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Class I HDAC imaging using [³H]CI-994 autoradiography

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[³H]CI-994, a radioactive isotopologue of the benzamide CI-994, a class I histone deacetylase inhibitor (HDACi), was evaluated as an autoradiography probe for ex vivo labeling and localization of class I HDAC (isoforms 1–3) in the rodent brain. After protocol optimization, up to 80% of total binding was attributed to specific binding. Notably, like other benzamide HDACi, [³H]CI-994 exhibits slow binding kinetics when measured in vitro with isolated enzymes and ex vivo when used for autoradiographic mapping of HDAC1–3 density. The regional distribution and density of HDAC1–3 was determined through a series of saturation and kinetics experiments. The binding properties of [³H]CI-994 to HDAC1–3 were characterized and the data were used to determine the regional $B_{max}$ of the target proteins. $K_r$ values, determined from slice autoradiography, were between 9.17 and 15.6 nM. The HDAC1–3 density ($B_{max}$), averaged over whole brain sections, was 12.9 picomol·mg⁻¹ protein. The highest HDAC1–3 density was found in the cerebellum, followed by hippocampus and cortex. Moderate to low receptor density was found in striatum, hypothalamus and thalamus. These data were correlated with semi-quantitative measures of each HDAC isoform using western blot analysis and it was determined that autoradiographic images most likely represent the sum of HDAC1, HDAC2, and HDAC3 protein density. In competition experiments, [³H]CI-994 binding can be dose-dependently blocked with other HDAC inhibitors, including suberoylanilide hydroxamic acid (SAHA). In summary, we have developed the first known autoradiography tool for imaging class I HDAC enzymes. Although validated in the CNS, [³H]CI-994 will be applicable and beneficial to other target tissues and can be used to evaluate HDAC inhibition in tissues for novel therapies being developed. [³H]CI-994 is now an enabling imaging tool to study the relationship between diseases and epigenetic regulation.

Introduction

Several enzymes control the chromatin and the accessibility of its associated DNA for transcription.¹ Of these, two families of enzymes modify the charge state of histone tails through acetylation or deacetylation of lysine residues. Although these enzymes can be promiscuous and are known to react with many non-histone proteins,² many of their primary biological functions have been ascribed to chromatin remodeling. Histone acetyltransferases (HATs) function by transferring the acetyl group of acetyl coenzyme A to the ε-amino group of certain lysines, which results in neutralization of the normally cationic amino acid. This in turn facilitates chromatin relaxation and can provide DNA access to the transcriptional machinery, as well as lead to the recruitment of acetyl-lysine binding proteins. HAT activity is opposed by histone deacetylases (HDACs), which trigger chromatin condensation and transcriptional repression.³ Dysfunctional regulation of histone acetylation has been implicated in the pathophysiology of several diseases including cancer, heart failure, inflammation and neurodegeneration.⁴ In some cases, the acetylation status can be “rescued” through chemical inhibition of certain HDACs, a strategy that has been successful in cancer therapy.⁵,⁷ The utility of HDACi in treating central nervous system (CNS) disorders is less clear;⁸ however, abnormal expression levels of class-I HDACs have been correlated with several CNS diseases, including Huntington disease,⁹ amyotrophic lateral sclerosis (ALS),¹⁰–¹² psychiatric disorders,¹³ and Alzheimer disease.¹⁴ Class I HDACs, consisting of HDAC isoforms 1, 2, 3 and 8, appear to have critical functions in brain development and CNS maintenance.¹⁵ For example, HDAC2 silences progenitor transcripts during neuronal differentiation of adult generated neurons¹⁶,¹⁷ and negatively regulates memory formation and synaptic plasticity. Class I HDAC inhibition can protect against epigenetic dysfunctions, such as contextual memory defects.¹⁸,¹⁹ HDAC2 and 3 enzymes have been implicated in neurotoxicity and HDAC inhibitors have potential as neuroprotective drugs.²⁰ HDAC1 and HDAC2 are indispensable for diverse aspects of neuronal development and control the progression of neural precursors to neurons during brain development.²¹

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Although class I HDACs are known to be expressed ubiquitously in the CNS and peripheral organs, information about distribution and density is limited to the relative abundance of its associated mRNA and relative HDAC protein density, as determined by techniques such as western blot and immunoprecipitation.\textsuperscript{23-26} Absolute protein densities and distributions of HDAC have not been determined, although these data would enlighten our understanding of HDAC function in both healthy and diseased tissue. Moreover, these data would provide a foundation for the development of in vivo imaging agents that could be used to characterize HDAC density, distribution, and ultimately function in humans.\textsuperscript{27,28} As a stepping stone to these goals, we are developing autoradiography probes that can be used for ex vivo tissue analyses. Development of an autoradiography probe for HDAC has certain advantages and provides complementary information when compared with other ‘visualization techniques’ for HDAC. For example, binding is stoichiometric and quantitative (in an absolute molarity sense); binding is displaceable and can be used to probe small-molecule interactions with HDAC; binding (for the ligand described herein) occurs in the active site and thus the images have direct relevance to the availability of the enzymes for catalysis, which can be important given that perhaps not all complex forms of HDAC can ‘receive’ a ligand or perform catalysis. Herein, we describe the development and validation of an autoradiography ligand, [\textsuperscript{3}H]CI-994, that can be used to characterize class I HDAC enzymes (specifically the sum of HDAC isoforms 1–3) ex vivo.

CI-994, (acetylamino)-(2-aminophenyl) benzamide, has demonstrated antitumor activity in vitro and in vivo against a broad spectrum of murine and human tumor models.\textsuperscript{29-35} CI-994 has also been pursued as a treatment for neurodegenerative diseases and for diseases outside of the brain.\textsuperscript{36,37} For example, it was recently reported that CI-994 inhibition of HDACs may have potential as for the treatment of memory/cognition and anxiety disorders (Tsai, et al, submitted) CI-994 is a class I HDACs inhibitor, selective for HDAC1, 2 and 3 with varying potency.\textsuperscript{39} Thus, a tritium-labeled version could provide a map of HDAC1–3 and be used to associate this class with disease, to develop class density provided that a ligand can be developed, which is both selective and specific (saturable). Ideally, a radioligand should have the following desirable properties: high affinity for its target protein(s), high specific radioactivity (which is obviously isotope-dependent), high specific to non-specific binding ratio (determined through saturation), and high selectivity for its target(s).

Our studies began by analyzing the structures and physicochemical properties of ligands known to bind HDAC enzymes with high affinity. We were particularly drawn to molecules known to be selective for class I HDACs due to the role of this class in brain disorders (but generally applicable in other tissues as well). We identified CI-994 as a tractable molecule for tritium labeling after first carefully analyzing its binding properties in vitro. This began with an analysis that we believed would be most predictive of non-specific binding, namely brain homogenate binding. CI-994 was compared with other benzamide-based HDAC inhibitors in this assay and found to be unique (Fig. 1). Only 12.9% of CI-994 was bound to mouse plasma protein when allowed to equilibrate with an equal volume of buffer; 14% was bound to mouse homogenate. Moreover, we note that the ligand has similarly good dissociation from human plasma proteins (23.7% bound at equilibrium). These data suggested to us that we would be able to wash away non-specifically bound CI-994 in autoradiography assays by using a series of washing steps.

The affinity of CI-994 was determined against the HDAC isoforms 1–3 using a kinetic assay. This provided us with not only the relevant dissociation constant (measured as K\textsubscript{D}, to be 55, 250, 24 nM, respectively), but also informative information about autoradiography protocol design. These K\textsubscript{D} values were similar to those calculated using a trypsin coupled assay with an H4K12Ac peptide substrate (50, 190, 500 nM, respectively, for HDAC1, 2, 3, with the greatest difference for HDAC3 (-20x)). Through these binding kinetics experiments we determined that the association of CI-994 was likely slow (inhibition was slow and time-dependent) and, thus, autoradiography incubation would need to be significantly longer than is common. The long dissociation half-life was, accordingly, very long (58–250 min at room temperature) and provided us with an opportunity for...
long free and non-specifically bound ligand removal. The affinity range provided that we would be able to measure areas of tissue with reasonably low HDAC1–3 density. We also surmised that the development of an HDAC1–3 ligand could provide a means to map each of the three isoforms selectively (if paired appropriately with a selective inhibitor). For example, the use of CI-994 with an HDAC1–2 inhibitor could provide a map of HDAC3 density. Isoform-selective inhibitors within this class are indeed known.\(^\text{40,41}\)

With the promising in vitro data in hand, we invested in the synthesis of \(^{[\text{H}]}\) CI-994, which was achieved through non-selective hydrogenation (proton exchange) using tritium-gas. The \(^{[\text{H}]}\) CI-994 was prepared in sufficiently high specific activity to proceed with initial autoradiography experiments. These experiments had the primary goal of optimization for specific vs. non-specific binding. Our major findings through these preliminary experiments were that: (1) incubation indeed needed to be long (hours to days), which necessitated tissue morphology stabilization; (2) specific binding was impacted by over-treatment with the fixative solution and morphology stabilization had to be rigorously controlled; (3) there was essentially no difference observed for samples incubated at room temperature vs. 37°C (room temperature was chosen because of increased tissue stability over long incubations); (4) washing needed to be accomplished at 4°C and was best when performed using multiple baths; (5) the identity, concentration and pH of the buffer for incubation and washing impacted the ratio of specific to non-specific binding; (6) DMSO must be used to increase the solubility of non-radioactive CI-994 in blocking experiments. In the end, using the optimized procedure outlined in the methods section, we were able to achieve up to 80\% specific binding; however, we must note that there can be significant variation from experiment to experiment and thus non-specific binding must be determined in each tissue-sample pair to extract quantitative data.

### Determination of specific binding, protein density, and equilibrium binding constant

Using our optimized protocol, we evaluated target saturation by observing total and non-specific binding as a function of \(^{[\text{H}]}\) CI-994 concentration (specific binding was calculated from these data). As seen in Figure 2A, regional distribution of \(^{[\text{H}]}\) CI-994 was heterogeneous and thus, we analyzed the saturation data both in terms of the whole brain (Fig. 2C) and in terms of individual brain regions (Fig. 3A). We determined that the whole brain \(B_{\text{max}}\) (assumed to represent HDAC1–3 density) was 12.9 picomol·mg\(^{-1}\) protein and the \(K_d\) calculated from saturation was 15.9 nM across the whole brain. In addition to curve fitting analysis, we formatted the data as a Scatchard plot, which was linear, as expected, and provided a \(B_{\text{max}}\) of 13.6 picomol·mg\(^{-1}\) protein and \(K_d\) 9.6 nM. These data indicate that the affinity of CI-994 for HDAC in tissue is actually higher than for the isolated recombinant enzymes in vitro and may suggest that HDAC in association with its known binding partners (e.g., in the CoREST complex) may exhibit different binding affinities (and perhaps different selectivity). Moreover, these data support that HDAC class I enzymes are of sufficient concentration in the brain to warrant non-invasive PET imaging (ultimately, in humans). PET imaging of HDAC enzymes is a current area of research; however, to date, the \(B_{\text{max}}\) was not known.

### Validation of imaging data

In order to provide proof that the autoradiography maps indeed relate to the HDAC1–3 density, we measured the HDAC1, HDAC2, and HDAC3 density in rodent brain regions using ‘semi-quantitative’ western blots. Brains from five different mice were dissected into nine major regions by the method of Glowinski and Iverson (1966)\(^\text{42}\) and processed separately as described under experimental procedures. Brain homogenate with known protein content was separated by electrophoresis and antibody binding to tissue sample and reference standards of known concentration was used to determine \(B_{\text{max}}\) ex vivo, Figure 4. A linear calibration was used to approximate the mass (ng) of HDAC in each brain sample. Using these data, we generated regional distribution plots of each isoform separately (Fig. 4C–E). Qualitatively, our data are consistent with the distribution of mRNA for each isoform provided by the Allen Brain Atlas.\(^\text{43}\) Of note, HDAC3 protein density is uniform across the
brain with a density typically twice that of HDAC1 or HDAC2. These data are in effect ‘average’ values within micro-dissected regions and thus variation that is observed as higher resolution is not detected. Also of note are the high densities of HDAC1 and HDAC2 in the cerebellum and the hippocampus.

Using the western-derived densities and the region-specific binding of $[^{3}H]$CI-994, we plotted correlation plots of individual isoforms vs. $[^{3}H]$CI-994, as well as all binary combinations. The best correlation, however, was observed by plotting the sum of isoform density by western (Fig. 3B) vs. $[^{3}H]$CI-994-derived B$_{max}$. These were remarkably well correlated and linear with respect to one-another ($R^2 = 0.98$). B$_{max}$ values determined by $[^{3}H]$CI-994 were slightly higher, on average, than those from western-derived values, but this was expected given the extreme differences in the protocols and analyses. From these data, we believe that $[^{3}H]$CI-994 represents the linear sum of the isoform densities and that the difference in K$_i$ for each isoform (determined in vitro) does not contribute to the selectivity between isoforms (i.e., the lower K$_i$ of HDAC2 in vitro seems to be inconsequential). Correlations alone cannot precisely determine the ratio of HDAC1, HDAC2 and HDAC3 binding by $[^{3}H]$CI-994. We are currently working to block individual isoforms with selective inhibitors to ascribe the precise ratio of isoform binding. For our preliminary analysis, we evaluated the density of ligand binding averaged across large brain regions. However, we note (as seen in Fig. 3C) that substructure can be observed. For detailed, high resolution assessment of sub-regions of the hippocampus, long film/phosphor screen exposure times (> 2 mo) will be required; however, with our 7 d exposure data, heterogeneous distribution is noted, for example, in portions of the dentate gyrus.

**Determination of ligand binding properties in tissue sections.** To rigorously determine the binding parameters of $[^{3}H]$CI-994 in tissue, we performed kinetic binding experiments. This allowed us to compare K$_i$ determined in our assay (assumed to be at equilibrium) to K$_i$ derived from the on- and off-rates. It also provided a means to analyze whether regional uptake was dominated by rate differences, which could be indicative of different HDAC forms (e.g., isoform or complex density differences). Figure 5A provides a subset of these data: association curves for the hippocampus and thalamus, which respectively had high and low B$_{max}$. The association rates were different in these two regions; however, we note a difference in the dissociation analysis (Fig. 5B). Of particular note is the remarkably slow kinetics of the benzamide ligand family, which dictates long incubation times to reach equilibrium binding (several hours, as opposed to only minutes for most ligand receptor interactions). The regional K$_i$ calculated from these regions are different (9.45 vs. 10.2 nM) and suggest to us that there may be a regional variation in isoform ratio (or perhaps HDAC-complex identity). Additional studies will need to be performed to fully understand the regional variation in $[^{3}H]$CI-994 affinity.

**Assessment of competitive binding.** We envisioned that the development of an autoradiography imaging tool for HDAC would be valuable beyond the characterization of protein density and could be applied to the study of novel ligands using competition experiments. As a demonstration of this potential, we validated that the IC$_{50}$ for CI-994 competition with $[^{3}H]$CI-994 matched the K$_i$ as anticipated (Fig. 6A). Interestingly, the IC$_{50}$ showed a parallel trend to the kinetic parameters between various brain regions. For example, the IC$_{50}$ difference in thalamus and hippocampus was consistent with the kinetically determined K$_i$ in those regions. Regional IC$_{50}$ differences using novel HDAC inhibitors could be used to improve our knowledge of the relationship between in vitro HDAC isoform affinity and in vivo binding (thus site of action) and efficacy. In order to characterize a well-studied and potent HDAC inhibitor, we determined the IC$_{50}$ of SAHA using $[^{3}H]$CI-994 (Fig. 6B). The IC$_{50}$ determined through our assay (1.3-1.5 nM) is consistent with the known potency of SAHA, which is relatively non-selective (as it also targets HDAC6, a class IIb HDAC), but yet highly potent at HDAC1, HDAC2 and HDAC3. We are currently using $[^{3}H]$CI-994 in competition experiments with HDAC3-selective and HDAC1,2-selective benzamides to explore additional ways to use this autoradiographic tool.

**Methods**

**Chemicals and supplies.** CI-994 was provided by the Broad Institute, (Cambridge, MA). Synthesis of $[^{3}H]$CI-994 (specific activity 47.45 Ci/mmol, radioactivity 1.0 mCi/mL, radiochemical purity > 97.7%) was synthesized by Perkin Elmer. Briefly, $[^{3}H]$$
CI-994 was synthesized by a non-selective catalytic hydrogen-tritium exchange reaction using tritium gas tuner catalyzed by a hydrogen transfer catalyst (Pd/C) under a carrier-free tritium gas atmosphere at 40 psi. The purity and specific radioactivity were determined by HPLC, TLC and mass spectrometry.

For autoradiography, DMSO was obtained from Sigma. TRIS-HCl and PBS buffer were obtained from Fisher Scientific, Inc. Paraformaldehyde (4% PFA) in phosphate-buffered saline was purchased from Boston Bioproducts. Scintillation cocktail, scintillation vials and filter paper were from Perkin Elmer. Slide incubation bath with lids, autoradiography cassette was from Fisher Scientific, Inc. For visualization, cyclone plus storage phosphor system, [³H] supersensitive screens were used (Perkin Elmer).

For western blot, reagent kits for protein characterization assay were obtained from Fisher Scientific, Inc. Primary antibodies: rabbit polyclonal to anti-HDAC1 antibody (ab53091), anti-HDAC2-chip grade antibody (ab7029) and anti-HDAC3 antibody (ab16047), each of which bind to mouse, rat and human HDAC, were purchased from Abcam. The secondary antibody (goat anti-rabbit HRP-conjugate) and most of the western blot experimental supplies e.g., criterion 4–15% precast gel, SDS loading, running and transferring buffers, PVDF membrane were purchased from Bio-Rad Laboratories, Inc. Biotin serum albumin was from Fisher Scientific, Inc. Non-fat dry milk was from Labscientific, Inc. Blue loading buffer was from New England Biolabs, Inc. Recombinant HDAC1, 2 and 3 purified proteins were purchased from BPS Bioscience, Inc. For imaging, Chemidoc TM XR+ was used (Bio-Rad Laboratories, Inc.). Micro-dissection tools, forceps, needles, spatulas, razor blade for rats and mice were purchased from Fine Science Tools.

In vitro rate and Kᵦ determination. Slow, tight-binding kinetics of CI-994 with HDACs 1, 2 and 3 were evaluated by the progression curves in inhibition and dilution experiments. Kinetic reactions were assembled in microtiter plate wells by adding 1.6 nM, 1.7 nM and 0.5 nM of recombinant, full length, human HDACs 1, 2 and 3 (BPS Biosciences, San Diego), respectively, into separate reaction mixtures containing 2 μM HDAC Caliper carboxyfluorescein (FAM)-labeled AcH4K12 peptide substrate (Broad Substrate A) and CI-994 at different concentrations. Plates were immediately placed into a LabChip EZ Reader II (Caliper/Perkin Elmer) and the wells were sampled continuously throughout a 3-h reaction period. The fluorescent product and substrate were separated based on charge difference between acetylated and deacetylated form, and monitored on the Caliper microfluidic instrument. The substrate conversion was calculated with Caliper software. The progression curves at different inhibitor concentrations were analyzed with a nonlinear regression program in Origin 8.5 and fit to the integrated rate equation for slow-binding inhibitors. The off-rate of CI-994 was determined from a dilution experiment. HDAC1 (0.1 μM), HDAC2 (0.1 μM) or HDAC3 (0.05 μM) were incubated with CI-994 at 0.6 μM, 2.5 μM or 0.6 μM respectively for 1 h. The mixtures were then diluted 100-fold into buffer containing 2 μM Broad Substrate A. HDAC-diluted samples were measured continuously over a 4-h period. The recovery of HDAC activity following dilution of the enzyme-[CI-994]-complex is an indication of the reversibility of CI-994. CI-994 off-rates were calculated from dilution progression curve fitting analogously to above.

Western blots. In order to investigate the expression level of class-I HDACs in different brain regions, nine different regions of the mouse brain were micro-dissected. A western blot assay with a known amount of purified human HDACs as standard was used (the affinity of the antibody used should be the same between human and mouse mice HDACs) to calculate the specific amount of HDACs in each brain region. Eight-week old adult WT C57BL/6 mice from Charles River Laboratories International, Inc. were sacrificed by decapitation in accordance with the animal care and use policies from the Subcommittee on Research Animal Care (SRAC) at Massachusetts General Hospital (MGH). The brains were quickly removed and then washed in ice-cold 1X PBS buffer to remove the blood. Water on the surface of the brain was carefully wicked dry using a Kimwipe tissue. The brain was then transferred to a microscope-slide

![Figure 4](image-url)
After weighing the tissues, olfactory bulb and cerebellum.

were dissected which included frontal cortex, parietal cortex, temporal cortex, hippocampus, thalamus, striatum, hypothalamus, olfactory bulb and cerebellum. The dissected tissues were placed in Eppendorf tubes and quickly transferred to dry ice. For micro-dissection in coronal orientation at room temperature as in the Leica dissection microscope in sequence. Slabs were then rinsed with PBS buffer at 4°C. The brain was mounted on a microscope slide and cut in coronal slabs from olfactory bulb to cerebellum with thickness of 1 cm and the slabs were arranged sitting on a bed of dry ice, loosely covered with aluminum foil and allowed to freeze for 10 min. Before sectioning the brain, it was rinsed with PBS buffer at 4°C. The brain was mounted on the microscope slide and cut in coronal slabs from olfactory bulb to cerebellum with thickness of 1 cm and the slabs were arranged in the Leica dissection microscope in sequence. Slabs were then micro-dissected in coronal orientation at room temperature as quickly as possible. Nine different regions of the mouse brain were dissected which included frontal cortex, parietal cortex, temporal cortex, hippocampus, thalamus, striatum, hypothalamus, olfactory bulb and cerebellum. The dissected tissues were placed in Eppendorf tubes and quickly transferred to dry ice. For long-term storage they were later transferred to a −80°C freezer.

Plasma and brain homogenate binding assays. Brain binding assays were performed by Sai Advantium Pharma Limited (Hinjewadi). Briefly, rapid equilibrium dialysis was performed with a rapid equilibrium dialysis (RED) device containing dialysis membrane with a molecular weight cut-off of 8,000 Daltons. Each dialysis insert contains two chambers. The red chamber is for plasma or brain homogenate while the white chamber is for buffer. A 200 μL aliquot of CI-994 in brain homogenate (triplicates) was added to the red chamber of dialysis inserts. A 350 μL aliquot of dialysis buffer was added to the buffer chamber of the inserts. Carbamazepine was used as positive control for brain tissue binding. After sealing the RED device with an adhesive film, dialysis was done at 37°C with shaking at 100 rpm for 4 h. A 50 μL aliquot of CI-994 and positive controls was separately treated with 100 μL aliquot of the carbamazepine and CI-994 were separately treated with 100 μL of MeCN containing internal standards. The carbamazepine and CI-994 were treated with 100 μL of MeCN containing internal standards. Each dialysis insert was incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:50,000) for 1 h, then developed with Bio-Rad Chem. luminescence. The amount of each HDAC in the different regions of mouse brains was determined and normalized to loaded protein mass. Quantitative analysis was performed densitometrically using an Image J analyzer (NIH) and data are reported in pmol·mg⁻¹ protein (not to be confused with pmol·mg⁻¹ tissue).

Protein preparation. After weighing the collected tissue sections, each tissue was homogenized by treating with 300 μL of lysis buffer (2 x RIPA) (25 mM TRIS-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). A homogenization pestle was used followed by vortex, incubation and centrifugation to remove insoluble materials. The supernatant was collected and frozen at −20°C; the debris was discarded. The frozen pellets of mouse brain homogenate were thawed on the day of assay. HDAC standard (reference) proteins and the clarified protein supernatant concentration were colorimetrically determined (at 562 nM by BCA protein assay for total protein concentration by following the protocol provided by the Pierce BCA protein assay kit). HDAC1, HDAC2 and HDAC3 levels in each of nine brain regions were analyzed by western blot in triplicate.

Proteins (15 μg/lane) were separated by SDS-PAGE and then transferred onto PVDF membrane. The membrane was blocked with 5% of non-fat dry milk in 1× Tris-buffered saline containing 0.5% tween-20 for 1 h. The blots were incubated with their respective primary antibody (1:10,000) for 16 h at 4°C. Blots were visualized after incubation with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:50,000) for 1 h, then developed with Bio-Rad Chem. luminescence. The amount of each HDAC in the different regions of mouse brains was determined and normalized to loaded protein mass. Quantitative analysis was performed densitometrically using an Image J analyzer (NIH) and data are reported in pmol·mg⁻¹ protein (not to be confused with pmol·mg⁻¹ tissue).
were subsequently sealed in autoradiography cassettes and moved tissue-side down on tritium-sensitive phosphor screens, which were then dried at reduced pressure (in a vacuum oven) at room temperature. The sections next for 16 h. After washing the sections in buffer, a deionized water bath (4°C) was used to remove buffer salts. The sections were transferred into two sequential baths in an attempt to remove free (unless otherwise noted). Following incubation, the slides were transferred into two sequential baths in an attempt to remove free and non-specifically bound \[^3\text{H}\text{CI-994}\] (10 nM Tris-HCl buffer, pH 7.5 at 4°C); the first rinse was performed for 30 sec and the next for 16 h. After washing the sections in buffer, a deionized water bath (4°C) was used to remove buffer salts. The sections were then dried at reduced pressure (in a vacuum oven) at room temperature for 24 h. Once dry, the microscope slide were placed tissue-side down on tritium-sensitive phosphor screens, which were subsequently sealed in autoradiography cassettes and moved to a leaded storage box for 1 week, unless otherwise stated. A Cyclone Phosphor Imager (Perkin Elmer) was used to record and visualize the phosphor-screen data.

**Binding optimization experiments.** Consecutive tissue sections in coronal and sagittal orientation throughout the rat brain (mounted and fixed as described above) were sequentially numbered divided into two groups: odd numbered tissue sections were used for non-specific binding and even numbered tissue sections were used for total binding. Odd numbered tissue section were treated with 100 μM cold CI-994 plus 10 nM \[^3\text{H}\text{CI-994}\] in 5% DMSO and 10 mM TRIS-HCl buffer; even numbered tissue section were treated with 10 nM \[^3\text{H}\text{CI-994}\] in 5% DMSO and 10 mM TRIS-HCl buffer. Optimization of incubation time, temperature and the rinsing protocol was achieved systematically and led to standard set of conditions above.

**Saturation binding assay.** Alternating adjacent sections (as above) were incubated with increasing concentrations of radioligand \[^3\text{H}\text{CI-994}\] from 1 nM to 100 nM, either with or without excess 100 μM unlabeled CI-994. Saturation experiments were simultaneously performed on different anatomical regions of the rat brain: the frontal cortex, parietal cortex, temporal cortex, striatum, hippocampus, thalamus, hypothalamus and cerebellum in coronal and sagittal planes, respectively. Sections from each individual brain were exposed to the same phosphor screen film. A calibration standard was used to determine the efficiency difference between each of the phosphor screens used. B\text{max} and K\text{d} were calculated by curve fitting and through a Scatchard plot analysis.

**Dissociation and association kinetics experiments.** Thaw mounted tissue sections in both coronal and sagittal orientations were treated with 10 nM \[^3\text{H}\text{CI-994}\] either with or without 100 μM unlabeled CI-994 according to the general method for 16 h. After transferring samples to the second washing bath (time zero), samples were removed from the baths at increasing time intervals from 1 to 48 h (according to Fig. 5B) to determine dissociation of both total and non-specifically bound \[^3\text{H}\text{CI-994}\]. The rate of dissociation for specific bound \[^3\text{H}\text{CI-994}\] was calculated from each time-point pair of sample (total – non-specific). To determine association rate, the incubation time with \[^3\text{H}\text{CI-994}\] was varied from 1 to 48 h (washing was held constant at 16 h). Specific binding association rate was determined in an analogous manner. Curve-fitting in GraphPad Prism was used to extract k\text{off} (dissociation rate), which was used in the determination of k\text{on} (association rate). Rate-derived K\text{d} was determined using these rate constants.

**Self- and SAHA-competitive (IC\text{50}) binding experiments.** Two parallel experiments were designed for competitive binding assay: CI-994 (self) and SAHA competition binding experiments. Thaw mounted tissue sections in both coronal and sagittal orientations were treated with unlabeled CI-994 or SAHA in a concentration range from 100 pM to 100 μM (according to Fig. 6) mixed with 10 nM \[^3\text{H}\text{CI-994}\] according to the general method. Competition curves were obtained by plotting total binding of \[^3\text{H}\text{CI-994}\] against the various (log) concentrations of the competing ligand (i.e., CI-994 or SAHA). IC\text{50} was determined by curve fitting in GraphPad Prism.

**Quantitative data analysis (calibration to protein density).** All autoradiography data were analyzed using densitometry using...
Image] Analyzer software (NIH). Specific binding measured in optical density (OD, non-normalized) was obtained by subtracting paired ROIs of non-specific binding and total binding. The non-specific and total binding ROIs had been corrected for background signal using a local non-tissue region of the slide area analyzed. All pairs analyzed through subtraction in this way were recorded adjacent on a phosphor screen. Each screen (and data set) was calibrated from OD to nCi-mg\(^{-1}\) plastic standard using a [\(^3\)H]-radioactive standard from American Radiolabeled Chemical, Inc. The standard samples were analogously treated in terms of ROI size and back subtraction and resulted in a calibration curve fit to a second order polynomial with \(R^2 = 0.99\) in Microsoft Excel. The polynomial equation was used for the conversion of OD to nCi-mg\(^{-1}\) plastic standard. To determine the relationship between nCi-mg\(^{-1}\) plastic standard and nCi-mg\(^{-1}\) protein, eight tissue samples of known size (2.1 cm \(\times\) 1.0 cm) from cryostat sectioning were cut and weighed. These samples were homogenized as outlined in the western blot analysis details to quantify the protein content in the samples. Using the known tissue section volume, mass, and protein density values that were determined, nCi-mg\(^{-1}\) plastic standard values were converted to nCi-mg\(^{-1}\) protein (for our analyses the conversion factor between these two values was 4.14). To convert from nCi to fmol, the specific radioactivity of [\(^3\)H]CI-994 was used. Thus, the final data were represented in fmol of CI-994 specific binding-mg\(^{-1}\) total protein. Assuming that all specific binding occurs at HDAC1–3, this value represents the total density of HDAC1–3 in the tissue sections.

**Conclusion and Outlook**

[\(^3\)H]CI-994 is now the first validated tool for imaging class I histone deacetylases by autoradiography and can now be used for probing regional variation in HDAC1–3 density in many experiment designs. For example, [\(^3\)H]CI-994 will be valuable for studying the affinity of novel HDAC inhibitors in tissue sections and it will afford an option for measuring (at least ex vivo) the change in HDAC1–3 expression as a function of normal aging processes and biological diversity (e.g., sex). [\(^3\)H]CI-994 can be used to characterize HDAC1–3 response to environmental inputs and will be valuable for exploring the relationships that exist between disease state and progression with respect to HDAC density. These studies can be accomplished on preserved human post mortem tissue provided that fixation of the tissue is mild to moderate. In preclinical settings, [\(^3\)H]CI-994 may be also valuable for exploring HDACi therapy response and mechanism. In preliminary experiments, we have been successful in using [\(^3\)H] CI-994 to study HDAC1–3 in other tissues (such as the heart) and found that the data provided herein for the brain are generally applicable to other organs.

Ultimately, we envision the development of probes to measure HDAC density in vivo and [\(^3\)H]CI-994 will be important for prescreening compounds to prioritize for PET imaging. As an in vivo imaging ligand itself, we have used [\(^3\)C]CI-994 and have determined that the in vivo blood-brain-barrier penetration of CI-994 is not sufficient for PET imaging in non-human primates; however, we are still characterizing its binding properties in other organs. We suspect that faster binding kinetic will be required for in vivo applications and are pursuing other classes of HDAC inhibitors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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24. Miller RH. Unwrapping HDAC1 and HDAC2 function: regulation of gene expression in physiological and pathological brain processes. Physiol Rev 2011; 91:603-49; PMID:21527735; http://dx.doi.org/10.1152/physrev.00012.2010


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