time of 8.1 min was collected and the pH was adjusted to 8 before solvent removal. Solvent was removed under reduced pressure and the product was formulated in 0.9% saline and used for studies. Purity of the product was analyzed using reverse phase C-18 Gemini-NX column (5 μ , 100 Å, 4.6 x 250 mm) using 0.1% trifluoroacetic acid (A) and acetonitrile (B) under gradient conditions given below with a flow rate of 1 mL/min. The gradient conditions used are 20% B for 0–2 min; 20–100% B from 2–15 min; 100% B for 15–20 min; 100–20% B from 20–25 min. The product has retention time of 10.1 min under these conditions (see Supp. Info.). The final isolated yield was around 19 $\pm$ 9% with specific activity of about 2.3 GBq/ μ mole (62 mCi/ μ mole).

2.4. LogP Determination

1 mL of octanol and 1 mL of phosphate buffered saline (PBS, pH 7.4) were pipetted into a standard cylindrical gamma counter tube. 1, 2, 4, or 8 μ Ci of [18 F]-FESAHA was then added via pipette to the tube (each experiment with a given amount of activity was performed in sextuplicate). Each tube was then vigorously vortexed for 20 min, centrifuged for 5 min to separate the immiscible layers, and allowed to settle on the bench-top for an addition 10 min. After this procedure, 500 μ L of each layer was removed with a micropipette and placed in separate, clean, pre-weighed gamma counter tubes. The tubes were then weighed against to determine the exact amount of solute in each tube. The activity in each tube was then measured using a Wizard 2 Gamma Counter (Perkin Elmer, Waltham, MA) with a 400 to 600-keV energy range, and the data was subsequently analyzed in Microsoft ExcelTM. LogP values were calculated using the following formula: $\log_{10}P = \log_{10}(\text{counts in 1 mL of octanol}/\text{counts in 1 mL of water})$.

2.5. Radiotracer Stability Measurements

1 mCi of [18 F]-FESAHA (in approximately 100 μ L) was added to 1 mL of PBS pH 7.4, PBS 8.2, FBS, RPMI 1640 media (supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 100 units/mL of penicillin and streptomycin), or F-12 Kaighn's media (supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 100 units/mL of penicillin and streptomycin) in 1.7 mL microcentrifuge tubes. After the addition of the radiotracer, each solution was incubated at 37 °C on a heat block (Fisher Scientific). At time points of 30 min, 60 min, 120 min, 240 min, and 480 min, small aliquots of each solution were removed and analyzed for decomposition via reverse phase HPLC. To this end, a C-18 Gemini-NX column (5 μ , 100 Å, 4.6 x 250 mm) was employed, using 0.1% trifluoroacetic acid (A) and acetonitrile (B) under gradient conditions given below with a flow rate of 1 mL/min. The gradient conditions used are 20% B for 0–2 min; 20–100% B from 2–15 min; 100% B for 15–20 min; 100–20% B from 20–25 min. The product has retention time of 10.1 min under these conditions. Any radioactive peaks observed on the HPLC were collected and their activity measured on a dose calibrator for comparison to the amount of radioactivity initially injected.

2.6. In Vitro Assays

2.6.1. Cell Culture—Human prostate cancer cell lines LNCaP and PC-3 were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA) and maintained by weekly serial passage in a 5% CO₂(g) atmosphere at 37 °C. Cells were harvested by using a formulation of 0.25% trypsin and 0.53 mM EDTA in Hank's Buffered Salt Solution (HBSS) without calcium or magnesium. LNCaP cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/

L glucose, 1.5 g/L sodium bicarbonate, and 100 units/mL of penicillin and streptomycin. PC-3 cells were grown in F-12 Kaighn's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 100 units/mL of penicillin and streptomycin.

2.6.2. MTT Cell Proliferation Assays—The cellular proliferation of LNCaP and PC-3 cells upon treatment with SAHA and FESAHA was quantified using an MTT assay obtained from American Tissue Culture Collection (ATCC, Manassas, VA). For the assay, LNCaP (30,000 cells/well) and PC-3 (20,000 cells/well) were seeded in 96 well plates (Costar, 3596) overnight (18–24 hrs). Then, after overnight incubation, the old media was aspirated and discarded, and new media mixed (via 1:20 dilution) with different drug concentrations were added to each well in 100 μ L aliquots. The drug-treated plates were then incubated for 24 or 72 hr at 37°C/5% CO₂. After drug treatment, 10 μ L of MTT reagent were added to each well to yield a final concentration of 5 mg MTT/mL, and the plates were then incubated at 37°C/5% CO₂ for three more hours. After this incubation, the cells were solubilized overnight at room temperature with the kit-provided detergent reagent. After solubilization, the absorbance of each well at 570 nm was measured using a SpectraMax M5 plate reader, and the amount of proliferation in each well was normalized to the amount of proliferation in a control, un-treated well.

2.6.3. HDAC Inhibition Assays—HDAC inhibition assays were performed by Reaction Biology Corp. (Malvern, PA). IC₅₀ values for each of the 11 HDAC sub-types were calculated for FESAHA, SAHA (generously provided by the laboratory of Dr. Paul Marks), and the well-studied HDAC inhibitor Trichostatin-A (TCA).

2.6.4. [¹⁸F]-FESAHA Cellular Uptake—LNCaP (400,000 cells/well) and PC-3 (150,000 cells/well) were seeded in 12-well plates (BD Falcon, 353043) 2 days prior to the assay (this disparity in initial seeding was purposeful, as the differing cell growth rates of the two lines meant that cell populations would approximately equalize in the time between seeding and assay). On the day of the assay, old media was aspirated and discarded. New media, mixed with [¹⁸F]-FESAHA was added to each well in 1 mL aliquots such that each well received 5 μ Ci of activity. After the addition of the radiotracer, the plates were then incubated at 37°C/5% CO₂ for 15, 30, 45, 60, 90, 120, 150, 180, and 240 min. At the end of each incubation, the media from each well was collected in a standard cylindrical gamma counter tube. Each well was then washed twice with PBS (pH 7.4) at room temperature, with each washing collected. The adherent cells were then detached from the plates by adding 500 μ L of 0.25 trypsin/0.53 mM EDTA to each well for 5–10 minutes, after which 500 μ L of media was added to each well, and the resultant cell suspension was harvested. The radioactivity associated with each collection was measured with a Wizard 2 Gamma Counter (Perkin Elmer, Waltham, MA) with a 400 to 600 keV energy window and decay correction. The amount of radioactivity associated with the cells was normalized to protein content using a standard BCA assay.

2.7 In Vivo Experiments

2.7.1. Rodents—All animal studies were conducted in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines. Male athymic *nu/nu* mice (NCRNU-M, 20–25 g, 6–8 weeks old) were obtained from Taconic Farms Inc. (Hudson, NY) and were allowed to acclimatize at the MSKCC vivarium for 1 week prior to implanting tumors. Mice were provided with food and water *ad libitum*. LNCaP tumors were induced on the right shoulder via sub-cutaneous (s.c.) injection of 5.0×10^6 cells in a 200 μ L cell suspension of a 1:1 v/v mixture of media with reconstituted basement membrane (BD Matrigel™, Collaborative Biomedical Products Inc., Bedford, MA) under 2% isoflurane

anesthesia. Palpable tumor developed after a period of 15–20 days, and the tumor volume (V/mm^3) was estimated by external vernier caliper measurements.

2.7.2. Acute Biodistribution Studies—Acute *in vivo* biodistribution studies were performed in order to evaluate the uptake of [^{18}F]-FESAHA in mice bearing s.c. LNCaP tumors ($100\text{--}200\text{ mm}^3$, 4 wks post-innoculation). Mice were randomized before the study and were warmed gently with a heat lamp for 5 min before administration of [^{18}F]-FESAHA ($6.6\text{--}7.4\text{ MBq}$ [$180\text{--}200\text{ uCi}$] in $150\text{ }\mu\text{L}$ 0.9% sterile saline) via intravenous tail-vein injection ($t = 0\text{ h}$). Animals ($n = 4$ per group) were euthanized by $\text{CO}_2(\text{g})$ asphyxiation at 30, 60, or 120 min post-injection and 12 organs (including tumor) were removed, rinsed in water, dried in air for 5 min, weighed, and counted in a gamma-counter with a 400 to 600 keV energy window and decay correction for ^{18}F -activity. Counts were converted into activity using a calibration curve generated from known standards. Count data were background and decay-corrected to the time of injection, and the percent injected dose per gram (%ID/g) for each tissue sample was calculated by normalization to the total activity injected.

2.7.3. Small-Animal PET Imaging—PET imaging experiments were conducted on a microPET Focus 120 rodent scanner (Concorde Microsystems). Mice bearing s.c. LNCaP tumors ($100\text{--}200\text{ mm}^3$) ($n = 3$) were administered [^{18}F]-FESAHA formulations ($14.8\text{--}16.6\text{ MBq}$ [$400\text{--}450\text{ }\mu\text{Ci}$] in $200\text{ }\mu\text{L}$ 0.9% sterile saline) via tail-vein injection. Approximately 5 minutes prior to the PET images, mice were anesthetized by inhalation of 2% isoflurane (Baxter Healthcare, Deerfield, IL)/oxygen gas mixture and placed on the scanner bed; anesthesia was maintained using 1% isoflurane/gas mixture. PET data for each mouse were recorded via a 60 min dynamic scan from $t = 0\text{ min}$ to $t = 60\text{ min}$ and a later, 5 min static scan at $t = 120\text{ min}$. For the static images, a minimum of 20 million coincident events were recorded.

An energy window of 350–700 keV and a coincidence timing window of 6 ns were used. Data were sorted into 2-dimensional histograms by Fourier re-binning, and transverse images were reconstructed by filtered back-projection (FBP) into a $128 \times 128 \times 63$ ($0.72 \times 0.72 \times 1.3\text{ mm}$) matrix. The image data were normalized to correct for non-uniformity of response of the PET, dead-time count losses, positron branching ratio, and physical decay to the time of injection but no attenuation, scatter, or partial-volume averaging correction was applied. The measured reconstructed spatial resolution for the Focus 120 is approximately 1.6 mm in full width at half maximum at the center of the field of view. The counting rates in the reconstructed images were converted to activity concentrations (percentage injected dose [%ID] per gram of tissue) by use of a system calibration factor derived from the imaging of a mouse-sized water-equivalent phantom containing ^{18}F . Images were analyzed using ASIPro VMTM software (Concorde Microsystems).

2.8. Statistical Analysis

Data were analyzed by the unpaired, two-tailed Student's t-test. Differences at the 95% confidence level ($P, 0.05$) were considered to be statistically significant.

3. Results and Discussion

3.1. Synthesis of Non-Radiolabeled FESAHA

Non-radiolabeled FESAHA was synthesized in three relatively facile steps from 2-(4-aminophenyl)ethanol and either monomethyl or monoethyl suberate (both esters were employed in order to maximize the likelihood of synthetic success) (Figure 3). In both cases, an initial HOBt/DCC-mediated peptide coupling reaction was followed by a DAST/ CH_2Cl_2

fluorination step. Subsequently, the final product could be obtained via the conversion of the methyl or ethyl esters to the hydroxamic acid with methanolic hydroxylamine following a transformation recently published by Gediya, *et al.*[51] It is important to note that while this method of forming the hydroxamic acid is high-yielding and simple, the preparation of the methanolic hydroxylamine solution can be fickle and, unless very carefully done, can dramatically effect product yields. All intermediates and the final FESAHA product were purified via column chromatography or reversed-phase HPLC and identified and characterized by ^1H -NMR, ^{13}C -NMR, UV-Vis, and ESI-MS (see *Experimental* for details). The FESAHA product was obtained in >99% purity via both routes and in 43% and 41% yield over three steps from the monomethyl and monoethyl suberate esters, respectively.

3.2. Radiosynthesis of [^{18}F]-FESAHA

Given the need for a suitable fluorination precursor, the radiosynthesis of [^{18}F]-FESAHA follows a slightly different route than the cold synthesis (Figure 4). Further, given the observation during the cold synthesis that the hydroxamic acid conversion proceeded well with either the ethyl or methyl ester intermediates, it was decided that the ethyl ester intermediate would be employed in the radiosynthesis. The initial HOBt/DCC coupling step between 2-(4-aminophenyl)ethanol and monoethyl suberate was identical to the first step in the cold synthesis. Subsequently, however, the synthesis diverged: the hydroxyethyl group of the intermediate was tosylated with TsCl /pyridine to form the radiofluorination precursor, ethyl 8-oxo-8-((4-(2-(tosyloxy)ethyl)phenyl)amino)octanoate (**4**). The fluorination precursor, like all other intermediates, was identified and characterized by ^1H -NMR, ^{13}C -NMR, UV-Vis, and ESI-MS (see *Experimental* for details), and it was produced in >99% purity in 79% yield over two steps.

The ^{18}F -radiolabeling reaction proceeded from **4** in a one-pot, two-step reaction to [^{18}F]-FESAHA. In the first step, the tosylate was substituted with ^{18}F via reaction with [^{18}F]- NBu_4F in CH_3CN at 90°C to produce the radiolabeling intermediate, ethyl 8-((4-(2-[^{18}F]-fluoroethyl)phenyl)amino)-8-oxooctanoate (**5**). Subsequently, the reaction vessel was cooled to 0°C and methanolic hydroxylamine was added to convert **5** into the final product, [^{18}F]-FESAHA (**6**). The final compound was purified from the fluorinated intermediates and any unreacted starting materials via reversed-phase HPLC to give the final product in >99% radiochemical purity (Figure 5, see *Experimental* for details). The overall synthesis time was approximately 70 minutes, including HPLC purification, and the isolated, decay corrected yield was $19 \pm 9\%$ ($n = 3$). The specific activity of the completed radiotracer — approximately $2.3 \text{ GBq}/\mu\text{mole}$ ($62 \text{ mCi}/\mu\text{mole}$) — was relatively low for an ^{18}F radiotracer; however, the abundance of HDAC in normal tissue and the even higher expression levels of the enzyme in tumor tissue render extremely high specific activity a much more minor concern in this case than it would be if targeting, for example, cell surface receptors of very low abundance. As discussed above, to the best of the authors' knowledge, this is the first PET radiotracer to employ the strategy of incorporating a hydroxamic acid after radiolabeling via any strategy, let alone an efficient one-pot procedure.

3.3 LogP Determination and Stability Measurements

The measured logP value of [^{18}F]-FESAHA at pH 7.4 in PBS was determined to be 1.01 ± 0.02 ($n=16$). This value is similar to those experimentally obtained for [^{18}F]-FAHA (1.39) and SAHA (1.04) and those simulated for other SAHA analogs bearing aniline ring substitutions (generally 1.0–2.0).[33,43,52]

Incubation of [^{18}F]-FESAHA at 37°C with PBS pH 7.4, PBS 8.2, FBS, complete RPMI 1640 media, and complete F-12 Kaighn's media revealed that the radiotracer is stable in all of the above solutions up to at least 8 hours (the latest time point assayed). Indeed, HPLC

analysis of each solution revealed that the radiotracers remained in >97% radiochemical purity for the entire time of incubation. Further still, in all cases, the amount of [^{18}F]-FESAHA activity isolated after HPLC was within 10% of the amount of activity injected into the column, strongly suggesting that very little of the [^{18}F]-FESAHA was becoming irreversibly bound to serum proteins (this, of course, only applies to the solutions with proteins present).

3.4 In Vitro Experiments

Two prostate cancer cells lines, LNCaP and PC-3, were employed for all *in vitro* experiments. Previously research has established that both LNCaP cells and PC-3 cells express HDACs in abundance.[24,53] Further, significant precedent exists for LNCaP cells being employed as a standard SAHA-sensitive prostate cancer cell lines, while PC-3 cells are widely known to be SAHA resistant, providing an interesting and potential useful pair of foils for the *in vitro* experiments at hand. Due to their sensitivity to HDAC inhibitors and the precedent of their use *in vivo* SAHA experiments, LNCaP cells were chosen for all *in vivo* [^{18}F]-FESAHA experiments

3.4.1 MTT Cell Proliferation and HDAC Inhibition Assays—Cell proliferation experiments performed using the standard MTT assay kit revealed that FESAHA has an GI_{50} value of $4.8 \pm 0.4 \mu\text{M}$ with the commonly employed LNCaP prostate tumor cell line (Figure 5a). The parent compound, SAHA, was shown to have an GI_{50} value ($5.2 \pm 0.3 \mu\text{M}$) consistent with those reported in the literature.[51] Further still, both SAHA and FESAHA were shown to be dramatically less effective at inhibit cell proliferation in PC-3 cells (Figure 5b). It has widely been reported that PC-3 cells are resistant to SAHA-induced cell death, most likely due to elevated Bcl-2 levels in the prostate cancer cell line.[54]

In order to further investigate the role of the fluoroethyl modification on the biological activity of FESAHA, *ex vivo* HDAC inhibition assays were performed to determine the IC_{50} values for SAHA and FESAHA with all eleven HDAC subtypes. The results clearly indicate that FESAHA is a potent inhibitor of all of the subtypes of HDAC, with IC_{50} values ranging from 3 nM (HDAC 6) to 1.7 μM (HDAC 4) (Table 1). More importantly, the HDAC inhibitory effects of FESAHA very closely mirror those of SAHA, both in the absolute values (SAHA IC_{50} values similarly range from 11 nM to 725 nM) and in the sub-type specific trends (*i.e.* both FESAHA and SAHA are most potent against HDAC6 and least potent against HDAC4). Interestingly, neither FESAHA nor SAHA inhibits the HDACs at concentrations as low as the well-studied HDAC inhibitor trichostatin-A (TCA, IC_{50} values from 2–514 nM). Taken together, the anti-proliferation and HDAC inhibition data strongly suggest that the fluoroethyl modification on the aniline ring of FESAHA has little effect on the intended biological effect of the molecule.

3.4.2 [^{18}F]-FESAHA Uptake Cellular Association—Cellular association experiments with [^{18}F]-FESAHA and PC-3 and LNCaP prostate cancer cells were performed to determine the relationship between incubation time and cellular uptake. The data, shown in Figure 6, clearly indicate association between [^{18}F]-FESAHA and both cell lines and, more importantly, reveal an increase in cellular association over time. In both cases, increases in association can be observed within the first 60–90 minutes of incubation, followed by a plateau of the incubation/association curve. Interestingly, the amount of association per μg protein appears to higher in the LNCaP cells than in the PC-3 cells, an effect that may relate to the well known resistance of PC-3 cells to SAHA and similar histone deacetylase inhibitors. Overall, the absolute degree of cellular association is not overwhelmingly high, but it is generally consistent with other small molecule ^{18}F and radiometal PET tracers.

3.5. In Vivo Experiments

3.5.1. Acute Biodistribution—In order to determine the fate of [^{18}F]-FESAHA *in vivo*, acute biodistribution studies were performed with mice bearing LNCaP tumors (Table 2). To this end, mice bearing s.c. LNCaP tumors ($100\text{--}200\text{ mm}^3$) were administered $180\text{--}200\text{ }\mu\text{Ci}$ [^{18}F]-FESAHA by tail-vein injection and euthanized at 30, 60 or 120 minutes post-injection; the animals' organs were subsequently harvested, washed, and counted on a gamma counter. At thirty minutes post injection, the biodistribution data reveal high levels of uptake in the liver ($13.0 \pm 2.7\text{ \%ID/g}$), small intestine ($15.3 \pm 2.9\text{ \%ID/g}$), and kidney ($17.9 \pm 8.7\text{ \%ID/g}$). Tumor levels were relatively low ($2.8 \pm 0.3\text{ \%ID/g}$ at 30 min) and decreased over the course of the experiment ($1.9 \pm 0.3\text{ \%ID/g}$ at 60 min and $1.5 \pm 0.4\text{ \%ID/g}$ at 120 min).

Over the course of 120 minutes, the amount of activity in the liver ($13.0 \pm 2.7\text{ \%ID/g}$ at 30 min vs. $4.9 \pm 0.6\text{ \%ID/g}$ at 60 min vs. $1.2 \pm 0.6\text{ \%ID/g}$ at 120 min) and kidney ($17.9 \pm 8.7\text{ \%ID/g}$ at 30 min vs. $5.0 \pm 0.7\text{ \%ID/g}$ at 60 min vs. $5.7 \pm 1.2\text{ \%ID/g}$ at 120 min) decreased substantially as the radiotracer was cleared from the animal. The activity in other lower uptake tissues (*i.e.* heart, lungs, and spleen) also cleared over the course of the experiment. The activity in the small intestine, however, did not decrease significantly from 30 to 60 to 120 min ($15.3 \pm 2.9\text{ \%ID/g}$, $16.7 \pm 1.2\text{ \%ID/g}$, and $12.8 \pm 3.9\text{ \%ID/g}$, respectively), indicating high and persistent levels of gut uptake. Significantly, bone activity starts at a high level and increases over the course of the experiment, from $9.6 \pm 2.1\text{ \%ID/g}$ at 30 min to $11.1 \pm 1.6\text{ \%ID/g}$ at 60 min to $13.4 \pm 0.6\text{ \%ID/g}$ at 120 minutes. This is almost certainly the consequence of the de-fluorination of [^{18}F]-FESAHA in the body by dehalogenase enzymes.

3.5.2. PET Imaging—Small-animal PET images of [^{18}F]-FEAHA were obtained in mice bearing LNCaP tumors ($100\text{--}200\text{ mm}^3$). Representative PET images are shown in Figure 7 ($t = 30$ and 120 min). The earlier time-point image ($t = 30$) is consistent with the biodistribution data. The tumor can be distinguished from the muscle and surrounding tissue on the right shoulder of the animal; however, there is significant non-tumor uptake in the liver, kidney, and small intestine. Other structures, including a substantial amount of bone activity, are also visible in the image. The later image ($t = 120$) clearly illustrates the problem of defluorination, as the spine and other bones are the most prominent structures visualized. Significant radioactivity is also still visible in the small intestine, large intestine, kidney, and bladder.

The low tumor-to-background organ ratios — tumor-to-intestine, tumor-to-liver, tumor-to-kidney, and tumor-to-bone, among others — observed in both the PET images and the biodistribution data could reflect a number of confounding factors. Dehalogenation of the radiotracer clearly and dramatically alters the background by producing a new species, $^{18}\text{F}^-$, that is readily trapped in bone and can distribute to other tissues. Also contributing may be the degree to which tumor tissue over-expresses HDACs. While it is known that tumor tissue, particularly prostate tumor tissue, over-expresses HDACs, most literature sources suggest that this overexpression is not by more than one order of magnitude. This degree of overexpression may not be sufficient to produce high target-to-background images. Also, the short half life of fluorine-18 ($t_{1/2} = 110\text{ min}$) precludes imaging at later time points ($t > 12\text{ h}$), when there can be sufficient clearance of the tracer from non-target tissue and thereby an enhancement of tumor to background ratios. The fact that tumor uptake of tracer in tumor does not decrease extremely rapidly after its maximum at 30 min is encouraging news, because SAHA analogs radiolabeled with longer half life PET isotopes (*e.g.* iodine-124, $t_{1/2} = 4.18\text{ d}$) might hold potential for imaging HDAC expression in tumors. These isotopes allow imaging at later time points (up to 96 h in some instances), when in many cases the tracer has largely cleared from non-target tissues. Further still, though perhaps less

satisfying, the answer could lie in the many peculiarities and peccadilloes of small molecule tracer chemistry. Regardless of the causes, non-tumor background radioactivity from [^{18}F]-FESAHA and its putative dehalogenation represent significant obstacles to the development of [^{18}F]-FESAHA as a viable HDAC radiotracer.

4. Conclusions

The *in vivo* experimentation clearly show that the non-tumor background uptake in the intestines, kidneys, liver, and, as a result of defluorination, bone present substantial, if not insurmountable, obstacles to the application of [^{18}F]-FESAHA as a clinical radiotracer for HDAC expression. Yet despite these limitations, the radiosynthesis, *in vitro* assays, and *in vivo* experiments in the investigation at hand still offer valuable lessons and can act as a spring-board for future investigations into PET radiotracers for HDAC expression.

First, the radiosynthesis of [^{18}F]-FESAHA produced the tracer in high radiochemical purity via an innovative one pot, two step synthesis that could potentially be employed in a wide variety of hydroxamic acid containing radiotracers. Molecules bearing hydroxamic acid moieties represent an increasingly promising group of targets for antineoplastic agents far beyond the histone deacetylase inhibitors discussed herein, including inhibitors of ribonucleotide reductase (RR), matrix metalloproteinases (MMP), and tumor necrosis factor alpha inhibiting enzyme (TACE).[28,55–58] Indeed, as more hydroxamic acid-based chemotherapeutics are developed, some of these molecules will inevitably become prime candidates for development as PET imaging agents. To this end, rapid and efficient protocols for the synthesis of hydroxamic acid groups under conditions that are compatible with ^{18}F radiochemistry will be of great value.

Second, the *in vitro* assays suggest that the slight modifications made to SAHA to create the radiotracer could be performed without a loss in growth inhibition, illustrating that the core SAHA structure can be modified with a pendant ^{18}F group without losing its cytostatic effects to create a radiotracer. While FESAHA's fluoroethyl appendage may appear structurally trivial at first glance, it is important to note that other seemingly innocuous modifications made to the SAHA core structure have rendered the resultant inhibitors orders of magnitude less active in both HDAC inhibition and anti-proliferation assays. [33,36,46,52] Thus, the *in vitro* success of FESAHA serves as an important reminder of two critical principles in the design of radiotracers that are based on known drugs: (1) be mindful when designing the attachment of the radionuclide to the parent molecule and (2) even if the modification seems harmless, perform assays comparing the biochemical behavior of the unlabeled daughter molecule to that of the parent. In this case, by studying published structure-activity relationships and performing some rudimentary molecular modeling, a modification strategy was chosen that interfered only minimally, if at all, with the HDAC inhibition and anti-proliferative activity of the parent molecule.

Finally, the biodistribution and PET imaging data make clear that despite the favorable *in vitro* biochemical properties of FESAHA, the *in vivo* instability of the link between the radionuclide and the SAHA core structure renders [^{18}F]-FESAHA unsuitable for the delineation of HDAC expression in tumors. Of course, this is far from an ideal outcome, yet it provides both another instructive example for radiotracer development and, more optimistically, a starting point for the creation of more stable and effective HDAC-targeting PET radiopharmaceuticals. The somewhat unexpected defluorination of [^{18}F]-FESAHA's fluoroethyl linkage to the phenyl ring presents the most important lesson. Fluoroethyl moieties have been employed without significant defluorination in a number of highly successful ^{18}F -based radiopharmaceuticals — including [^{18}F]-fluorodeschydroxycholeline, [^{18}F]-fluoroethylcholine, and [^{18}F]-fluoroethyltyrosine — yet in this case, the linkage

proved unstable, providing a cautionary reminder that the previous successes of a radio-fluorination strategy do not exempt similarly-modified tracers from defluorination or deleterious metabolism. More important, however, is that the limitations of [^{18}F]-FESAHA will act as a spring board for the development of further HDAC imaging agents featuring different attachments between the radionuclide and SAHA core structure. Indeed, armed with a newfound understanding of both the successful design of an active SAHA analog and the ultimate instability of the [^{18}F]-fluoroethyl linkage, efforts are currently underway toward the development of novel, biologically active, and physiologically stable PET radiopharmaceuticals for the delineation HDAC expression in cancer. .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
The structures of SAHA, [^{18}F]-FAHA, and [^{18}F]-FESAHA

Figure 2.
Model of FESAHA bound to the active site of HDLP (based on the crystal structure published by Finnin, *et al.*)[32,59]

Figure 3.
The synthetic route to FESAHA

Figure 4.
The synthetic route to $[^{18}\text{F}]$ -FESAHA

Figure 5.

Dose-response curves for the growth inhibitory action of SAHA and FESAHA with LNCaP (top, A) and PC-3 (bottom, B) prostate cancer cells. Cells were treated with compounds for 3 days, and cell proliferation was measured by MTT assays. Absorbance values are normalized to the untreated control and are the mean $\pm$ SEM from 6 experiments.

Figure 6. Time-dependent cell association assay of [^{18}F]-FESAHA and LNCaP and PC-3 prostate cancer cells. Cells were doses with $4\ \mu\text{Ci}$ [^{18}F]-FESAHA in 1 mL media and allowed to incubate at $37\ ^\circ\text{C}$ for various amounts of time. After incubation, the radioactive media was removed, and the cells were washed twice before being trypsinized and counted for radioactivity.

Figure 7.

Representative coronal and sagittal small animal PET images of nude mice bearing LNCaP xenografts in the right shoulder imaged with [^{18}F]-FESAHA. Images A and B were the result of a 60 minute dynamic scan ($t = 30$ minutes) following injection of the mouse with $\sim 400 \mu\text{Ci}$ [^{18}F]-FESAHA. Images C and D were the result of a 5 minute static scan at $t = 120$ minutes. In A and B, the tumor is marked with a white arrow, and the anomalous activity in the lower quartiles of the images are PET artifacts. Images C and D serve to illustrate both the high levels of non-tumor background uptake and the extent of bone activity due to the putative defluorination of the radiotracer. In order to display maximal bone uptake in images C and D, an image projection was chosen that does not include tumor. Maximum and minimum values for the above images are 3.5 and 0.0.

Sub-type specific IC₅₀ values (nM) for FESAHA, SAHA, and TCA (Trichostatin A) with the eleven HDAC subtypes. Standard deviations are typically ± 5%, and none exceeds ± 10%.

Table 1

Compound	HDAC Subtype-Specific IC ₅₀ (nM)										
	HDAC1	HDAC2	HDAC3	HDAC4	HDAC5	HDAC6	HDAC7	HDAC8	HDAC9	HDAC10	HDAC11
TCA	16	25	18	514	15	2	48	242	30	16	18
SAHA	209	830	336	725	136	11	646	468	261	287	353
FESAHA	56	446	146	1755	67	3	85	115	473	169	155

Table 2

Biodistribution profile of [^{18}F]-FESAHA in nude mice with LNCaP xenografts at 30, 60, and 120 min after tail vein injection. Values are mean $\pm$ SD of mean (n = 4) for each time point.

	30 min	60 min	120 min
Blood	3.3 $\pm$ 0.4	1.5 $\pm$ 0.2	0.7 $\pm$ 0.1
Tumor	2.8 $\pm$ 0.3	1.9 $\pm$ 0.3	1.5 $\pm$ 0.4
Heart	4.3 $\pm$ 0.7	1.6 $\pm$ 0.3	1.0 $\pm$ 0.7
Lungs	3.5 $\pm$ 0.5	2.4 $\pm$ 0.5	1.6 $\pm$ 0.4
Liver	13.0 $\pm$ 2.7	4.9 $\pm$ 0.6	1.2 $\pm$ 0.6
Spleen	4.4 $\pm$ 1.1	1.7 $\pm$ 0.4	0.9 $\pm$ 0.2
Stomach	2.1 $\pm$ 0.6	1.5 $\pm$ 0.6	1.0 $\pm$ 0.3
Small Intestine	15.3 $\pm$ 2.9	16.7 $\pm$ 1.2	12.8 $\pm$ 3.9
Large Intestine	4.3 $\pm$ 1.2	4.8 $\pm$ 0.9	5.1 $\pm$ 1.8
Kidney	17.9 $\pm$ 8.7	5.0 $\pm$ 0.7	5.7 $\pm$ 1.2
Muscle	1.9 $\pm$ 0.3	1.4 $\pm$ 0.3	1.2 $\pm$ 0.6
Bone	9.6 $\pm$ 2.18	11.1 $\pm$ 1.6	13.4 $\pm$ 0.6
Brain	1.0 $\pm$ 0.12	0.7 $\pm$ 0.1	0.4 $\pm$ 0.1