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Mercaptoacetamide-based class II HDAC inhibitor lowers A β levels and improves learning and memory in a mouse model of Alzheimer's disease

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Abstract

Histone deacetylase inhibitors (HDACIs) alter gene expression epigenetically by interfering with the normal functions of HDAC. Given their ability to decrease A β levels, HDACIs are a potential treatment for Alzheimer's disease (AD). However, it is unclear how HDACIs alter A β levels. We developed two novel HDAC inhibitors with improved pharmacological properties, such as a longer half-life and greater penetration of the blood-brain barrier: mercaptoacetamide-based class II HDACI (coded as W2) and hydroxamide-based class I and II HDACI (coded as I2) and investigated how they affect A β levels and cognition. HDACI W2 decreased A β 40 and A β 42 *in vitro*. HDACI I2 also decreased A β 40, but not A β 42. We systematically examined the molecular mechanisms by which HDACIs W2 and I2 can decrease A β levels. HDACI W2 decreased gene expression of γ -secretase components and increased the A β degradation enzyme Mmp2. Similarly, HDACI I2 decreased expression of β - and γ -secretase components and increased mRNA levels of A β degradation enzymes. HDACI W2 also significantly decreased A β levels and rescued learning and memory deficits in aged hAPP 3x Tg AD mice. Furthermore, we found that the novel HDACI W2 decreased tau

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phosphorylation at Thr181, an effect previously unknown for HDACIs. Collectively, these data suggest that class II HDACIs may serve as a novel therapeutic strategy for AD.

Keywords

Amyloid- β ; Alzheimer's disease; Amyloid Precursor Protein; Epigenetics; Histone deacetylase inhibitors

Introduction

While significant progress has been made in understanding Alzheimer's disease (AD) pathology, treatment options for AD still remain limited. Two major pathological hallmarks of AD include aggregated amyloid peptides (A β) and neurofibrillary tangles (Golde, et al., 2010). A β peptides are neurotoxic products released following the cleavage of amyloid precursor protein (APP) by β - and γ -secretase cleavage (De Strooper, et al., 2010). Abnormally hyperphosphorylated tau causes disruption of microtubules and results in the progressive neurodegeneration characteristic of AD (Iqbal and Grundke-Iqbal, 2005). Since A β and tau pathologies are known to act synergistically, a drug that targets both pathologies may be more effective in treating AD (Golde, et al., 2010).

Inhibitors of HDAC (HDACI) have been identified as therapeutic drugs in cancer due to their role in chromatin remodeling (Kazantsev and Thompson, 2008; Marks and Dokmanovic, 2005). Interestingly, several recent studies reported that the commercially available HDACIs alter A β levels by an unknown mechanism (Kilgore et al., 2010; Ricobaraza et al., 2011). In the present study, we developed two novel HDACIs (W2 and I2) with improved solubility, permeability and plasma stability, as compared to other commercially available HDACIs (Konsoula and Jung, 2008). Here, we found that W2 and I2 reduced A β levels and further investigated the molecular mechanisms by which this occurs. Interestingly, HDACIs W2 and I2 regulate cell surface levels of APP and modulate the levels of A β synthesis and A β degradation enzymes. Moreover, W2-treated AD mice had decreased brain A β levels, decreased tau phosphorylation, and improved learning and memory. These results strongly suggest that Class II HDACIs are novel agents that may be effective as AD therapeutics.

Material and Methods

Mice

The 3xTg AD mice were originally generated by co-microinjection of human APP (K670M/N671L) and tau (P301L) transgenes under the control of the Thy 1.2 promoter into mutant PS-1 (M146V) knock-in mice (Oddo, et al., 2003; Oddo, et al., 2006). All animal experiments were approved by the Institutional Animal Care and Use Committees at each institute.

Brain extract preparation

Homogenization of brains from 3xTg AD mice were performed in 50 mM Tris-HCl buffer (pH 7.6) supplemented with 250 mM sucrose and protease inhibitor cocktail (Sigma, St. Louis, MO). Soluble and insoluble A β was extracted in 0.4% DEA and FA (formic acid), as described (Nishitomi et al., 2006). Crude brain homogenate (10%) was mixed with 0.4% DEA, sonicated, and ultracentrifuged 1 hour (100,000g). Supernatant was neutralized with 10% 0.5 M Tris base (pH 6.8). Next, 200 μ L formic acid was added to the pellet of the DEA fractionation. This mixture was sonicated, centrifuged, and 105 μ L of this solution was added to 2 mL of the FA neutralization solution (1M Tris-HCl, 0.5M Na₂HPO₄, 0.05% NaN₃). The DEA fraction was

used to measure soluble A β , and the FA fraction was used to measure insoluble A β using an A β ELISA.

Immunoblotting

To measure the levels of sAPP α and sAPP β , DEA fractionations from 3xTg AD mice were conducted as described above. After DEA fractionation, 20 μ g of protein was separated by Tris-glycine polyacrylamide gel electrophoresis under denatured and reduced conditions. Proteins were then transferred onto PVDF membranes at 130 mA for 1 hour, and blocked with 5% nonfat dry milk. Membranes were then probed with antibodies directed toward sAPP α (2B3 antibody, IBL Co.) and sAPP β (sAPP β Swedish antibody, IBL Co.).

A β ELISA

To examine the effect of HDACIs on A β levels, a hAPP overexpressing N2a stable cell line (N2a-APP) was treated with HDACI I2, W2, or control (10% DMSO) for 24 hours. After 24 hours of incubation, conditioned medium was collected and human A β 40 and A β 42 levels were measured by A β ELISA (Invitrogen, Carlsbad, CA). Additionally, endogenous full-length rodent A β 40 and A β 42 levels from the media from primary cortical neurons were quantified with sandwich ELISA as previously described (Nishitomi et al., 2006). A 96-well plate (Maxisorp) was coated with an anti-A β 40 antibody (clone 1A10) overnight at 4°C. Standards (synthetic mouse A β peptide 1-40) and samples were loaded and incubated overnight at 4°C after blocking for 2 hours. The plate was visualized using a 3,3',5,5'-tetra methyl benzidine (TMB) substrate after HRP-coupled detection antibody (14F1) incubation (Immuno-Biological Laboratories, IBL). To examine the effect of HDACIs *in vivo*, 3x Tg AD mice were injected with HDACI or control for 2 or 4 weeks. After 2 or 4 weeks, we conducted DEA or FA fractionations and measured soluble or insoluble A β (Invitrogen, Carlsbad, CA).

Primary neuron culture and transfection

Primary hippocampal and cortical neurons were prepared from embryonic day (E) 18–19 Sprague-Dawley rat embryos dissociated with trypsin. Neurons were placed onto 12 well plates or 18 mm poly-L-lysine (1mg/ml in 0.1 M borate buffer, pH 8.5) coated coverslips at a density of ~ 750 cells mm^{-2} for hippocampal neurons and ~ 150 cells mm^{-2} for primary cortical neurons. Primary hippocampal and cortical neuronal cultures were grown in Neurobasal medium (Gibco BRL) supplemented with B27 (Gibco BRL), 0.5 mM glutamine, and 12.5 μ M glutamate. Primary hippocampal neurons were transfected with GFP and APP at 18 or 20 DIV using Lipofectamine 2000. Two to three days post-transfection, live cell surface staining was performed.

Live cell surface staining

To measure the cell surface levels of APP, primary hippocampal neurons were transfected with GFP and APP at 18–20 DIV and treated with control or W2 and conducted cell surface live staining as described previously (Hoe, et al., 2009). After transfection and treatment, primary hippocampal neurons were incubated with anti-APP (Sigma-Aldrich) for 10 minutes, which recognizes the N-terminal of APP (10 μ g/mL in conditioned medium). Neurons were then lightly fixed in 4% paraformaldehyde in non-permeabilizing conditions. After fixation, cells were incubated with Alexa Fluor 555-conjugated anti-rabbit secondary antibodies for 2 hours. Three to four dendrites per neuron were quantified using Metamorph analysis software with a Zeiss LSM510 confocal microscope.

Quantitative Real-time PCR (qPCR)

Total RNAs were extracted from rat primary neuronal cells using RNazol® RT (Molecular Research Center, Cincinnati, OH) with Polyacryl carrier (Molecular Research Center). RNAs were reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). qPCR was performed with Power SYBR® Green PCR Master Mix (Applied Biosystems) in ABI 7500 and ABI 7900HT instrument (Applied Biosystems) using the default thermal cycling program with the following primers: glyceraldehyde-3-phosphate dehydrogenase (Gapdh) forward primer: AGGTCGGTGTGAACGGATTTG and reverse primer: TGTAGACCATGTAGTTGAGGTCA; ADAM metalloproteinase domain 10 (Adam10) forward primer: TTAAAGACCGAATCCTGCCG and reverse primer: CCTTCCTCTACTCC-AGTCATTTG; ADAM metalloproteinase domain 17 (Adam17) forward primer: CGGGTACAGGACGT-AATTGAG and reverse primer: GTGGACAAGAATGCTGAAAGG; beta-site APP-cleaving enzyme 1 (Bace1) forward primer: CAGTGGGACCAACCTTC and reverse primer: GCTGCCTTGATGGA-CTTGAC; presenilin 1 (Psen1) forward primer: TGCACCTTTGCTCTACTTCC-A and reverse primer: GCTCAGGGTTGTCAAGTCTCTG; presenilin 2 (Psen2) forward primer: GAGCTGACCCTCAAGTA-TGG and reverse primer: GTGAAGGGCGTGTAGATGAG; nicastrin (Ncstn) forward primer: TGAATGGGTATTGACGGATGG and reverse primer: TGAATTGGGCTTGGCTAGAG; anterior pharynx defective 1b (Aph1b) forward primer: CTGTATCCAAGAGCTGTTTCAGG and reverse primer: CATGATTCCAAAGCCCAAGC; presenilin enhancer 2 (Pen2) forward primer: TGTCATTTGGGTCT-TGCTCTG and reverse primer: CAACCAAAGAAAAGGCAGAAATG; Neprilysin (Nep) forward primer: AGAAGCTCCGAGAAAAGGTG and reverse primer: AATGAGTTGGACTGCCGAG; Endothelin converting enzyme 1 (Ece1) forward primer: TTTTATACCCGCTCTTCGCC and reverse primer: GTTCCCATCCTTGTCATACTCC; Matrix metalloproteinase 2 (Mmp2) forward primer: GCTGATACTGACACTGGTACTG and reverse primer: CACTGTCCGCCAAATAAACC; cathepsin B (Ctsb) forward primer: GGCTACTCCACATCCTACAAG and reverse primer: ACACAGTAAAAGCA-CCCTCC; cathepsin D (Ctsd) forward primer: TCCTGGGCGATGTCTTTATTG and reverse primer: GGCTTTCTCTACTGGACTCTG; cystatin C (Cst3) forward primer: CTAAGTGTCTTTCCACGACC and reverse primer: ATTGGCATGGTCCTATGAGAC. Rat Gapdh control was used as a normalization reference. To confirm the specificity of qPCR reactions, dissociation curves were analyzed at the end of qPCR assays. ABI7500 Software (Version 2.0.5) and 7900HT sequence Detection Systems (Version 2.4) was used to obtain Ct values. To eliminate a potential issue with cross-plate normalization, relative gene expression levels were compared between HDACi and control groups within the same PCR plate. Relative mRNA levels, heat map and Principal component analysis were analyzed with GenEx 5.3.2 (Multid analyses, Göteborg, Sweden) and Partek® Genomics Suite™ (Partek, St. Louis MO).

Morris Water Maze

To examine the effects of W2 on cognitive performance, we injected 2-3 month old wild-type mice, and 9-10 month old hAPP 3x Tg AD mice, with W2 daily (i.p.) for 4 weeks, then began behavior testing as described previously (Minami, et al., 2011). Briefly, spatial learning and memory were assessed by measuring the amount of time required for animals to find a Plexiglas platform hidden beneath opaque water in a large circular pool. During training, the platform was hidden 14 inches from the wall in one quadrant of the maze. The animals were gently placed randomly into one of the four quadrants (separated by 90 degrees) facing the wall. The time required (latency, limited to 90 seconds) to locate the hidden platform was tracked via TOPSCAN software. Animals failing to locate the platform within 90 seconds were guided to reach the platform. Animals were allowed to remain on the platform for 15 seconds on the first trial, and 10 seconds on subsequent trials. A probe trial of 90 seconds was given 24 hours after

the final learning trial. The percentage of time spent in the quadrant where the platform was previously located was recorded. Animals were required to locate a clearly visible black platform (placed in a different location) raised above the water surface at least 12 hours after the last trial.

Statistical analyses

For analyses of biochemical assays in N2a cells, primary cortical neurons, and primary hippocampal neurons, all data were analyzed using either a 2-tailed t-test or 1-way ANOVA with Tukey's Multiple Comparison test for post-hoc analyses (Graphpad Prism 4 software, GraphPad, La Jolla, CA). Significance was determined as $p < 0.05$. To analyze the Morris Water Maze escape latencies during the training phase, we used 2-way ANOVA with Tukey's Multiple Comparison test for post-hoc analyses. Descriptive statistics were calculated with StatView 4.1 and expressed as mean $\pm$ SEM.

Results

Class II HDACIs W2 and I2 decrease A β levels in vitro

The accumulation and aggregation of the A β cleavage products of amyloid precursor protein (APP) is causally linked to AD (De Strooper, et al., 2010). Several recent studies have shown that HDACIs, including suberoylanilide hydroxamic acid (SAHA) and phenylbutyrate (PBA), decrease A β levels and improve cognitive performance (Govindarajan, et al., 2011, Ricobaraza, et al., 2011, Ricobaraza, et al., 2010). However, these commercially available HDACIs have poor pharmacological properties. Thus, we developed two novel HDAC inhibitors, mercaptoacetamide-based Class II HDACI W2 and hydroxamide-based Class I and II HDACI I2, with improved blood-brain barrier penetration, physicochemical, and pharmacological properties (Tables 1 and 2; Konsoula and Jung, 2008, Konsoula, et al., 2009, Konsoula, et al., 2011).

To examine whether our novel class II HDACI W2 and class I and II HDACI I2 can alter A β levels, a stable cell line of N2a cells overexpressing hAPP (N2a-APP) were treated with control (10% DMSO), W2 (1 or 5 μ M), or I2 (1 or 5 μ M) for 24 hrs and human A β 40 and A β 42 levels were measured. HDACI W2 treatment resulted in a 20% decrease in human A β 40 and A β 42 levels (Figs. 1A,B; $n=6$). Interestingly, 5 μ M I2 significantly decreased human A β 40 levels in N2a-APP cells; however, there were no changes in human A β 42 levels compared to control (Figs. 1C,D; $n=6$). Additionally, we examined rodent A β levels in primary cortical neurons following HDACI treatment, and found that HDACI W2 or I2 significantly decreased rodent A β 40 (by 20%, Figs. 1E, F; $n=6-8$).

APP trafficking is an important pathway in modulating A β production. α -secretase cleavage occurs primarily at the cell surface, while β - or γ -secretase cleavage predominates at the endosomal membrane (De Strooper, et al., 2010). Thus, APP located at the cell surface is more likely to undergo α -secretase cleavage to produce sAPP α , thereby reducing A β production.

To examine whether our novel HDACIs can alter cell surface levels of APP, N2a-APP cells were treated with 5 μ M HDACIs (W2 or I2) or control for 2 hrs or 24 hrs. Cell surface proteins were biotinylated, isolated with avidin beads, and immunoblotted for APP. Quantification of immunoblot results demonstrated that W2 (5 μ M) significantly increased cell surface APP within 2 hrs ($n=3$; by 33 %, $*P < 0.05$, Figs. 2A, B). However, I2 (5 μ M) did not alter cell surface APP compared to control at 2 hrs of treatment (Figs. 2A, B). After 24 hrs of treatment, both W2 and I2 (5 μ M) significantly increased cell surface APP by two fold ($n=3$; $**P < 0.01$) (Figs. 2C, D).

Since we observed a significant effect on cell surface levels of APP by treating with W2 in N2a-APP cells, we further investigated the effect of W2 on APP trafficking in primary neurons. For this experiment, primary cortical neurons were treated with W2 (5 μ M) or control for 24 hrs and the cell surface level of APP was measured using a cell surface biotinylation assay, as described above. We observed a similar increase in surface levels of APP with HDACI W2 (n=3, Figs. 2E, F).

To verify the effects of HDACI W2 on cell surface of APP, we conducted live cell surface staining. Primary hippocampal neurons were transfected with GFP and APP and treated with W2 (1 or 5 μ M) or control for 24 hrs. 1 μ M or 5 μ M W2 significantly increased cell surface APP by two fold (n=10; ***P<0.001), compared to control (Figs. 2G, H). Taken together, these data suggest that our novel HDACI affects A β production by modulating APP trafficking.

Transcriptional regulation of A β synthesis and degradation enzymes by HDACIs W2 and I2

While it is known that some commercially available HDACIs can decrease A β levels, the mechanism underlying this beneficial effect is unknown. Thus, to understand the molecular mechanisms by which our novel HDACIs W2 and I2 decrease A β levels, we examined the expression of genes involved in A β synthesis and degradation. Primary cortical neurons were treated with 5 μ M HDACIs (W2 or I2) or control for 24hrs. Gene expression levels were measured by qPCR. In order to efficiently visualize the complex gene expression profiles, we reduced the dimension of the data set using the principal component analysis (PCA) method and analyzed data with heat map clustering. Interestingly, we found that most genes involved in A β synthesis were down-regulated while genes related to A β degradation were up-regulated in HDACI W2 or I2 treated groups (Fig. 3A). According to PCA-based classification, gene expression profile was grouped into four major categories (Fig. 3B). Psen2 and Nicastrin were commonly down-regulated by both W2 and I2. In addition, several A β degrading enzymes were up-regulated by W2 and I2, with the exception of Neprilysin, which was only increased by I2. Further experiments demonstrated that W2 significantly decreased mRNA levels of γ -secretase components (Psen1, Psen2, and Nicastrin), while I2 significantly repressed expression of β - (BACE1) and γ -secretase components (Nicastrin and Psen2) (Figs. 3C, D). Additionally, we observed that HDACI W2 treatment significantly increased Mmp2 expression, while HDACI I2 increased Nep, Ece1, Mmp2, Ctsd and Ctsb expression (Figs. 3E, F). Taken together, our data suggest that our novel compounds have dual effects of down-regulating A β production pathways while simultaneously up-regulating A β clearance mechanisms.

Class II HDACI W2 decreases A β levels in hAPP 3xTg AD mice

Since we observed a greater effect on A β levels by treating with HDACI W2 compared to I2 *in vitro*, we continued to examine the effect of HDACI W2 on A β production *in vivo*. We injected 9-10 month old hAPP 3x Tg AD mice with 50 mg/kg W2 or control (daily i.p. for 2 weeks). After 2 weeks, mouse hemi-brains were dissected and subjected to DEA extraction to measure soluble A β levels. HDACI W2 treatment significantly decreased soluble A β 40 levels compared to control-injected mice, but there was no change in soluble A β 42 levels (Figs. 4A, B, n=10/group). We then examined whether W2 can alter A β levels with longer treatment duration. For these experiments, we injected 9-10 month old hAPP 3x Tg AD mice with W2 or control (daily i.p. for 4 weeks). HDACI W2 treatment dramatically decreased soluble and insoluble A β 40 and A β 42 levels, compared to control-injected mice after daily 4 week treatments (Figs. 4C, D, n=10/group, data shown as percentage of soluble A β levels in control injected mice).

Next, we examined whether our novel class II HDACI W2 regulates APP processing *in vivo* by injecting 50 mg/kg W2 or control in hAPP 3x Tg AD mice (daily i.p. for 4 weeks). After 4

weeks, we measured the levels of sAPP α , an α -secretase cleavage product, and sAPP β , a β -secretase cleavage product. HDACI W2 treatment significantly increased sAPP α and significantly decreased sAPP β , compared to the control group (Figs. 4E, F, n=3/group).

Class II HDACI W2 decreases tau phosphorylation at Thr181 in hAPP 3xTg AD mice

In addition to A β , tau plays critical role in AD pathogenesis. However, it is unknown whether HDACIs can alter tau phosphorylation. Thus, to examine the effect of our novel HDACI W2 on tau phosphorylation, we injected hAPP 3x Tg AD mice for 4 weeks with W2 (50 mg/kg, daily i.p.) or control (10% DMSO). After 4 weeks, the hemi-brains of these mice were isolated and immunoblots were performed with antibodies directed toward P-CDK5, CDK5, P-GSK3 β (Y216), and GSK3 β . We found that W2 did not change levels of these tau kinases (Figs. 5A-C, n=4). We then asked whether HDACI W2 could alter tau phosphorylation. To test this, we similarly immunoblotted brain lysates with the anti-phospho-tau antibodies AT270 and AT180, which recognize the phosphorylated residues of Thr181 and Thr231 of tau, respectively. Additionally, we performed immunoblots with AT8 (Ser202/Thr205), p-tau (Ser262), p-tau (Ser356), and anti-5E2 (total tau). Interestingly, HDACI W2 specifically reduced phosphorylation of tau at the Thr181 site (Figs. 5D-F, n=4). However, W2 did not alter tau phosphorylation at the Thr231 site, other serine residues, or the total level of tau (Figs. 5D-F). Quantification of these blots revealed that the HDACI W2 induced an approximately 50% decrease in levels of the Thr181 phospho-tau protein (Fig. 5F, n=4/group). Collectively, these data suggest that our novel HDACI W2 not only affected A β levels, but also modulated tau phosphorylation at Thr181 *in vivo*.

Class II HDACI W2 improves learning and memory in hAPP 3x Tg AD mice

Based on our data demonstrating that W2 decreases A β levels *in vitro* and *in vivo*, we further examined whether W2 could affect cognitive performance using the Morris Water Maze. We initially injected 2-3 month old wild-type mice for 4 weeks with W2 (50 mg/kg, daily i.p.) or control (10% DMSO), and found that W2 treated wild-type mice had improved learning and memory as evidenced by increased time spent in the target quadrant and number of platform crossings without altering swim speed (Figs. 6A-D, n=12/group).

We then examined whether class II HDACI W2 can rescue the learning and memory deficits seen in a mouse model of AD. To test this, we injected 9-10 month old hAPP 3x Tg AD mice (with mild cognitive impairment) for 4 weeks with W2 (50 mg/kg, daily i.p.) or control. We found that W2 treatment improved learning and memory in 9-10 month old hAPP 3x Tg AD mice as evidenced by increased number of platform crossings without altering swim speed (Figs. 6E-H, n=12/group). This data suggests that our novel HDACI W2 can rescue the memory deficits exhibited in aged hAPP 3x Tg AD mice.

Discussion

Reduced histone acetylation has been implicated in the pathogenesis of several neurodegenerative diseases, and in particular, previous studies have assessed the effects of HDAC inhibitors on A β levels in several mouse models of AD (Cha, et al., 2001, Robakis, 2003, Yacoubian, et al., 2008, Kozikowski, et al., 2009, Lockett, et al., 2010). Our study examines the effects of novel Class II HDACI W2 on A β production, tau phosphorylation, and cognitive performance in mouse models of AD.

A previous study demonstrated that HDACIs decreased A β levels and increased levels of sAPP α , the cleavage product of α -secretase, in primary cortical neurons (Kozikowski, et al., 2009). Chronic administration of sodium 4-phenylbutyrate (4-PBA), a known HDACI, decreased A β levels in hAPP Tg AD mice (Ricobaraza, et al., 2011). Valproic acid, another

known HDACI, was shown to significantly reduce A β plaque number in the APP23 transgenic mouse model of AD (Qing, et al., 2008). However, these studies provide limited insight into the underlying molecular mechanism of HDAC inhibitors on A β levels and cognitive performance (Kilgore et al., 2010; Ricobaraza et al., 2011; Qing et al., 2008).

Therefore, we developed two novel HDAC inhibitors, mercaptoacetamide-based Class II HDACI W2 and hydroxamide-based Class I and II HDACI I2, with improved physicochemical, and pharmacological properties compared to commercially available HDACIs, such as SAHA (Konsoula and Jung, 2008, Konsoula, et al., 2009, Konsoula, et al., 2011). In our initial study of these two novel HDACIs, Class II W2 and Class I and II I2, we found that the Class II HDACI W2 was more effective in reducing A β levels *in vitro*. Therefore, we selected Class II HDACI W2 for further examination regarding its effects on A β levels *in vivo*. Class II HDACI W2 dramatically decreased soluble and insoluble A β levels in hAPP 3xTg AD mice. These findings led us to investigate the mechanisms by which HDACIs are able to regulate A β levels.

Given the critical role that HDACs play in regulating gene expression, we determined the expression of genes implicated in A β synthesis and degradation in the presence or absence of our two novel HDAC inhibitors. Interestingly, several genes facilitating A β production were repressed, whereas a number of A β degradation enzymes were significantly up-regulated. The two novel HDACIs, Class II W2 and Class I and II I2, may have better therapeutic potential compared to other HDACIs, due to their ability to modulate both the production and the degradation of A β . We hypothesize that our novel HDACIs affect A β -regulatory enzymes via induction of transcription and/or alteration of mRNA stability. One potential mechanism of HDACI-mediated regulation of mRNA is via the inhibition of HDAC interaction with various binding partners involved in mRNA metabolism. One such binding partner is heterogeneous nuclear ribonucleoprotein H (hnRNP H), which forms complexes with RNA polymerase II transcripts to provide the substrate necessary for pre-mRNA processing (Krecic and Swanson, 1999). hnRNP H regulates alternate splicing of BACE1, and knockdown of hnRNP H decreases the production of functional BACE1, leading to decreased A β in transfected HEK 293 cells (Fisette, et al., 2012).

HDACIs can also confer neuroprotection via the regulation of microtubule stability, which is dependent on post-translational modifications such as acetylation of α -tubulin. Neurons in the AD brain exhibit lower levels of acetylated α -tubulin, suggesting a link between the neurofibrillary tangles characteristic of AD pathology (caused by hyperphosphorylated tau) and the loss of microtubule stability. Interestingly, the HDACI nicotinamide increases the level of acetylated α -tubulin, and selectively decreases Thr231-phosphotau in 3x-Tg AD mice (Green, et al., 2008, Hempen and Brion, 1996). It is possible that our novel HDACIs, W2 and I2, function in a similar manner.

In addition to these *in vitro* studies, several *in vivo* findings strongly suggest that HDACIs may be a valuable treatment option for learning and memory deficits associated with neurodegenerative diseases, including AD (Fischer, et al., 2010, Kazantsev and Thompson, 2008). Here, we found that our novel HDACI W2 enhanced cognitive performance in wild-type mice and rescued cognitive decline in 9-10 month old 3x Tg AD mice. We are currently investigating the lowest optimal dose of HDACIs as well as any additional molecular mechanisms by which HDAC inhibitors affect cognitive performance, including the transcriptional regulation of memory-associated genes. These ongoing efforts may lead to an effective therapeutic tool to combat neurodegenerative disorders such as AD.

Conclusions

Our study demonstrates that the novel Class II HDACI W2 decreases A β levels and improves learning and memory. HDAC inhibition decreased expression of genes involved in A β production and induced expression of A β degradation enzymes. In addition, Class II HDACI W2 selectively decreases tau phosphorylation. Taken together, these data demonstrate that our novel compound Class II HDACI W2 may have the potential to alleviate critical neuropathogenic processes in dementias, including AD, providing broad-spectrum therapeutic benefits.

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Abbreviations

Aβ	amyloid-beta
AD	Alzheimer's disease
HDACI	histone deacetylase inhibitors
APP	amyloid precursor protein

Highlights

- We developed novel HDACIs with improved pharmacological properties.
- Our HDACIs decreased A β levels by altering A β synthesis and degradation enzymes.
- Our novel HDACIs improved learning and memory in the Morris Water Maze.
- Our novel HDACIs selectively decreased tau phosphorylation.

Fig. 1. HDACIs decrease Aβ levels in N2a-APP cells and primary neurons. (A, B) N2a-APP cells were treated with class II HDACI W2 (1 μM or 5 μM) or control (10% DMSO) for 24 hrs, and human Aβ40 (A) and Aβ42 (B) levels in the conditioned media were determined by human Aβ ELISA (n=6). HDACI W2 resulted in 20% decrease in Aβ40 and Aβ42 in N2a-APP cells. (C, D) N2a-APP cells were treated with class I and II HDACI I2 (1 μM or 5 μM) or vehicle control for 24 hrs, and human Aβ40 (C) and Aβ42 (D) levels were measured (n=6). (E, F) Primary cortical neuronal cells were treated with class II HDACI W2 (E), class I and II HDACI I2 (F), or control (10% DMSO) for 24 hrs, and rodent Aβ40 levels in the conditioned media were determined by rodent Aβ ELISA (n=6-8). HDACI W2 and HDACI I2 resulted in 20% decrease in Aβ40 in primary cortical neurons.

Fig. 2.

Class II HDACI W2 increases cell surface levels of APP in N2a-APP cells and primary neurons. (A-D) N2a-APP cells were treated with I2 (5 μ M), W2 (5 μ M), or vehicle control for the indicated times (2 hrs (A-B), 24 hrs (C-D)). Cell surface proteins were biotinylated, isolated with avidin-conjugated beads, and immunoblotted with 6E10 antibody against N-terminal of APP (upper panel, n=3). Total APP levels were measured in cell lysates with anti-c1/6.1 (lower panel). (E-F) Cultured cortical neurons were treated with W2 (5 μ M) or vehicle control for 24 hrs and cell surface APP was detected with anti-22C11 (n=3). (G) Primary hippocampal neurons were transfected with GFP and APP and treated with W2 (1 μ M or 5 μ M) or control for 24 hrs. Cell surface APP intensity was measured using Metamorph (n=10). (H) Quantification of cell surface APP intensity showed that 1 μ M or 5 μ M W2 increased cell surface APP compared to control.

Fig. 3.

Transcriptional regulation of genes involved in A β synthesis and degradation by HDACs W2 and I2. (A) Expression levels of genes involved in A β metabolism were measured using q-PCR. Relative expression levels were analyzed by an unsupervised hierarchical clustering method. Each cell indicates log₂ fold change in the relative mRNA levels compared to the DMSO control group. Red cells indicate an up-regulation of gene expression and blue cells indicate a down-regulation of gene expression. (B) Score plot of PCA classification using log₂ of qPCR relative quantification values. (C-F) Analyses of genes involved in A β synthesis and degradation. mRNA levels of genes associated with A β synthesis (W2: Psen1, Ncstn and Psen2; I2: Bace1, Ncstn and Psen2) were significantly decreased (n=4, * p <0.05, *** p <0.001). In contrast, mRNAs associated with A β degradation (W2: Mmp2, I2: Nep, Ece1, Mmp2, Ctsc and Ctsb) were significantly increased (n=4, * p <0.05, ** p <0.01, *** p <0.001). Data are presented as mean $\pm$ SEM.

Fig. 4.

Class II HDACI W2 decreases $A\beta$ levels in 9-10 month old hAPP 3x Tg AD mice. (A, B) $A\beta$ 40 (A) and $A\beta$ 42 (B) levels were measured in hAPP 3x Tg AD mice injected with W2 (50 mg/kg) or control (n=10) for 2 weeks. (C, D) Soluble and insoluble $A\beta$ 40 (C) and $A\beta$ 42 (D) levels were measured in hAPP 3x Tg AD mice injected with control (n=10) or W2 (50 mg/kg) for 4 weeks. Levels are presented as a percentage of soluble $A\beta$ levels in control injected mice (E) hAPP 3x Tg AD mice were injected with W2 (50 mg/kg) or control daily for 4 weeks. After 4 weeks, sAPP α , sAPP β , and β -actin were measured. (F) Quantification of data shows that W2 significantly increased sAPP α (0.5 fold 58%, n=3, *P<0.05) and significantly decreased sAPP β (0.6 fold, n=3, **P<0.01) in hAPP 3x Tg AD mice.

Fig. 5.

Class II HDACI W2 decreases tau phosphorylation at Thr181 in 9-10 month old hAPP 3x Tg AD mice. (A, B) Relative levels of p-CDK5 and p-GSK3 β were determined in hAPP 3x Tg AD mice injected with W2 (50 mg/kg) or control for 4 weeks by immunoblotting with the indicated antibodies: p-CDK5, CDK5, p-GSK3 β , and GSK3 β . (C) Quantification of immunoblots revealed that W2 did not alter the levels of p-CDK5 and p-GSK3 β ($n=4$). (D, E) Relative levels of tau epitopes were determined in hAPP 3x Tg AD mice injected with W2 (50 mg/kg) or control for 4 weeks by immunoblotting with the indicated antibodies: AT270, AT8, AT180, p-tau (pS262), and p-tau (pS356) to measure phospho-tau epitopes; and anti-5E2, a measure of total Tau ($n=4$). (F) Quantification of immunoblots revealed that W2 significantly decreased tau phosphorylation at Thr181 ($n=4$, $*p < 0.05$).

Wild-Type mice

hAPP 3xTg AD mice

Fig. 6.

HDACI W2 rescues learning and memory in 9-10 month old hAPP 3xTg AD mice. Spatial learning was assessed by the Morris Water Maze paradigm. (A-D) 2-3 month old wild-type mice. (E-H) 9-10 month old hAPP 3x Tg AD mice. (A, E) Escape latencies during the training phase for wild-type or hAPP 3x Tg AD mice are shown. (In each panel, the lower curve shows the behavior of W2-treated mice, $n=12$, $*p<0.05$.) (B, F) Time spent in the target quadrant was measured during the probe test on day 5. (C, G) After the platform had been removed during the probe test on day 5, the number of times each mouse swam over the target

site was recorded (n=12, * $p<0.05$). (D, H) The swim speeds of control and W2 treated mice were not significantly different from one another in WT or hAPP 3xTg AD mice.

HDAC isoform inhibition activity *in vitro* using recombinant proteins. HDAC4 and HDAC9 are known to be inactive. Therefore, data obtained using these proteins are not included. Additionally, commercially available HD5* is not full-length. HD and HDAC refer to histone deacetylase. The partition coefficient (CLogP) refers to the calculated lipophilicity of a compound.

Table 1

Compound	CLogP	IC50 Pan [μ M]	IC50 [μ M] HDAC isomer								
			HD1	HD2	HD3	HD5*	HD6	HD7	HD8	MD10	HD11
W2	2.43	0.04	3.22	1.25	1.32	0.89	0.021	2.78	6.12	10.65	0.61
I2	1.4	0.18	0.58	2.58	1.56	0.31	0.073	1.29	6.8	0.27	0.19

Table 2

Plasma pharmacokinetic parameters after i.p. administration of I2 and W2 (400 mg/Kg) in nude athymic mice. Pharmacokinetic data demonstrated that I2 reached peak plasma concentrations within 2 h, and exhibited a half life of 11.17 ± 0.87 h, maximum plasma concentration ($C_{\max}$) of 6.88 ± 0.71 μM , and area under the curve of 8.08 ± 0.91 $\mu\text{M} \times \text{h}$. $C_{\max}$, the maximum plasma concentration; $t_{\max}$, the time to reach maximum plasma concentration; AUC, the area under the compound concentration vs. time curve; $t_{1/2}$, the elimination half-life; CL, the clearance.

PK parameters	Compounds	
	I2	W2
$C_{\max}$ (μM)	6.86 ± 0.71	1.31 ± 0.34
$t_{\max}$ (h)	2	0.5
AUC ($\mu\text{M} \times \text{h}$)	8.08 ± 0.9	4.97 ± 0.6
$t_{1/2}$ (h)	11.17 ± 0.87	2.2 ± 0.33
CL (L/h)		4.05 ± 0.15