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Invited review

Crebinostat: A novel cognitive enhancer that inhibits histone deacetylase activity and modulates chromatin-mediated neuroplasticity

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ABSTRACT

Long-term memory formation is known to be critically dependent upon *de novo* gene expression in the brain. As a consequence, pharmacological enhancement of the transcriptional processes mediating long-term memory formation provides a potential therapeutic strategy for cognitive disorders involving aberrant neuroplasticity. Here we focus on the identification and characterization of small molecule inhibitors of histone deacetylases (HDACs) as enhancers of CREB (cAMP response element-binding protein)-regulated transcription and modulators of chromatin-mediated neuroplasticity. Using a CREB reporter gene cell line, we screened a library of small molecules structurally related to known HDAC inhibitors leading to the identification of a probe we termed crebinostat that produced robust activation of CREB-mediated transcription. Further characterization of crebinostat revealed its potent inhibition of the deacetylase activity of recombinant class I HDACs 1, 2, 3, and class IIb HDAC6, with weaker inhibition of the class I HDAC8 and no significant inhibition of the class IIa HDACs 4, 5, 7, and 9. In cultured mouse primary neurons, crebinostat potently induced acetylation of both histone H3 and histone H4 as well as enhanced the expression of the CREB target gene *Egr1* (early growth response 1). Using a hippocampus-dependent, contextual fear conditioning paradigm, mice systemically administered crebinostat for a ten day time period exhibited enhanced memory. To gain insight into the molecular mechanisms of memory enhancement by HDAC inhibitors, whole genome transcriptome profiling of cultured mouse primary neurons treated with crebinostat, combined with bioinformatic analyses of CREB-target genes, was performed revealing a highly connected protein–protein interaction network reflecting modules of genes important to synaptic structure and plasticity. Consistent with these findings, crebinostat treatment increased the density of synapsin-1 punctae along dendrites in cultured neurons. Finally, crebinostat treatment of cultured mouse primary neurons was found to upregulate *Bdnf* (brain-derived neurotrophic factor) and *Grn* (granulin) and downregulate *Mapt* (tau) gene expression—genes implicated in aging-related cognitive decline and cognitive disorders. Taken together, these results demonstrate that crebinostat provides a novel probe to modulate chromatin-mediated neuroplasticity and further suggests that

Abbreviations: AcH4K12, acetylated histone H4 lysine 12; AcH3K9, acetylated histone lysine 9; *Bdnf*, brain-derived neurotrophic factor; CRE, cAMP response element; CREB, cAMP response element-binding protein; CBP, CREB binding protein; *Egr1*, early growth response 1; *Grn*, granulin; HDAC, histone deacetylase; *Mapt*, tau; SAHA, suberoylanilide hydroxamic acid.

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pharmacological optimization of selective of HDAC inhibitors may provide an effective therapeutic approach for human cognitive disorders.

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1. Introduction

Numerous studies have described a key role for the transcription factor CREB (cAMP response element-binding protein) in neuroplasticity underlying learning and memory (Silva et al., 1998; Benito and Barco, 2010; Sakamoto et al., 2011). Early work in *Drosophila melanogaster* showed that CREB is required for olfactory memory (Yin et al., 1994). Knockout of CREB in mice impairs fear conditioning memory (Bourtchuladze et al., 1994). Finally, enhancement of hippocampal-dependent memory by histone deacetylase (HDAC) inhibitors depends on CREB and its interaction with the coactivator CBP (CREB-binding protein) (Vecsey et al., 2007). CBP has lysine acetyltransferase activity, and can acetylate lysines in histone N-terminal tails. Pointing to the importance of these mechanisms to human cognition, loss of function of CBP is known cause the human genetic disorder Rubinstein–Taybi syndrome (Rubinstein and Taybi, 1963; Petrij et al., 1995), a congenital neurodevelopmental disorder defined by characteristic postnatal growth deficiencies, dysmorphology and intellectual disability. The identification of dysregulated histone acetylation due to loss of CBP in Rubinstein–Taybi syndrome patients (Murata et al., 2001), and in the corresponding mouse models (Alarcón et al., 2004; Korzus et al., 2004; Wood et al., 2005), serves as one of the first examples of a now growing list of human diseases with cognitive deficits that can be considered as 'chromatinopathies' due to causally involved mutations in regulators of the structure or function of chromatin and gene expression (reviewed in Levenson and Sweatt, 2005; van Bokhoven, 2011; Haggarty and Tsai, 2011). In addition to these primary chromatinopathies, it is also increasingly recognized that certain neurodegenerative disorders with cognitive deficits, such as Alzheimer's disease (Gräff et al., 2012) and Huntington's disease (Giralt et al., 2012), involve a significant component of epigenetic dysregulation as a downstream consequence of disease pathophysiology. Taken together, these findings point to CREB-mediated transcription as being of paramount importance to the study of human cognitive disorders and efforts to develop novel cognitive enhancers.

The CREB transcriptional pathway is activated by intracellular signaling triggered by increases in intracellular cAMP concentration, or a variety of other signaling pathways (Silva et al., 1998; Johannessen et al., 2004; Benito and Barco, 2010). Typically, the final effector of these signaling pathways is a kinase that phosphorylates CREB at serine 133. The coactivator histone acetyltransferase CBP is then recruited to phospho-(S133)-CREB, which is bound to cyclic-AMP response elements (CREs) in gene promoters. The complex of CREB-CBP then interacts with the general transcriptional machinery to induce activation of transcription of CREB target genes (Goldman et al., 1997). Termination of this transcriptional pathway is mediated by phosphatases that dephosphorylate CREB (Mauna et al., 2011). Finally, the pathway can also be downregulated by proteasome-mediated degradation of CREB (Garat et al., 2006).

Inhibitors of HDACs regulate CREB-dependent transcription (e.g. Fass et al., 2003) and enhance cognition (reviewed in Haggarty and Tsai, 2011). In the case of contextual fear conditioning, enhancement of cognition by HDAC inhibitors is dependent on the functioning of CREB (Vecsey et al., 2007). HDACs are a family of 18 isoforms that catalyze the deacetylation of the ε-amino group of lysine side chains in histone N-terminal tails, and also in numerous other intracellular proteins (Grozinger and Schreiber, 2002; Choudhary et al., 2009). The catalytic mechanism of 11 of these HDACs is critically dependent on a divalent zinc cation coordinated by conserved histidine and aspartate residues within the catalytic site (Bressi et al., 2010). Zinc-dependent HDAC isoforms have been categorized into classes based on structural analyses of the active sites and other domains in these proteins and inhibitor sensitivity (Bradner et al., 2010): class I (HDACs 1, 2, 3, and 8); class IIa (HDACs 4, 5, 7, and 9); class IIb (HDACs 6 and 10); and class IV (HDAC11).

The HDAC isoforms that regulate CREB-dependent transcription and cognition have not been fully identified (reviewed in Fischer et al., 2010). HDACs could potentially regulate CREB-dependent transcription via several mechanisms. First, HDACs could reverse CBP-catalyzed histone acetylation events that mediate transcriptional activation (e.g Valor et al., 2011). Indeed, an HDAC inhibitor failed to enhance cognition in mice with a focal depletion of CBP in the hippocampus (Barrett et al., 2011). Second, HDACs could counteract CBP and the related lysine acetyltransferase p300 activation by auto-acetylation (Thompson et al., 2004). Also, HDAC1- and HDAC8-protein phosphatase 1 complexes have been shown to mediate CREB dephosphorylation (Canettieri et al., 2003; Gao et al., 2009). Further work will be required to fully identify the HDAC isoforms that regulate CREB-dependent transcription and cognition, and their mechanisms of action.

Here, we describe a chemical–genetic approach to identify novel HDAC inhibitors that regulate CREB-dependent transcription, neuronal histone acetylation, and enhance learning and memory *in vivo*.

2. Materials and methods

2.1. CREB reporter gene assay

PC12 cells with a stably incorporated 6X-CRE-β-galactosidase CREB reporter gene (Meinkoth et al., 1990) were seeded into poly-D-lysine coated 384-well plates at a density of 25,000 cells per well. The following day, cells were treated with HDAC inhibitors (20 μM) in the absence or presence of forskolin (10 μM) for 4 h. Cells were then processed for a β-galactosidase assay (Applied Biosystems Galacto-Star) according to the manufacturer's instructions, and luminescence was read on an Envision plate reader (Perkin–Elmer).

2.2. In vitro HDAC enzymatic assays

Assays were run in 384-well plates with recombinant HDACs 1–9 (BPS Bioscience) under conditions appropriate for Michaelis–Menten kinetic analysis with the use of acetylated tripeptide substrates at K_m that are amide-coupled to 7-amino-4-methylcoumarin that can detect either class I/IIb (substrate MAZ1600) or class IIa/HDAC8 (substrate MAZ1675) HDAC activity exactly as described in Fass et al. (2010).

2.3. Neuronal histone acetylation assays

Mouse primary neuronal cultures were generated in factory precoated poly-D-lysine black/clear bottom 96-well plates (BD Biosciences #BD356692) treated overnight with 75 μl/well of laminin [0.05 mg/ml] (Sigma #L2020) in PBS buffer. E17 embryonic mouse forebrain was dissociated into a single cell suspension by gentle trituration following trypsin/DNAse digestion (trypsin: Cellgro #25-052-CI; DNAse: Sigma #D4527). Cells were plated at a density of 12,500 cells per well in 100 μl of Neurobasal medium (Gibco #21103-049) containing 2% B27 (Gibco #17504-044), 1% Penicillin/Streptomycin/Glutamine (Cellgro #30-009-CI) and cultured at 37 °C with 5% CO_2. After 13 days, cultures were treated with HDAC inhibitors by pin transfer of compound (185 nl per well) using a CyBi-Well vario pinning robot (CyBio Corp., Germany) and subsequently incubated for 24 h at 37 °C with 5% CO_2. Cells were then fixed with 4% formaldehyde for 10 min. Following two washes with phosphate-