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Lysine Deacetylation by HDAC6 Regulates the Kinase Activity of AKT in Human Neural Progenitor Cells

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Abstract

AKT family of serine-threonine kinases functions downstream of phosphatidylinositol 3-kinase (PI3K) to transmit signals by direct phosphorylation of a number of targets, including the mammalian target of rapamycin (mTOR), glycogen synthase kinase 3 β (GSK3 β) and β -catenin. AKT binds to phosphatidylinositol (3,4,5)-triphosphate (PIP₃) generated by PI3K activation, which results in its membrane localization and subsequent activation through phosphorylation by phosphoinositide-dependent protein kinase 1 (PDK1). Together, the PI3K-AKT signaling pathway plays pivotal roles in many cellular systems, including in the central nervous system where it governs both neurodevelopment and neuroplasticity. Recently, lysine residues (Lys¹⁴ and Lys²⁰) on AKT, located within its pleckstrin homology (PH) domain that binds to membrane-bound PIP₃, have been found to be acetylated under certain cellular contexts in various cancer cell lines. These acetylation modifications are removed by the enzymatic action of the class III lysine deacetylases, SIRT1 and SIRT2, of the sirtuin family. The extent to which reversible acetylation regulates AKT function in other cell types remains poorly understood. We report here that AKT kinase activity is modulated by a class IIb lysine deacetylase, histone deacetylase 6 (HDAC6), in human neural progenitor cells (NPCs). We find HDAC6 and AKT physically interact with each other in the neuronal cells, and, in the presence of selective HDAC6 inhibition, AKT is acetylated at Lys¹⁶³ and Lys³⁷⁷ located in the kinase domain, two novel sites distinct from the acetylation sites in the

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This paper is dedicated to Prof. Stuart L. Schreiber on the occasion of his 60th birthday.

PH-domain modulated by the sirtuins. Measurement of the functional effect of HDAC6 inhibition on AKT revealed decreased binding to PIP₃, a correlated decrease in AKT kinase activity, decreased phosphorylation of Ser⁵⁵² on β -catenin, and modulation of neuronal differentiation trajectories. Taken together, our studies implicate the deacetylase activity of HDAC6 as a novel regulator of AKT signaling and point to novel mechanisms for regulating AKT activity with small-molecule inhibitors of HDAC6 currently under clinical development.

Acetylation and deacetylation of the ϵ -amino group on lysine residues modulate the functioning of various proteins^{1, 2}. Initial studies of such posttranslational modifications focused on acetylation of histone proteins and on the role of HDACs and HATs (histone acetyltransferases) in regulating chromatin function^{3, 4}. The role of reversible acetylation in the function of non-histone proteins is increasingly recognized and studied^{1, 5–10}. One such instance is the acetylation of lysine residues on AKT.

AKT is a serine/threonine protein kinase that plays a pivotal role in many cellular processes, including important regulatory functions in the central nervous system¹¹. AKT has been implicated in the disease biology of neuropsychiatric disorders and has been shown to be involved in mediating the effects of various psychotropic agents^{12–14}. AKT functions downstream of the PI3K/insulin signaling pathway¹⁵. When activated, PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce PIP₃, which resides in the plasma membrane. Since AKT binds to PIP₃ through its pleckstrin homology (PH) domain, increased PIP₃ results in membrane localization of AKT where it is phosphorylated by PDK1¹⁶. AKT phosphorylates a number of downstream targets, including the two N-terminal serine residues Ser⁹ and Ser²¹ on GSK3 β and GSK3 α respectively as well as Ser⁵⁵² on β -catenin^{17–19}. AKT has been shown to be acetylated by the HATs p300 and PCAF in tumor cell lines and in tissues lysates from mice. Proteomics studies have revealed that the acetylated residues are Lys¹⁴ and Lys²⁰ are in the pleckstrin homology (PH) domain²⁰. Previous research has implicated the NAD⁺-dependent deacetylases SIRT1 and SIRT2 in the deacetylation of Lys¹⁴ and Lys²⁰ sites on AKT²¹. The first report showed that SIRT1 deacetylated AKT1 in tumor cell lines and in murine tissue, leading to activation of AKT²⁰. A subsequent study in tumor cell lines showed that SIRT2 bound to AKT in insulin-responsive cells and sensitized the cells to insulin²².

Here, we report on studies examining the effect of HDAC inhibitors on AKT in human neural progenitor cells (NPCs) generated from human induced pluripotent stem cells (iPSCs). We tested a set of isoform-specific HDAC inhibitors to identify the HDAC isoform that deacetylates AKT in the human NPCs. We show that only small molecules that inhibit HDAC6 resulted in increased acetylation. We found that the lysine residues Lys¹⁶³ and Lys³⁷⁷ in the kinase domain of AKT are acetylated in the presence of an HDAC6 inhibitor, which are distinct from the PH-domain sites previously reported to be deacetylated by SIRT1 and SIRT2^{20–22}. HDAC6 inhibition resulted in a decreased ability of AKT to bind PIP₃ and was accompanied by decreased ability to phosphorylate downstream targets, even in the presence of Ser⁴⁷³ phosphorylation, which usually potentiates AKT catalytic activity^{23, 24}. Moreover, we found that HDAC6 inhibition resulted in decreased phosphorylation of Ser⁵⁵² on β -catenin, which is phosphorylated by AKT and mediates

subcellular localization of β -catenin¹⁹. Our studies show that AKT is a critical regulator of PI3K-AKT signaling with implications for understanding the pharmacological effects of HDAC6-selective inhibitors currently under clinical development.

RESULTS AND DISCUSSION

Effect of HDAC (lysine deacetylase) inhibitors on AKT acetylation in human neural progenitor cells.

We carried out experiments with lysine deacetylase inhibitors in human iPSC-derived NPCs to study the nature and consequence of AKT acetylation in the human neuronal context for the first time. We treated human NPCs for 24 hours with prototypical small molecules that inhibit target class I HDACs (CI-994), class IIb HDACs (ACY-1215 - Ricolinostat), SIRT1 (EX-527) and SIRT2 (AGK-2) (Fig. 1). We chose these particular HDAC inhibitors since they are highly selective for specific HDAC isoforms²⁵. Whole-cell extracts from the NPCs were immunoprecipitated with an antibody targeting AKT and probed with an antibody that recognized acetyl-lysine (Ac-Lys) residues. The treatments had no effect on overall levels of AKT (Fig. 2A). In the presence of DMSO alone, AKT immunoprecipitated from the NPCs did not have any detectable Ac-Lys. Among the small molecules tested, the only deacetylase inhibitor that showed increased Ac-Lys in immunoprecipitated AKT was ACY-1215 (Fig. 2A), a selective HDAC6 inhibitor²⁶. Consistent with these pharmacological findings and previous studies in mouse fibroblasts²⁷, probing of AKT immunoprecipitates from human NPCs with an anti-HDAC6 antibody demonstrated that HDAC6 and AKT indeed physically associate with each other (Fig. 2B).

Effect of HDAC6 inhibitor on AKT phosphorylation.

Loss of HDAC6 has been reported to increase AKT phosphorylation in mouse embryonic fibroblasts and in HCT-116 tumor cell lines, though it was not clear which sites had increased phosphorylation²⁸. We examined the effect of HDAC6 inhibition on AKT phosphorylation in human NPCs. Treatment with 5 μ M ACY-1215 for 24 hours resulted in increased phosphorylation of both Ser⁴⁷³ and Thr³⁰⁸ on AKT (Fig. 3A).

Functional effects of HDAC6 inhibition-induced AKT acetylation on PIP₃ binding and AKT kinase activity.

We examined whether HDAC6 inhibition affected the ability of AKT to bind to PIP₃. We treated human NPCs with ACY-1215, immunoprecipitated the lysates with PIP₃-beads, and probed the immunoprecipitated samples with antibodies targeting AKT and PDK1. We found that treatment with ACY-1215 resulted in reduced levels of AKT bound to PIP₃, without impacting the levels of PDK1 bound to PIP₃ (Fig. 3B). We next examined if treatment with ACY-1215 affected the kinase activity of AKT, using an assay measuring phosphorylation of AKT target sites Ser⁹ on GSK3 β and Ser²¹ on GSK3 α . We treated human NPCs with ACY-1215 and immunoprecipitated lysates with anti-phos-AKT (Ser⁴⁷³). Treatment with ACY-1215 resulted in increased levels of Ac-AKT in the immunoprecipitated lysates (Fig. 3C). When the immunoprecipitated lysates were tested for AKT kinase activity, we found that ACY-1215 treated lysates resulted in a marked decrease

in the phosphorylation of Ser⁹ on GSK3 β and Ser²¹ on GSK3 α , compared to levels seen with DMSO treated samples (Fig. 3D), indicating that AKT kinase activity was diminished.

Effect of HDAC6 inhibitor on β -catenin phosphorylation.

A physiologically important substrate for AKT in the neuronal context is β -catenin^{29–31}. AKT phosphorylates Ser⁵⁵² on β -catenin, which results in its dissociation from complexes at the plasma membrane and translocation to the nucleus, resulting in increased β -catenin-mediated transcriptional activity¹⁹. In neuronal cells, it has been shown that decreased AKT activation results in reduced phosphorylation of Ser⁵⁵² on β -catenin^{32, 33}. We investigated the effect of HDAC6 inhibition on phosphorylation of this serine residue and found that HDAC6 inhibition resulted in decreased phosphorylation of Ser⁵⁵² on β -catenin (Fig. 3E). This result suggests that HDAC6 inhibition-mediated decrease in AKT kinase activity would promote localization of β -catenin at the plasma membrane, as we have previously reported in human NPCs²⁵.

Association of AKT with acetylated α -tubulin in the presence of HDAC6 inhibition.

To determine the generalizability of our findings to other HDAC inhibitors and rodent neuronal cells, we also studied additional small-molecule inhibitors, including three HDAC6 inhibitors with distinct chemical scaffolds, in murine Neuro2a cells transfected with FLAG-tagged constructs for AKT1. We treated cells with the compounds for 6 hours, lysed the cells and immunoprecipitated AKT1 using anti-FLAG M2 magnetic beads. The collected samples were probed with anti-AKT and anti-Ac-Lys antibodies. We observed that treatment with the HDAC6-selective inhibitors ACY1215, tubastatin A and tubacin resulted in increased detection of an acetylated protein that migrated in the same size range of ~50kD as AKT1 itself acetylation while treatment with CI-994, EX-527 and AGK-2 did not have this effect (Fig. 4A). However, we recognized though that given the nature of the anti-Ac-Lys antibody recognizing multiple epitopes we could not unambiguously determine if the protein detected was acetyl-AKT1 or rather an associated protein that co-migrated at the same size.

To probe this question further, as HDAC6 is known to deacetylate HSP90 and α -tubulin^{5, 34, 35} and AKT is known to be present in macromolecular complexes with HSP90^{36, 37}, with acetylation of lysine residues on α -tubulin and HSP90 known to regulate their binding to AKT and modulate its kinase activity^{36, 37} we sought to determine if the physical association of these proteins with AKT changed upon HDAC6 inhibition. To do so, the same Neuro2a cells were again transfected with FLAG-tagged constructs for AKT1, treated with the HDAC6 inhibitor ACY-1215 or DMSO, lysed, and FLAG-AKT1 immunoprecipitated from the lysate using anti-FLAG M2 magnetic beads. Using fairly stringent wash conditions consisting of RIPA buffer, we found that α -tubulin co-immunoprecipitated with AKT1 with or without HDAC6 inhibition, while HSP90 was present in neither condition demonstrating specificity of the AKT1/ α -tubulin interaction in these cells (Fig. 4B). Strikingly, upon HDAC6 inhibition with ACY-1215, ac- α -tubulin (K40) co-immunoprecipitated with the AKT1 complex (Fig. 4B). Further analysis of these immunoprecipitations with the cocktail of pan-acetyl-Lys antibodies revealed a band co-migrating with the ac- α -tubulin (K40)/tubulin band suggesting that the elevation of acetyl-

Lys we observed noted with AKT immunoprecipitation is likely due to its association with acetyl- α -tubulin (K40) itself rather than directly reporting on AKT acetylation.

Identification of novel lysine residues on AKT in the presence of HDAC6 inhibitor.

Since the immunoprecipitation studies we performed could not definitely determine if AKT was acetylated upon HDAC6 inhibition due to its differential associated we observed of ac- α -tubulin (K40), in order to ascertain whether AKT is acetylated in the presence of HDAC6 inhibition, we undertook mass spectrometry experiments with immunoprecipitated AKT1 from Neuro2a cells that had been treated with 5 μ M ACY-1215. We found that AKT1 was indeed acetylated at two novel lysine residues - Lys¹⁶³ and Lys³⁷⁷ – that are located within the AKT kinase domain and distinct from the lysine residues in the PH domain that are regulated by SIRT1 and SIRT2 (Fig. 5)³⁸.

Effects of HDAC6 inhibition on neuronal differentiation.

Since *in vivo* studies in rodents show that decreasing AKT activation in NPCs during cortical development affects neuronal differentiation^{32, 33}, we examined whether HDAC6 inhibition affected proliferation and differentiation of human NPCs. When NPCs were cultured under proliferation conditions in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), exposure to ACY-1215 did not have any impact on the proliferation rate. AKT is known to play a major role in neuronal differentiation and activated p-AKT(Ser⁴⁷³) is present in cortical neural precursors^{33, 39}. Prior studies in murine cells had shown that broadly selective class I HDAC inhibitors biased NPC differentiation along the neuronal lineage at the expense of glial lineage^{40, 41}. We tested the effects of ACY-1215 during NPC differentiation, in the absence of EGF and bFGF, for six weeks. In the presence of DMSO alone, as expected, the differentiated cultures had greater proportion of neurons positive for β -III-tubulin (TUJ1) when compared to glial cells positive for glial fibrillary acidic protein (GFAP) (Fig. 6). The presence of ACY-1215 in the culture media impacted this ratio dramatically, resulting in a much higher proportion of glial cells compared to neurons (Fig. 6). Our results suggest that HDAC6 inhibition in human NPCs has the opposite effect of class I HDAC inhibitors in promoting differentiation along the glial lineage.

AKT plays important roles in multiple cellular contexts but much remains to be learned about the regulation of AKT in the different cellular contexts, including in the human central nervous system^{17, 42, 43}. Multiple lines of evidence suggest an important role for AKT in the disease biology of neuropsychiatric disorders such as autism, schizophrenia and bipolar disorder^{44–47}. The PI3K-AKT-GSK3 pathway has been shown to be pivotal in mediating the biological effects of antipsychotic medications and mood stabilizers^{12, 13, 48, 49}. This pathway is an important modulator of synaptic biology and AKT has been shown to be necessary for the induction of long term depression (LTD) in drosophila⁵⁰. In addition, AKT knockdown has been shown to decrease dendritic spines and adversely impact synaptogenesis in rodent hippocampal neurons^{51, 52}.

Human iPSCs can be differentiated along the neuronal lineage to allow the study of signaling processes and disease biology in human neurons^{53, 54}. Given the pivotal role of

AKT in neurobiology related to human diseases and their treatments, we sought to get a better understanding of the regulation of AKT biology in human neurons. We set out to identify small molecule probes that can help uncover new mechanisms underlying AKT regulation and lead to the discovery of potential new therapeutic leads.

Posttranslational modifications play important roles in the regulation of AKT localization and activation but many such posttranslational modifications have been studied primarily in the context of cancer using cancer cell lines⁵⁵. Recently, two lysine residues in the PH domain, Lys¹⁴ and Lys²⁰, were shown to be acetylated in cancer cell lines in rodent tissue²⁰. SIRT1 and SIRT2 have been shown to modulate these acetylation sites in different contexts^{21, 22}. While these studies implicated the sirtuins in the modulation of AKT acetylation and activity, an earlier study had indicated that HDAC inhibitors affected AKT activity through histone acetylation-independent effects⁵⁶. While broadly selective HDAC inhibitors that targeted both class I and II HDACs were shown to modulate AKT activity, inhibitors specific for class I HDACs did not have such effects. They found that the effects of the HDAC inhibitors on AKT closely mirrored their potency in inducing Lys⁴⁰ α -tubulin acetylation, which is a surrogate for HDAC6 activity⁵⁶. Recently, studies of tumor cell survival have indicated that HDAC6 may modulate AKT phosphorylation indirectly through PTEN²⁸. Studies in rodent neurons have suggested that HDAC6 protects against neuronal death from prion peptide by regulating the PI3K-AKT-mTOR pathway⁵⁷. However, the studies did not examine the acetylation of AKT in these systems.

We found acetylation of two heretofore-unknown acetylation sites Lys¹⁶³ and Lys³⁷⁷ on AKT in the presence of HDAC6 inhibition. Both of these lysine residues and surrounding sequences are conserved in human and mouse AKT. Lys¹⁶³ is in the vicinity of the ATP binding pocket in the kinase domain and acetylation of Lys¹⁶³ in this context may interfere with recruitment of ATP and limit the activity of the enzyme, consistent with our results (Fig. 7)⁵⁸. Lys¹⁶³ is in the turn-motif of the AKT kinase domain and mutation of Lys¹⁶³ to methionine has been shown to result in AKT destabilization⁵⁹. There are additional models that can be consistent with our findings. For example, in the activated state, AKT is phosphorylated at Thr³⁰⁸ and Ser⁴⁷³. Phosphorylation of Thr³⁰⁸ induces an ordered conformation of the activation loop and the α C-helix, while Ser⁴⁷³ phosphorylation allows the hydrophobic motif (HM) at the C-terminus to fold back into PDK1-interacting fragment-pocket with the ordered α C-helix being part of it⁶⁰. In addition, AKT is known to be phosphorylated at Thr⁴⁵⁰ within the turn/zipper-motif, and p-AKT(Thr⁴⁵⁰) is supposed to stabilize AKT and facilitate binding of the HM to the PIF-pocket by interacting with the N-lobe immediately above the glycine-rich loop⁵⁹. It is possible that Lys¹⁶³ on the N-lobe forms contacts to p-AKT(Thr⁴⁵⁰) in the non-acetylated state⁵⁹. Upon acetylation, this interaction might be disrupted resulting in the destabilization of the active conformation and decreased catalytic activity, consistent with the results presented in this study (Fig 3). On the other hand, the second acetylation site Lys³⁷⁷ is in a region of the kinase domain that is in spatial proximity to the PH domain and Lys³⁷⁷ acetylation may interfere with PIP₃ binding to the PH domain⁶¹. While mass spectrometry data showed the presence of acetylation at Lys¹⁶³ and Lys³⁷⁷ on AKT in the presence of an HDAC6 inhibitor (Fig. 5), AKT immunoprecipitation samples probed with pan-ac-Lys antibodies showed a close alignment of the ac- α -tubulin (K40) band with the band for acetyl lysine. This confounds a simple

interpretation of the AKT immunoprecipitation results due to the possibility that the acetylation observed with AKT immunoprecipitation may be due to its association with ac- α -tubulin (K40) rather than directly reporting on AKT acetylation (Fig. 4B). Further studies with antibodies raised against AKT acetylated at these lysine residues as well as site directed mutagenesis will be needed to unambiguously determine their response to HDAC6 inhibition.

While investigating the effects of HDAC6 inhibition on AKT acetylation, we also discovered an unexpected physical association of ac- α -tubulin with AKT that was present only in the setting of HDAC6 inhibition. While ac- α -tubulin has been described to modulate AKT activity through its association in a complex with HSP90³⁶, we report here an association of AKT with ac- α -tubulin, without involvement of HSP90 in the presence of an HDAC6 inhibitor. AKT had been reported to regulate microtubule stability in fibroblasts⁶² and was recently shown to control microtubules organization during neuronal migration, likely through regulation of microtubule binding to accessory proteins⁶³. These effects of an HDAC6 inhibitor on the binding and localization of AKT and microtubules may be mediating the differential trajectory of NPC differentiation that we observed (Fig. 6).

In summary, we report our finding that HDAC6 deacetylase modulates AKT activity in human neuronal cells. AKT is acetylated at Lys¹⁶³ and Lys³⁷⁷ in the presence of an HDAC6 inhibitor, and these residues are different from acetylation sites modulated by SIRT1 and SIRT2. HDAC6 inhibition resulted in decreased ability of AKT to bind PIP₃ and decreased ability to phosphorylate downstream targets, even in the presence of Ser⁴⁷³ phosphorylation, which usually potentiates AKT activity^{23, 24}. Moreover, upon HDAC6 inhibition, we find decreased phosphorylation of the AKT phosphorylation site Ser⁵⁵² on β -catenin, accompanied by a higher proportion of glial cells during neuronal differentiation. Our studies point to new mechanisms for regulating AKT activity with small-molecule inhibitors of HDAC6.

METHODS

Cell culture.

Human neural progenitor cells (NPCs) derived from human iPSCs (HIPTM) were obtained from GlobalStem, Rockville, MD. NPCs were cultured in plates coated with 20 μ g/mL poly-L-ornithine (Sigma-Aldrich, St. Louis, MO) solution in ddH₂O followed by 5 μ g/mL laminin (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, CA). NPCs were cultured in media containing 70% DMEM with high glucose (Life Technologies, Carlsbad, CA), 30% Ham's F12 with L-glutamine (Cellgro/Mediatech, Manassas, VA), penicillin/streptomycin, and B27 supplement (Life Technologies, Carlsbad, CA) as well as 20 ng/mL epidermal growth factor (EGF) (Sigma-Aldrich, St. Louis, MO), 20ng/mL basic fibroblast growth factor (bFGF) (Stemgent, Cambridge, MA), and 5 μ g/mL heparin (Sigma-Aldrich, St. Louis, MO). For the differentiation experiments, NPCs were cultured in the same media but in the absence of EGF and bFGF. Neuro2a cells were cultured in DMEM [supplemented with 10% Fetal Bovine Serum (Thermo Fisher Scientific), penicillin (50 units/ml), and streptomycin (50 μ g/

ml)] and transfected overnight using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Antibodies.

Antibodies recognizing α -tubulin (T9026), acetylated K-40 α -tubulin (T7451), β -tubulin (T8328), β -actin (A5441), and FLAG (F1804) were obtained from Sigma-Aldrich (St. Louis, MO). The antibody recognizing HDAC6 (SC-11420) was obtained from Santa Cruz Biotechnology. Antibodies recognizing acetyl-lysine (#9814 and #6952), PDK1 (#3062), phos-GSK3 β (Ser9) (#5558), phos-GSK3 α (Ser21) (#4070), phos-AKT (Ser473) (#3653), pan-AKT (#4691), AKT1 (#2938) and HSP90 (#4877) were from Cell Signaling Technology.

Compounds and plasmids.

EX-527 and AGK-2 were purchased from Tocris Bioscience and CI-994, ACY-1215 (Ricolinostat), tubastatin A and tubacin were purchased from Selleck Chemicals. Mouse AKT1 was cloned into the EcoR1 and NotI sites of the p3XFLAGCMV-7.1 plasmid (Sigma-Aldrich).

Western Blots and Immunoprecipitation.

For western blotting, cells were collected by scrapping in 1x Laemmli sample buffer (Bio-Rad, Hercules, CA) supplemented with 5% 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Equal amount of proteins were separated by SDS-PAGE (polyacrylamide gel electrophoresis) and transferred to a polyvinylidene difluoride (PVDF) or nitrocellulose membrane. Membranes were blocked in TBST (tris-buffered saline and 0.1% Tween 20) supplemented with 5% nonfat powdered milk and probed with the indicated primary antibody at 4°C overnight. Next, after washes with TBST, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and visualized using ECL (enhanced chemiluminescence) reagents according to the manufacturer's guidelines (Pierce, Thermo Fisher Scientific). For immunoprecipitation experiments, lysates were incubated with primary antibody and A/G Plus-Agarose beads (Santa Cruz, Dallas, TX) overnight at 4°C. Cell fractionation was performed using standard protocols as described in the Cell Fractionation Kit obtained from Cell Signaling (Danvers, MA).

AKT immunoprecipitation in Neuro2a cells.

Neuro2a cells grown in 100 mm plates were transfected with 3X FLAG-tagged mouse wild-type AKT1 for 24–36h. To allow accumulation of acetylated AKT, transfected cells were treated for 6 hours with 5 μ M ACY-1215 before lysis in ice-cold radioimmunoprecipitation assay buffer [50 mM tris-HCl (pH 8.0), 300 mM NaCl, 0.5% Igepal-630, 0.5% deoxycholic acid, 0.1% SDS, 1mM EDTA] supplemented with a cocktail of protease inhibitors (Complete Protease Inhibitor without EDTA, Roche Applied Science) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail A, Santa Cruz Biotechnology). Immunopurification of FLAG-tagged AKT was performed using anti-FLAG M2 antibody conjugated to magnetic beads (Sigma-Aldrich, M8823) and overexpressed 3X FLAG-AKTs

were eluted from the FLAG antibody by incubating the beads with 25 µg of 3X FLAG peptide (Sigma-Aldrich, F4799) for two hours at room temperature with gentle agitation. Equal volume of immunopurified material was separated using SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Subsequent steps were carried out according to the Western blot procedure described above. .

AKT Kinase Assay.

The activity of AKT was determined with an AKT Kinase Assay Kit purchased from Cell Signaling (#9840). Data were analyzed according to the manufacturer's instructions.

PIP₃ Binding Assay.

Lysates from human iPSC-derived NPCs were incubated with PIP₃-conjugated agarose beads overnight. AKT bound to PIP₃ was detected by immunoblotting with an antibody against AKT (#9272) purchased for Cell Signaling. PIP₃-conjugated agarose beads were purchased from Echelon Biosciences (#PB003a). The PIP₃ binding assay was analyzed according to the manufacturer's instructions.

Mass spectrometry.

To identify acetylated lysine residues in AKT1, 3X FLAG-tagged AKT1 was overexpressed in Neuro2a cells, immunoprecipitated, and eluted from the anti-FLAG M2 antibody conjugated to magnetic beads as described above. Eluates from 4 separate immunoprecipitation were combined and concentrated by ethanol precipitation. The sample was then separated by SDS-PAGE and the gel stained with Coomassie blue solution according to manufacturer's guidelines (Bio-Rad, #161-0436). Finally, the 3X FLAG AKT1 band was excised from the stained gel and submitted for mass spectrometry analysis.

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CI-994

EX-527

AGK-2

ACY-1215

Tubacin

Tubastatin A

FIGURE 1. Small molecule inhibitors used in the study.

Structures of the small molecule inhibitors used in the study. The HDAC specificity of the small molecules are as follows: CI-994 (HDAC 1,2,3), ACY-1215 (HDAC6), EX-527 (SIRT1), AGK2 (SIRT2). IC₅₀ values (μM) of the small molecules for inhibition of different HDAC isoforms are showed in Table 1.

A.

B.

FIGURE 2. HDAC6 inhibition increases lysine acetylation on AKT.

A. Immunoblot analysis after immunoprecipitation with anti-AKT antibody of cell lysates from human NPCs that had been treated with different deacetylase inhibitors at 5 μ M for 24 hours with western blot using antibodies against Ac-Lys and AKT. Only lysates from NPCs treated with ACY-1215 show increased lysine acetylation in the immunoprecipitated AKT. Antibodies against Ac-Lys⁴⁰- α -tubulin are shown in the input to indicate the level of HDAC6 inhibitory activity of each small molecule. B. Immunoblot analysis of human NPC lysates immunoprecipitated with anti-AKT antibody and probed with anti-HDAC6 antibody.

A.

B.

FIGURE 3. HDAC6 inhibition results in decreased AKT kinase activity.

A. Immunoblot analysis for nuclear (N) and membrane/cytoplasmic (M/C) fractions for NPCs treated with HDAC6 inhibitor ACY-1215 (5 μ M) or DMSO for 24 hours. Histone H2 is shown as a nuclear marker and HSP90 as a cytoplasmic marker. B. Human NPCs treated with ACY-1215 (5 μ M) or DMSO for 24 hours were immunoprecipitated with PIP₃ beads and probed by Western blot for levels of AKT and PDK1. ACY-1215 treatment resulted in decrease in AKT bound to PIP₃. C. Human NPCs treated with ACY-1215 (5 μ M) or DMSO for 24 hours were immunoprecipitated with anti-p-AKT⁴⁷³, probed for levels of Ac-Lys and

ability to phosphorylate AKT targets in GSK3 peptides. Treatment with ACY-1215 resulted in a decrease in AKT's ability to phosphorylate Ser⁹ on GSK3 β and Ser²¹ on GSK3 α . D. Time-course of ACY-1215 (5 μ M) treatment on ability of AKT to phosphorylate Ser⁹ on GSK3 β . E. Lysate from human NPCs treated with ACY-1215 (5 μ M) or DMSO for 24 hours were probed with anti-p- β -catenin (Ser⁵⁵²). Treatment with ACY-1215 resulted in a decreased phosphorylation of Ser⁵⁵² on β -catenin.

FIGURE 4. AKT immunoprecipitation after HDAC6 inhibition shows increased association with acetylated α -tubulin.

A. Neuro2a cells were transfected with FLAG-tagged constructs for AKT1, treated different deacetylase inhibitors at 5 μ M for 6 hours and the AKT1 fusion protein immunoprecipitated using anti-FLAG M2 magnetic beads. The samples were probed in western blot using antibodies against Ac-Lys and AKT. Antibodies against tubulin, ac-a-tubulin (K40) and FLAG are shown in the input and β -actin is shown as loading control. B. Neuro2a cells were transfected with FLAG-tagged constructs for AKT1, treated with 10 μ M ACY-1215 for 6 hours and the AKT fusion proteins immunoprecipitated using anti-FLAG M2 magnetic

beads. The samples were probed in western blot using antibodies against AKT, Ac-lys, HSP90, α -tubulin and ac- α -tubulin (K40). Antibodies against FLAG, HSP90 and ac- α -tubulin (K40) are shown in the input and β -actin is shown as loading control.

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FIGURE 5. AKT1 is acetylated at two novel lysine residues in the presence an HDAC6 inhibitor. A. Schematic representation showing distribution of AKT1 acetylated lysine residues in Neuro2a cells treated with 5 μ M ACY-1215 identified by mass spectrometric analysis over protein domains (PH, pleckstrin homology domain; ATP, ATP-binding motif; KD, kinase domain). B. & C. Representative product ion spectrum of MS/MS fragmentation supporting acetylation of AKT1 at Lys¹⁶³ (B) and Lys³⁷⁷ (C). D. Lys¹⁶³ and Lys³⁷⁷ (arrowheads) are conserved between AKT 1/2/3 protein isoforms.

A.

B.

FIGURE 6. HDAC6 inhibition promotes NPC differentiation along glial lineage.

A. Representative images of neuronal cultures that were differentiated for 6 weeks in the presence of DMSO, ACY-1215 (600 nM) and ACY-1215 (1.2 μ M), shown at 10X magnification. Scale bar; 500 μ m. Nuclear marker Hoechst is shown in blue, neuronal marker β -III tubulin (TUJ1) is labeled green and glial marker glial fibrillary acidic protein (GFAP) is shown in red. B. Quantification of cell distribution of neuronal cells positive for β -III-tubulin and GFAP in the 6-week differentiation cultures. One way ANOVA with Bonferroni multiple comparison test, $p < 0.001$, $n = 16$ fields of view (FOV) from 3 wells.

FIGURE 7. Site of lysine residues acetylated on AKT in the presence of an HDAC6 inhibitor. Schematic model showing the location of Lys¹⁶³ and Lys³⁷⁷ that are acetylated in the presence of ACY-1215 in a structure of the AKT2 catalytic cleft bound to the ATP analog AMPPNP (PDB 1o6k)⁵⁸. Lys¹⁶³ is located in the nucleotide-binding pocket in close proximity to the ATP analog.

Table 1IC₅₀ Values (μM) of the Small Molecules for Inhibition of Different HDAC Isoforms^{26, 64–66}

	HDAC1	HDAC2	HDAC3	HDAC6	SIRT1	SIRT2	HDAC Selectivity
CI-994	0.05	0.19	0.55				1,2,3
ACY-1215	0.058	0.048	0.051	0.004			6
Tubastatin A	3.2	3.5	4.9	0.018			6
Tubacin	0.028	0.042	0.275	0.016			6
EX-527					0.038		SIRT1
AGK-2					>50	3.5	SIRT2