Review

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Epigenetic drugs in Alzheimer's disease

Abstract: Epigenetic processes, such as DNA methylation and histone acetylation, regulate the genome-environment interactions that may play important roles in a wide range of brain disorders, including Alzheimer's disease (AD). Indeed, the role of epigenetic machinery in learning and memory processes is well documented. In this review, we will focus on the most recent literature on tools that target epigenetic mechanisms, particularly on histone acetylation, and we will discuss the use of chemical probes to validate these targets in therapeutic strategies for AD.

Keywords: amyloid; histone acetylation; memory; tau phosphorylation.

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterised by memory loss and cognitive impairment, which is the most common form of dementia in the elderly, with over 35 million cases worldwide (1). Pathologically, AD is characterised by the presence of extracellular plaques of aggregated amyloid-β peptide in the brain and intracellular neurofibrillary tangles that mainly contain hyperphosphorylated tau protein. These pathological features are associated with neuronal dysfunction that ultimately leads to neuronal loss, as observed in the atrophic brain of AD patients. No effective treatment for AD exists, and the effectiveness of current FDA-approved AD treatments that target cholinergic and glutamatergic neurotransmission to improve symptoms is debatable. Thus, it is critical to develop new disease management/treatment strategies, particularly given the increasing prevalence of AD among an ageing population. The search for effective AD management strategies has largely centred on the amyloid-β (Aβ) hypothesis, focusing mainly on reducing the number of senile plaques. This approach has had little success to date and there is a growing belief that current AD treatments are prescribed far too late to significantly slow disease progression or delay the onset of the most severe symptoms. The continued failure of these therapies indicates that new alternatives, non-amyloid-based strategies, must be considered to restore memory function in AD.

Sporadic or late-onset forms AD are associated with ageing and they represent the majority of AD cases (90–95%), with familial forms associated with mutations in amyloid precursor protein (APP) or presenilin (PS1 and PS2) genes accounting for the remaining 5% (2). The majority of AD cases are thus of a multifactorial nature, and they are likely to involve complex gene-gene and gene-environment interactions (3). In line with this, environmental factors are now thought to epigenetically modify the expression of genes that contribute to the pathogenesis of AD through different mechanisms. Moreover, as epigenetic processes are involved in both ageing and cognitive functions (4, 5), and gene transcription and protein synthesis are required for the formation of new synapses associated with memory formation (6), it is possible that memory deficits in AD result from altered chromatin plasticity mediated by epigenetic mechanisms, such as histone acetylation. Epigenetic processes are dynamic and can be manipulated by both environmental factors and pharmacological tools. Moreover, gene expression can be modulated by epigenetic events, including histone modifications and DNA methylation, or epigenetic

modifiers such as microRNAs and long non-coding RNAs, either individually or in combination. Here, we discuss the literature on DNA methylation and microRNAs in the context of age-associated neurodegeneration and AD. Specifically, we focus on histone acetylation, which is the most studied marker of epigenetic changes in AD. Finally, we review the most recent literature on epigenetic drugs that may be relevant to develop novel therapeutic strategies to treat AD.

Epigenetics in Alzheimer's disease: therapeutic approaches

DNA methylation

In differentiated cells DNA methylation is a more stable epigenetic marker than histone modifications. DNA methyltransferases (DNMTs) are enzymes that establish and maintain DNA methylation, catalysing the transfer of a methyl group from S-adenosyl-methionine to cytosine residues within CpG-rich regions of the genome. DNMT1 is expressed strongly in neurons and it acts as a maintenance methyltransferase, whereas DNMT3a and DNMT3b act as *de novo* methyltransferases (7, 8).

Approximately 70% of CpG dinucleotides within the human genome are methylated. Methylated cytosine residues impair the transcription machinery and they are usually associated with the silencing of gene expression (9). DNA methylation in the brain is a reversible and dynamic process, and it is altered during physiological processes such as memory acquisition. Furthermore, given its reversible and dynamic nature, DNA methylation can be modified pharmacologically [review in (10)]. However, before using DNA methylation as a therapeutic option it is important to understand the alterations in DNA methylation involved in a given disease.

Some AD-related genes, such as the APP gene, undergo methylation in AD patients (11). Age-linked decreases in methylcytosine levels have been reported in the APP promoter, which has a guanine-cytosine (GC) content of 72% (12). DNA methylation also mediates the expression of β-secretase (BACE) and presenilin 1 (PS1) genes, secretases involved in Aβ production, and consequently, this manifestation influences amyloid levels (13). A decrease in methylcytosine and DNMT1 immunoreactivity was recently reported in AD patients, suggesting that the loss of DNA methylation promotes aberrant APP expression, which in turn contributes to AD pathology (14). An inverse correlation between methylcytosine levels and neurofibrillary tangles has also been reported in neurons, suggesting that DNA methylation is attenuated in AD (14). Moreover, twin studies of late-onset-AD revealed significantly lower levels of DNA methylation in the neuronal nuclei of the temporal neocortex of the AD twin. These findings are consistent with the hypothesis that epigenetic mechanisms, at the molecular level, mediate the effects of life events on AD risk, and they provide a potential explanation for AD discordance despite genetic similarities (15). However, a study using a quantitative assay to measure DNA cytosine methylation reported no significant differences in the relative methylation of CCGG sites in brain DNA from 44 AD patients compared with 20 controls (16). By contrast, the expression of a variety of epigenetic markers is reduced in neurons within layer II of the entorhinal cortex, both in AD patients and APP transgenic mice (14).

Vitamin B12 and folate play important roles in DNA methylation as both coenzymes are required for the synthesis of methionine and S-adenosyl-methionine from homocysteine. S-adenosyl-methionine is a methyl donor required for the maintenance of DNA methylation. A lack of dietary vitamin B12 and folate enhances homocysteine and DNA hypomethylation in both rats and humans (17, 18), and deficiency in these nutrients during pregnancy increases the risk of neural tube defects (e.g., spina bifida) due to aberrant DNA methylation (19). Epidemiological studies also indicate that low folate and high homocysteine levels are risk factors for cognitive decline or AD in later life (20, 21). Folate levels are decreased in the CSF of AD patients (22), and the age-associated decrease in APP promoter methylation (12) has been linked to decreases in folate and vitamin B12 levels. In line with these findings, folate deprivation *in vitro* induces DNA hypomethylation that promotes the expression of BACE and PS1 (13), while vitamin B deprivation accelerates the progression of Alzheimer's-like features in APP transgenic mice (23).

An attempt to improve the status of overall one-carbon metabolism by dietary vitamin B supplementation had no positive effects in AD patients (24, 25). However, dietary supplementation with a cocktail of folate, vitamin B6, S-adenosylmethione, N-acetyl cysteine and acetyl-L-carnitine has been shown to improve memory and the performance of daily activities in AD patients (26).

Therapeutic approaches that target DNA methylation have been applied to cancer and other diseases but, to date, they have not been extended to the treatment of neurological disorders. It was originally thought that the pattern of DNA methylation remained stable and that *de novo* methylation did not occur in fully differentiated cells

like neurons, and thus, that DNA methylation inhibitors would have no impact on the brain [reviewed in (27)]. However, it now appears that a balance exists between methylation and demethylation in postmitotic cells, including neurons. Therefore, the development of small-molecule DNMT inhibitors that interact directly with DNMTs and that are not incorporated into DNA, such as the classic inhibitor 5-AZA, could have significant potential as modulators of DNA methylation, even in neurons.

Non-coding RNAs

Non-coding RNAs (ncRNAs) regulate chromatin architecture and gene expression, and they have recently been attributed a role in AD as they participate in important functions in brain development and cognition. Differences in miRNA expression profiles have been demonstrated between sporadic AD patients and age-matched controls [reviewed in (28)], although it remains to be determined whether these changes are a cause or consequence of the disease process. The regulation of APP expression through its 3'UTR is influenced by several miRNA-binding sites, *cis*-acting regulatory elements and binding proteins, and single-nucleotide polymorphisms (SNPs). The levels of miRNA-101, which negatively regulates APP, are reduced in the cortex of AD patients' brains (29, 30). BACE1 β-secretase mRNA expression is regulated by both miRNAs (miRNA-107) and long ncRNAs, such as BACE1-antisense (BACE1-AS) (29). BACE1-AS improves BACE stability and it is upregulated in AD brains (31). Systemic injection of targeted exosomes was recently used successfully to deliver siRNAs that silence BACE1 expression in the mouse brain (32). Although the efficacy of RNA interference in the central nervous system (CNS) must still be determined, recent findings point to non-coding RNAs as a new class of potential drug targets in neurodegenerative diseases. However, before investigating the therapeutic potential of miRNAs, it is necessary to develop new delivery methods to ensure stable expression and minimal toxicity, particularly for chronic treatment regimens such as those required for neurodegenerative disorders.

Histone modifications

Histones are basic proteins that regulate chromatin compaction and that undergo post-translational epigenetic modifications via acetylation, methylation, phosphorylation, ubiquitination or sumoylation. Histone acetylation and phosphorylation have been linked to transcriptional activation, while trimethylation of histone-3K4 is associated with gene silencing. In this section we will focus on histone acetylation, as this is the most extensively studied epigenetic modification in AD.

Histone acetylation is controlled by the opposing actions of two different types of enzyme that modulate gene expression: HATs and HDACs (33–35). Histone acetylation results in a less tightly packaged chromatin structure that is associated with transcriptional activation, as well as learning and memory processes (36). Increases in histone acetylation have been described following exposure to different learning paradigms (37–41) and HDAC inhibition enhances hippocampus-dependent memory formation (33, 37, 42, 43). Moreover, dysregulation of histone acetylation has been described in the brains of cognitively impaired AD mouse models (44, 45) and human AD patients (19).

Histone deacetylases

The induction of histone acetylation following the inhibition of histone deacetylases (HDACs) has been proposed as a potential therapeutic strategy to treat memory decline in AD (46, 47), as successfully demonstrated in several AD mouse models (44, 48–52). However, it remains unclear which HDAC subtypes are involved in the pathophysiology of AD. HDAC enzymes are divided into four classes: class I consists of HDAC1, 2, 3 and 8; class II is divided into two subclasses, class IIa (HDAC4, 5, 7 and 9) and class IIb (HDAC6 and 10); class III is composed of a group of proteins known as sirtuins (1–7); and the sole member of class IV is HDAC11. The function of class I and II HDACs in the nervous system has been the subject of much research (53) and most HDAC inhibitors tested in AD mouse models target both classes (Table 1 summarises the different HDAC inhibitors tested in AD to date and their selectivity for different classes of HDACs).

Class I and class II HDACs

Although the distribution of individual HDACs in the CNS is not well defined and their role in memory varies, it is clear that class I HDACs do influence memory processes. Moreover, both HDAC2 and HDAC3 have been shown to regulate learning and memory. Indeed, memory functions are enhanced when mice lack HDAC2 and HDAC3 in the hippocampus, while those that lack HDAC1 have no obvious phenotype (54, 55), although a recent study implicated HDAC1 in the extinction of contextual fear memories (56). No role has been demonstrated for HDAC8 in learning

Table 1 HDAC inhibitors tested in AD.

HDAC inhibitor	HDAC target[a]	Findings
Sodium phenylbutyrate (PBA[b])	Class I (HDAC1, 2, 3, 8) Class IIb (HDAC6, 10) (66)	Memory restoration in Tg2576 and APP/PS1 mice. Decreased pTau levels *in vitro* and *in vivo* (via GSK3β inactivation), decreased C99 and Aβ levels, and decreased amyloid burden (Tg2576 and APP/PS1 mice) (44, 48, 49, 68, 103)
Sodium butyrate (NaBu)	Class I (HDAC1, 2, 3, 8) (66)	Contextual fear and associative memory restoration in APP/PS1 mice (51, 52). Decreased pTau levels *in vitro* (68)
Valproic acid (VPA)	Class I (HDAC1, 2, 3, 8) (104)	Memory restoration in APP23 mice (65). Decreased Aβ and pTau levels (via GSK3β) and CDK5 inactivation (67). Contextual fear memory restoration in APP/PS1 mice (51)
Vorinostat (SAHA)	Class I (HDAC1, 2, 3, 8) and Class IIb (HDAC6) (66)	Contextual fear memory restoration in APP/PS1 mice (51)
Trichostatin A (TSA)	Class I (HDAC1, 2, 3), Class IIa (HDAC4, 7, 9) and Class IIb (HDAC6) (105)	Rescue of CA3-CA1 LTP in slices from APP/PS1 mice (45)
Ms-275	Class I (HDAC1, 2, 3) (66)	Decreased amyloid burden and improved nesting behaviour in APP/PS1 mice (19)
Mercaptoacetamide-based class II (W2)	Class IIb (HDAC6) (68)	Decreased Aβ40 and Aβ42 *in vitro*. Memory restoration, and decreased Aβ and pTau levels in hAPP 3xTg-AD mice (69)
Hydroxamide-based inhibitors of class I and II HDACs (I2)	Class I (HDAC1), Class IIa (HDAC5) and Class IIb (HDAC6, 10) (68)	Decreased Aβ40 *in vitro* (69)
Nicotinamide	Class III (SIRT1-7) (78)	Memory restoration and decreased pTau and Aβ levels in 3xTg-AD mice (78)

[a]Inhibition within 1.0 log units of the next most potent isoform.
[b]PBA is also a chemical chaperone.

and memory. Concerning the class IIa HDACs, Agis-Balboa et al. (57) showed that impaired spatial memory formation was evident in HDAC5$^{-/-}$ mice although inhibition of HDAC5 failed to improve memory deficits or pathogenesis in a mouse model of amyloid pathology. In contrast, no changes in learning and memory in HDAC5$^{-/-}$ mice have been reported by other authors, despite defining spatial memory, impairment in conditional brain-specific HDAC4 knockout mice was encountered (58). No causative role in cognition has been described for other HDACs, although inhibition of HDAC6, a class IIb HDAC, ameliorates cognitive deficits in a mouse model of AD (59). HDAC6 is a unique member of the HDAC family that acts on cytoplasmic non-histone substrates, primarily α-tubulin (60), and has been implicated in cytoskeletal stability and intracellular transport (61).

There are higher levels of HDAC2, but not HDAC1 or HDAC3, in the hippocampus of AD mouse models and in the hippocampus and entorhinal cortex of AD patients (62). There is also more HDAC6 expression levels in post-mortem tissue samples from AD patients (63), consistent with the decreased tubulin acetylation concentration observed in neurons from the brains of AD patients (64). These findings indicate that HDAC2 is one of the main targets of pan-HDAC inhibitors to counteract cognitive decline in AD, although effects on non-histone proteins such as α-tubulin via HDAC6 inhibition may also be involved. Many HDAC inhibitors tested in AD transgenic mice are non-selective and ameliorate or even reverse memory deficits in multiple AD mouse models, including sodium butyrate (NaBu), sodium phenylbutyrate (PBA), valproate and tricostatin A (see Table 1 for details) (44, 49, 51, 52, 65).

The influence of HDAC inhibition on spatial and contextual fear memory was first tested effectively in a mouse model of AD (Tg2576) using PBA, a pan-HDAC inhibitor (HDACi) that specifically inhibits class I and IIb HDACs and that also acts as a chemical chaperone (44, 49). Both chaperone activity and HDAC6 inhibition may be at least partially involved in memory restoration in PBA-treated Tg2576 mice. However, the fact that similar effects are observed in different AD mouse models (Table 1) treated with NaBu, Ms-275 or valproate (19, 51, 52, 65, 66), specific inhibitors of class I HDACs, strongly suggests that the inhibition of class I HDACs, and more specifically the HDAC2 that is upregulated in AD, are involved in the recovery of memory. Thus, although amyloid-β and pTau may not be affected by HDAC2 inhibition, restoring the transcription of plasticity-related genes appears to ameliorate the symptoms of dementia, even after disease onset in AD patients.

It should be noted that although AD is a multifaceted disorder in which memory decline is the main symptom, the aggregation of misfolded proteins such as amyloid-β and pTau ultimately leads to the loss of neurons, the main cause of AD pathology. The effect of HDACis on amyloid and/or pTau pathology has been addressed in different studies using a variety of *in vivo* and *in vitro* models of AD (see Table 1 for details). Beneficial effects have been observed with non-selective HDACis (targeting class I and II HDACs) that decrease the levels of both pTau and amyloid-β levels in different models of AD such as PBA and valproate (44, 49, 50, 65, 67, 68). *In vitro* assays provide better opportunities to elucidate the mechanism of action of different drugs. In a recent study, we compared the effects of PBA (which inhibits class I and IIb HDACs) with those of NaBu (a selective inhibitor of class I HDACs) in primary cultures of neurons from Tg2576 mice. Decreased levels of $A\beta_{42}$ and its precursor C99 were observed in the conditioned media from PBA-treated but not NaBu-treated neurons (68). As NaBu selectively inhibits class I HDACs, these findings suggest that amyloid pathology is ameliorated by the inhibition of class IIb HDACs (including HDAC6 and HDAC10) but not class I HDACs. While the chemical chaperone activity of PBA may influence the amyloid pathology (68), a recent study reported that a mercaptoacetamide-based class II HDACi diminished Aβ levels *in vitro* and *in vivo* by modulating APP processing (69). Thus, class II HDAC inhibitors, particularly those that target class IIb HDACs like PBA, may also modulate amyloid pathology and should be considered as potential novel agents to treat AD.

Interestingly, both PBA and valproate modulate GSK3β activity and reduce tau hyperphosphorylation, the latter representing another key event in AD pathogenesis (44, 49, 67, 68). Both of these compounds produce chaperone-like effects, reducing endoplasmic reticulum stress (49, 67, 68). Although the role of HDAC inhibition on tau phosphorylation remains unclear, as tau phosphorylation was significantly decreased in primary cultures of neurons of Tg2576 mice exposed to either PBA or NaBu (a selective class I HDACi) for 3 days (68), the modulation of tau phosphorylation through the inhibition of class I HDACs cannot be ruled out. Decreased pTau levels have also been reported in 3xTgAD mice treated with selective inhibitors of class II HDACs (69). HDAC6 (class IIb) mediates microtubule stability by increasing α-tubulin acetylation, a mechanism potentially involved in the decrease in tau phosphorylation detected following exposure to pan-HDACis that target this enzyme. It is important to emphasise that tau interacts with HDAC6, inhibiting its deacetylase activity and leading to increases in tubulin acetylation (70). However, treatment with tubacin (a selective HDAC6 inhibitor) does not impair this interaction but attenuates tau phosphorylation (63). Taken together, these findings point to HDAC6 as a promising therapeutic target in AD.

Finally, given that neuronal loss is one of the key features of AD in the human brain, the neuroprotection putatively offered by HDACis suggests they have additional relevant properties. VPA is neuroprotective in several models of neurodegenerative diseases [reviewed in (71)] and long-term treatment with PBA but not NaBu prevents neuronal loss in the CA1 hippocampal layer of $TghAPP^{WT}$ mice (68). The multimodal action of PBA and VPA, as pan-HDACis and chemical chaperones, may contribute to this effect (68, 71). Thus, the evidence available suggests that HDAC inhibition is a promising and novel strategy for AD therapy, acting through a multi-target mechanism that involves epigenetic regulation, chromatin remodeling (promoting memory restoration) and regulation of proteostasis (affecting signalling cascades triggered by Aβ and pTau: Figure 1).

Class III HDACs

Class III HDACs, also known as sirtuins, have attracted considerable attention over the last decade because of their role as epigenetic regulators of ageing. Unlike other classes of HDACs, sirtuins do not require zinc as a co-factor but they are dependent on NAD+ for catalysis (72). SIRT1 is the best characterised sirtuin, and it is necessary to maintain synaptic plasticity, learning and memory. Pharmacological regulation of SIRT1 can effectively reverse the ageing process and lower the incidence of age-related complications in rodent models. Indeed, recent studies have demonstrated the therapeutic potential of dietary compounds that increase the activity of SIRT1, including resveratrol, leptin and curcumin [reviewed in (73)]. In the inducible p25 transgenic mouse, increased SIRT1 activity caused by lentiviral overexpression of SIRT1 or resveratrol treatment protects against hippocampal neurodegeneration, and prevents learning and memory deficits (74). Moreover, SIRT1 is decreased in the parietal cortex of AD patients, an alteration that may be associated with their amyloid-β and pTau accumulation (75). By contrast, the role of sirtuins in ameliorating AD-like symptoms in animal models remains controversial. Overexpression of SIRT1 is reported to reduce Aβ production and amyloid burden in a mouse model of AD (76), and several studies have provided evidence of anti-amyloidogenic properties of the SIRT1 activators resveratrol or curcumin [reviewed in (77)]. However, nicotinamide, a competitive sirtuin

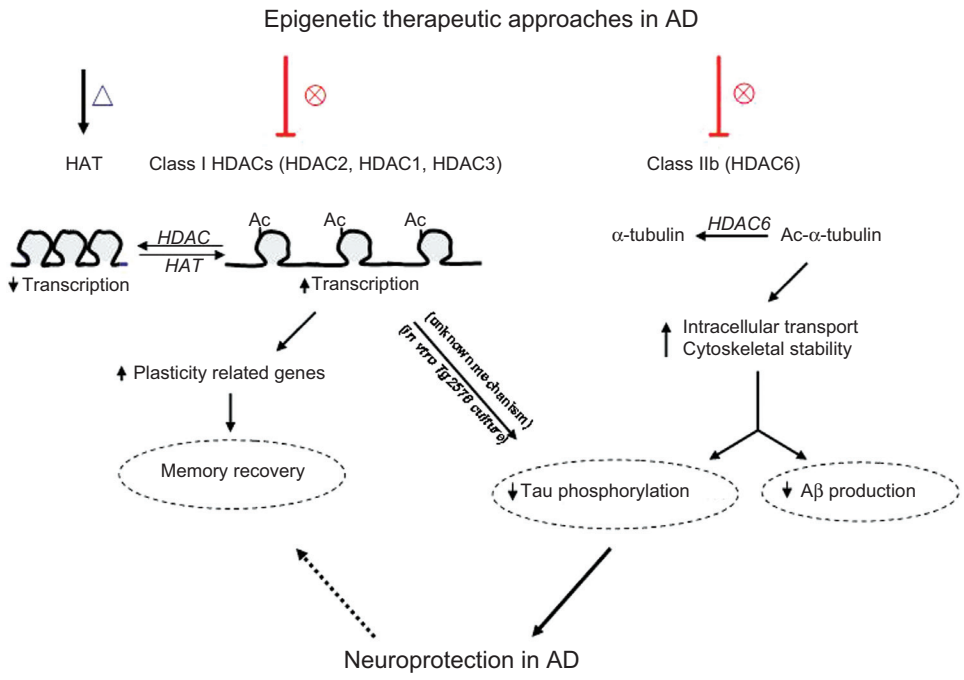


Figure 1 Scheme showing potential benefits obtained by modifying histones and α-tubulin acetylation in AD. HDAC, histone deacetylase; HAT, histone acetyltransferase; Ac, Acetylation; △ activation; ⊗ inhibition.

inhibitor, restores cognition and decreases tau phosphorylation in the 3xTgAD mouse model without affecting amyloid levels (78), indicating that the decrease in tau phosphorylation may be related to SIRT2 inhibition given that it has been shown to act as an α-deacetylase (79). Thus, activation of SIRT1 and inhibition of SIRT2 by distinct mechanisms appears to modulate AD-like features (amyloid-β and pTau) in mouse models of this disease (80).

Histone acetyltransferases (HAT)

The use of pan-HDAC inhibitors is limited by their toxicity, although an alternative means of restoring the transcriptional balance and protein acetylation would be to use histone acetyltransferase (HAT) activators. Thus, stimulation of acetyltransferase activity is another potential tool to treat neurodegenerative diseases and the specificity in restoring chromatin may be enhanced by targeting HATs than by inhibiting HDACs (81).

HATs involved in memory formation include p300, the cAMP-response element binding protein (CBP), and the p300/CBP-associated factor (PACAF). The intrinsic HAT activity of P300 and CBP, and their recruitment of the basal transcriptional machinery to the promoter indicates that these genes regulate gene expression directly. CBP loss of function has been reported in several diseases characterised by neurological deficits, including Rubinstein-Taybi syndrome and polyglutamine-related pathologies (e.g., Huntington's disease) (82, 83). Morris water maze training induces CBP, p300 and PCAF mRNA expression in the rat hippocampus, supporting the important role of these HATs in memory processing [reviewed in (81)]. Furthermore, CBP overexpression restores memory function in 3xTg-AD triple transgenic mice (84).

Together, these data suggest that targeting these HATs (CBP, p300 and PCAF) is a more specific means of enhancing memory than the use of the currently available non-selective HDAC inhibitors (see Figure 1). Nonetheless, the HAT activators described to date exhibit poor solubility and membrane permeability, and they are therefore unsuitable candidates to treat neurodegenerative and/or neurological disorders. The best characterised HAT activator is N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide (CTPB) (85), a small-molecule modulator of the p300 histone acetyltransferase that induces structural alterations in p300 acetyltransferase. However, CTPB is cell impermeable and must be bound to carbon spheres (CSP) if it is to be used in cell systems, in which it promotes p300 autoacetylation and transcription (86). Furthermore, intra-peritoneal injection of CSP-CTPB induces hyperacetylation of histone 3 in the mouse brain, indicating its ability to cross the blood-brain barrier

(BBB) (86). A recent patent application covers the use of HAT activators to enhance learning and memory, as well as cognition, and to treat neurodegenerative disorders and diseases involving accumulation of the amyloid-beta peptide and tau protein (87).

By contrast, there is evidence that HAT inhibition is beneficial in models of AD. While the p300 inhibitor, C646, reduces levels of acetylated and phosphorylated tau *in vitro* (88), gallic acid and/or curcumin, two polyphenol HAT inhibitors, activate SIRT1 (89) and ameliorate amyloid pathology by alleviating inflammatory progression (90–92). By modulating different signalling pathways, such as those dependent on NF-kappaB and mitogen-activated protein kinase, polyphenols exert antioxidant and anti-inflammatory effects that may be beneficial in neurodegenerative disorders such as AD [reviewed in (89)].

Epigenetics: chemical tools

A lack of mechanistic rigour in the selection and validation of therapeutic targets has contributed to a crisis in drug discovery. It is essential for chemical probes to be used to investigate the relationship between such targets and the biological processes involved in disease pathogenesis to achieve rigorous preclinical target validation (93). Medicinal chemistry can provide selective chemical probes to assess target engagement, the corresponding functional pharmacology, and the relevant phenotypes when targets have not yet been validated for clinical applications.

As illustrated above, certain epigenetic processes are promising targets for the treatment of AD, although the identification and validation of these targets requires an investment of time, resources and money before any drug discovery programmes can be launched. Therefore, the development of selective chemical probes and relevant assays is critical to ensure the success of this strategy and to develop new AD therapies. In this section we will discuss some the most important epigenetic-based chemical tools.

DNA methylation inhibitors

Many DNMT inhibitors have been described to date, compounds that can be divided into two families: nucleoside analogues that have been studied for many years; and non-nucleoside inhibitors whose structure varies according to their inhibitory mechanism. While nucleoside-like inhibitors have been approved by the FDA, their lack of specificity and strong secondary effects highlight the urgent need for more selective, novel DNMT inhibitors.

In recent years, non-nucleoside molecules have emerged as potential candidates to be used in the CNS, as their mechanism of action does not rely on their incorporation into DNA (94). Several novel DNMT inhibitors of different origins and structures have been described in recent years. Curcumin derivatives are particularly potent, and they have been shown to inhibit the bacterial C5 DNA methyltransferase M. SssI [**1**] (95) and its derivatives with an IC_{50} of ~30 nM (Figure 2). RG-108 [**2**] (96), a compound identified through virtual screening, inhibits M. SssI and human DNA methylation in HCT116 and NALM6 (leukaemia) cells at 100 μM (Figure 2). After bisulfite conversion of the genome of RG108-treated cells, demethylation of gene promoters could be detected by sequencing after treatment. Moreover, unlike other DNMT inhibitors, RG108 is neither genotoxic nor cytotoxic (94, 96). Finally, by competing with S-adenosylmethionine (SAM) in the methylation reaction and acting as a competitive inhibitor of the SAM co-factor, SGI-1027 [**3**] (97) mediates the selective degradation of DNMT1, producing few or no effects on DNMT3A and DNMT3B (Figure 2). Because of the highly conserved I and X motifs involved in the recognition of the SAM co-factor (94, 97), all DNMTs are inhibited by SGI-1027 with a comparable IC_{50} (6–13 μM).

Most known non-nucleoside inhibitors are compounds with demonstrated biological activity against targets other than DNMTs, although none have yet entered clinical development (94). Thus, further studies will be necessary to identify novel, selective (in terms of off-target promiscuity and isoform selectivity) and potent

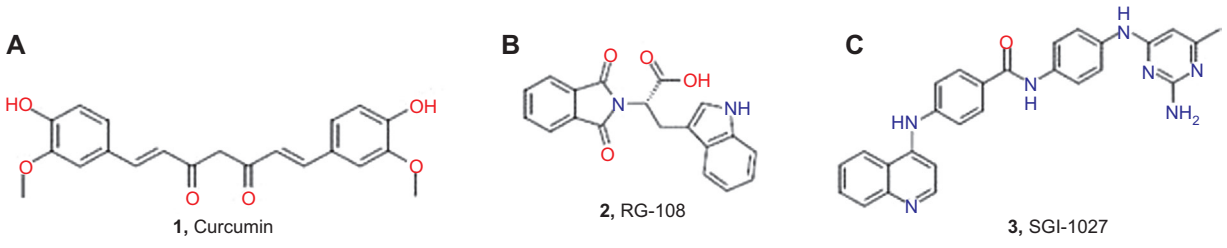


Figure 2 Non-nucleoside inhibitors of DNMTs.
(A) Curcumin, (B) RG-108 and (C) SGI-1027.

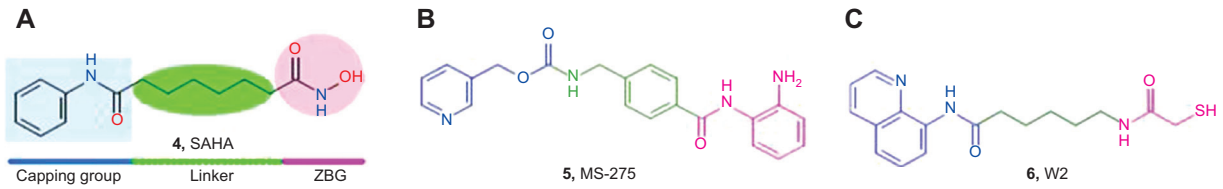


Figure 3 The three key pharmacophore features of inhibitors of class I and II HDACs.
(A) SAHA (also known as Vorinostat or Zolinza®, approved by the FDA); (B) MS-275; and (C) W2. Structures are colour-coded according to the features of each region.

non-nucleoside DNMT inhibitors in order to validate DNMTs as therapeutic targets.

HDAC inhibitors

A large set of HDAC inhibitors have already been tested in AD mouse models (Table 1) and *in vitro* biochemical profiling has demonstrated that these compounds display distinct selectivity for different HDAC classes. For example, SAHA [**4**] targets class I and IIb HDACs (IC_{50} of 30–410 nM for all isoforms), MS-275 [**5**] is a selective class I inhibitor that targets HDAC1, HDAC2 and HDAC3 (IC_{50}<370 nM) and W2 [**6**] is a selective class IIb inhibitor with an IC_{50} for HDAC6 of 21 nM (>1.5 log units difference compared to the next most active isoforms: HDAC5 and HDAC11) (50). These inhibitors display a common pharmacophore that consists of a chelator (zinc-binding group, ZBG), a linker domain and a surface recognition domain (Figure 3). While the zinc-binding domain is critical for the catalytic activity, different structural motifs may confer selectivity to specific classes of HDACs.

Assessing the activity of these compounds *in vivo* may allow us to elucidate the optimal means to treat AD, although given that it is most likely that chronic treatment will be necessary, greater selectivity may be required in order to determine the specific effects of each isoform, in terms of both efficacy and safety. A new generation of selective chemical probes will therefore be required to minimise the polypharmacology (unwanted off-target effects) of HDACs, and to identify and validate the most relevant HDAC isoform(s) for AD treatment. Potent HDAC6 inhibitors have recently been described (98–100) that have a selectivity ~2 log units better than the next most active isoforms, such as tubastatin A [**7**] (Figure 4). The selectivity of tubastatin A is attributed to the specific interactions between the unique capping motif and the surface topology of HDAC6. Moreover, tubastatin A [**7**] has recently been used as a chemical probe, and in this way HDAC6 was shown to be a unique potential therapeutic target for AD and related neurodegenerative tauopathies (101).

TFMO-compound 1 is a selective inhibitor of class IIa HDACs, with a novel zinc-binding mode of action [**8**] (Figure 4) that circumvents the selectivity and pharmacologic liabilities of hydroxamates (102). These cell-active chemical probes are important tools in the HDAC inhibitor field, and they help precisely define class IIa HDAC catalytic and/or acetyl-lysine activity.

HAT activators

A recent patent describes compound I [**9**] as a HAT activator (Figure 5) and *in vitro* assays show this molecule to target CBP with an EC_{50} of 2.75 µM. Compound I [**9**] is over 1 log unit more selective than PCAF and GCN5, and it does not inhibit any of the HDACs tested (10 isoforms assayed from classes I, IIa, IIb and III) (87). Given

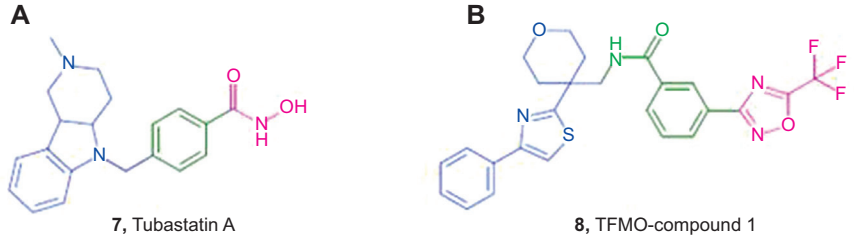
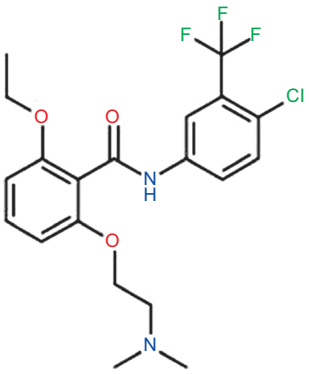


Figure 4 Inhibitors of class IIb HDACs.
(A) Tubastatin A and (B) TFMO-compound 1. Structures are colour-coded according to the pharmacophore features of each structural region.



9, Compound I

Figure 5 HAT activators.

its pharmacokinetic profile and ability to cross the BBB, together with its pharmacological activity *in vivo* (increasing histone 3 acetylation levels in the hippocampus) and lack of toxicity after chronic treatment (87), compound I [**9**] appears to represent an important pharmacological tool for use *in vivo*, promising to be exceedingly useful for validating HAT targets (in this case CBP) in AD mouse models. However, further work is required to elucidate the role of each HAT in AD, for which more potent and selective chemical probes will be required.

Conclusions

In conclusion, an increasing number of chemical tools have emerged that may aid the validation of epigenetic targets. However, careful characterisation of chemical probes is essential to ensure that accurate biological conclusions are reached. A new generation of selective chemical probes will be required to unequivocally validate targets, thereby facilitating the development of potent and selective compounds with minimal unwanted off-target effects. The generation of information relevant to human disease requires chemical probes with optimised pharmacokinetics that are capable of crossing the BBB and that meet the current critical safety criteria. These probes will be essential pharmacological tools for *in vivo* target validation in the search for AD pharmacotherapies.

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