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REVIEW

Targeting the histone orthography of cancer: drugs for writers, erasers and readers

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Gene expression is dynamically controlled by epigenetics through post-translational modifications of histones, chromatin-associated proteins and DNA itself. All these elements are required for the maintenance of chromatin structure and cell identity in the context of a normal cellular phenotype. Disruption of epigenetic regulation is a common event in human cancer. Here, we review the key protein families that control epigenetic signalling through writing, erasing or reading specific post-translational modifications. By exploiting the leading role of epigenetics in tumour development and the reversibility of epigenetic modifications, promising novel epigenetic-based therapies are being developed. In this article, we highlight the emerging low MW inhibitors targeting each class of chromatin-associated protein, their current use in preclinical and clinical trials and the likelihood of their being approved in the near future.

LINKED ARTICLES

This article is part of a themed section on Epigenetics and Therapy. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-11

Abbreviations

AML, acute myeloid leukaemia; FDA, Food and Drug Administration; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; HMT, histone methyltransferase; KMT, protein lysine methyltransferase; LSD1, lysine-specific demethylase 1; MDS, myelodysplastic syndrome; PHD, plant homeodomain fingers; PRC2, polycomb repressive complex 2; PRMT, protein arginine methyltransferase; PTM, post-translational modification; SAM, S-adenosylmethionine; TSA, trichostatin A

Table of Links

TARGETS	LIGANDS	
DNA methyltransferases	Anacardic acid	GSK126
DOT1L, histone methyltransferase	5-azacytidine (azacitidine)	Panobinostat
EZH2, histone methyltransferase	5-aza-2′-deoxycytidine (decitabine)	Quisinostat (JNJ-26481585)
HAT, histone acetyltransferases	Belinostat	Romidepsin
HDAC, histone deacetylase	BIX-01294	SAM, S-adenosyl methionine
HDAC1	Butyrate	Trichostatin A
HDAC2	C646	Vorinostat (SAHA)
HDAC3	Curcumin	UNC0638
HDAC6	E11	
Histone demethylases	EPZ-5676	
HMT, histone methyltransferase	EPZ004777	
KMT, protein lysine methyltransferase	Garcinol	
LSD1, lysine-specific demethylase 1	Givinostat	

This Table lists key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Cells in an organism contain identical genetic material but all of them have the ability to maintain the specific phenotypes and biological functions of the tissues and organs in which they are embedded. This capability is ensured by the chromatin-associated proteins and the heritable chemical modifications of histones and DNA sequence: the epigenome (Waddington, 1952; Berger *et al.*, 2009). Although these modifications do not involve changes in the linear DNA sequence, they are fundamental to maintaining cell identity and regulating processes such as differentiation, development, proliferation and genome integrity (Kouzarides, 2007). Several factors influence epigenetic gene regulation through changes in chromatin structure, but the covalent modifications of histones and DNA are possibly the most decisive elements coordinating this process (Segal and Widom, 2009).

Chromatin modifications are responsible for changes in the conformation of chromatin through their effect over interactions between DNA sequence and histones, which determine accessibility to specific loci, or through the creation of docking sites for the recruitment of epigenetic regulators. This map of the combinations of covalent modifications is known as the histone code and is defined by four different DNA modifications (Baylin and Jones, 2011; Wu and Zhang, 2011; Pfaffeneder *et al.*, 2014) and at least 16 types of histone modification, such as phosphorylation, acetylation, methylation, ubiquitination and sumoylation (Kouzarides, 2007). Research in the past decade has significantly increased our knowledge of the proteins related to these post-translational modifications (PTMs). These are chromatin-interacting proteins that catalyse, recognize and remove the specific chemical modifications. They are popularly known as writers, readers and erasers respectively (Figure 1). Most of these proteins possess specialized domains that are able to recognize particular regions within the genome, being guided by a specific histone code.

Deregulation of epigenetic control has been frequently associated with several human diseases, such as cancer (Baylin and Jones, 2011). Tumour cells suffer global epigenetic reorganization resulting in the CpG-specific hypermethylation of tumour suppressor gene promoters and a generalized loss of DNA methylation at microsatellite regions, repetitive sequences and oncogene promoters (Esteller, 2008). Moreover, genes encoding epigenetic regulators undergo several aberrations in cancer, such as point mutations, translocations, amplifications and deletions (Simó-Riudalbas and Esteller, 2013). Depending on the protein involved and the pathway affected, these alterations could lead to changes in gene transcription or to more global changes in chromatin structure.

In contrast with the irreversible genomic mutations that inactivate tumour suppressor genes or activate oncogenes in cancer, epigenetic modifications can be reversed. Thus, the dynamism of the epigenome allows the correction of aberrant epigenetic profiles by therapeutic manipulation. Epigenetic therapies targeting some chromatin regulators have already been approved by the Food and Drug Administration (FDA). This is the case for 5-azacytidine and 5-aza-2′-deoxycytidine, nucleoside analogues that irreversibly inhibit the DNA methyltransferases DNMT1 and DNMT3B and which are currently used as first-line treatment for patients with myelodysplastic syndrome (MDS; Garcia-Manero and Fenaux, 2011; Wells *et al.*, 2014). Not only drugs targeting epigenetic writers are used in clinics at present, but also drugs against epigenetic erasers. Vorinostat (SAHA) and romidepsin are inhibitors of histone deacetylases (HDAC) approved for the treatment of refractory cutaneous T-cell lymphoma (Foss *et al.*, 2011; Khan and La Thangue, 2012). Although the introduction of these compounds into clinics has been a success for the field, their precise mechanism of action remains unclear, and no reliable biomarkers are available for the prediction of their clinical activity.

New generation epigenetic therapies are being designed that take into account the abnormal expression levels of chromatin-associated proteins present in tumours. In this

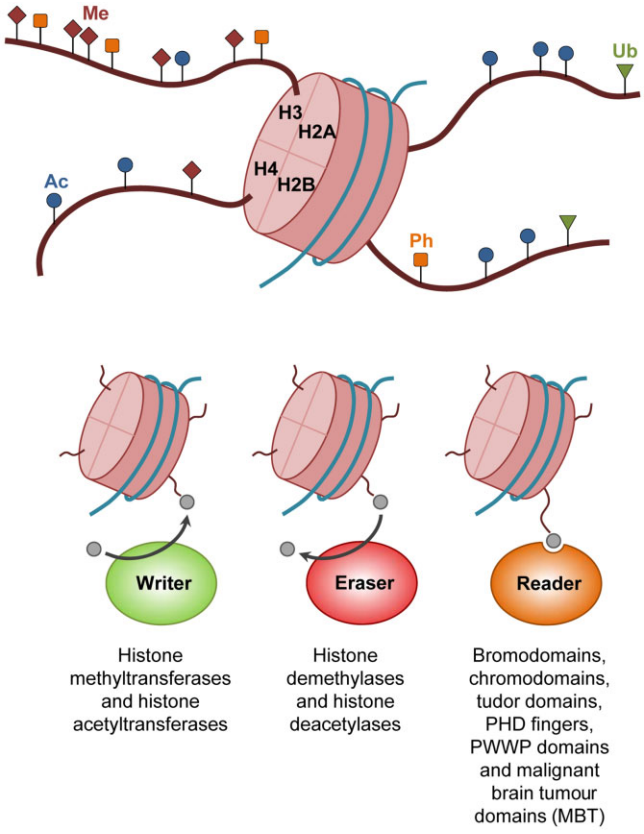

Figure 1

Writers, erasers and readers. The basic functional unit of chromatin is the nucleosome, which is composed of DNA wrapped around histones (H2A, H2B, H3 and H4). Core histone tails are projected from nucleosomes and are subject to PTMs. These include methylation (Me), acetylation (Ac), phosphorylation (Ph) and ubiquitination (Ub). The main epigenetic regulators can be categorized as writers, erasers and readers of PTMs. Epigenetic writers are responsible for the addition of chemical modifications. Epigenetic erasers catalyse the removal of the covalent modifications. Epigenetic readers are proteins with specific domains that recognize and bind to particular modifications.

review, we focus on the proteins involved in depositing, removing or binding to PTMs, as well as the latest advances in the development of specific inhibitors to these proteins associated to chromatin.

Targeting epigenetic writers

Epigenetic writers encompass enzymes such as histone acetylases (HAT), kinases, histone methyltransferases (HMT) and ubiquitin ligases. These chromatin-associated proteins catalyse the deposition of the PTMs on proteins and introduce dynamic modifications that respond rapidly to environmental changes, like histone acetylation.

HMTs

A clear association between histone methylation, transcriptional regulation and tumour phenotype has encouraged the design of specific low MW inhibitors of distinct histone arginine and lysine methyltransferases. The lysine residues can exist in monomethylated, dimethylated and trimethylated states, while arginine residues are either monomethylated or dimethylated. Demethylation of arginine residues can occur symmetrically (via monomethylation of both terminal guanidine nitrogens) or asymmetrically (via dimethylation of one of the terminal guanidine nitrogens; Chesworth *et al.*, 2014).

Protein arginine methyltransferases (PRMTs) and protein lysine methyltransferases (KMTs) are able to transfer a methyl group from the cofactor S-adenosylmethionine (SAM) to arginine or lysine residues respectively. The catalytic domains of PRMT and KMT proteins are structurally different. All the enzymes of the KMT class, with the exception of DOT1L, share a conserved catalytic domain known as the SET domain. The catalytic domain of DOT1L shares structural homology with the PRMT class (Richon *et al.*, 2011).

Histone methylation does not affect chromatin structure directly because this chemical modification does not change the charged state of an aminoacidic residue. Instead, each type of methyl mark represents a specific modification that is recognized as a docking site for chromatin-associated proteins that maintain chromatin architecture (Trojer *et al.*, 2007) or regulate gene expression (Lee *et al.*, 2007). Depending on each specific residue, methylation is associated with activated euchromatic genes (H3K4, H3K36 and H3K79) or with silenced heterochromatic genes (H3K9, H3K27 and H4K20; Barski *et al.*, 2007; Bannister and Kouzarides, 2011).

Aberrant activity of HMTs, due to chromosomal translocation, amplification, deletion, overexpression or silencing of their corresponding genes, has been discovered in cancer (Ryan and Bernstein, 2012; Shih *et al.*, 2012). The recent discovery of HMT disruption in cancer suggests a strategy for targeting patient populations with these alterations using low MW compounds designed to selectively inhibit oncogenic methyltransferases. Drug discovery efforts are giving rise to low MW inhibitors that can reduce HMT activity and reverse the abnormal transcription patterns of tumour cells (Figure 2).

DOT1L

Over recent decades, an increasing number of non-random chromosomal abnormalities have been described in different subtypes of haematological malignancies. Approximately 10% of adult acute myeloid leukaemias (AMLs) and 70% of infant leukaemias involve rearrangements of the *MLL* (also known as *KTM2A*) gene, resulting in fusion of *MLL* with several different protein partners (De Boer *et al.*, 2013). The artificial fusion complexes recruit DOT1L, a HMT that specifically methylates Lys^{79} of histone H3 (H3K79). After this abnormal recruitment to unusual localizations, DOT1L enhances the expression of genes required for leukaemia initiation (Okada *et al.*, 2005; Deshpande *et al.*, 2013).

As DOT1L is a key protein in the development of MLL-rearranged leukaemia, there is an increasing interest in its therapeutic targeting. EPZ004777 is selective inhibitor of DOT1L H3K79 methyltransferase activity, which acts by mimicking the cofactor SAM (Table 1; Daigle *et al.*, 2011). In MLL-rearranged cell lines, EPZ004777 decreases global H3K79 methylation levels and has anti-proliferative effects after blocking the expression of MLL-fusion target genes (Daigle *et al.*, 2011). The specificity of its mechanism of action ensures that the DOT1L inhibitor only affects cells with MLL

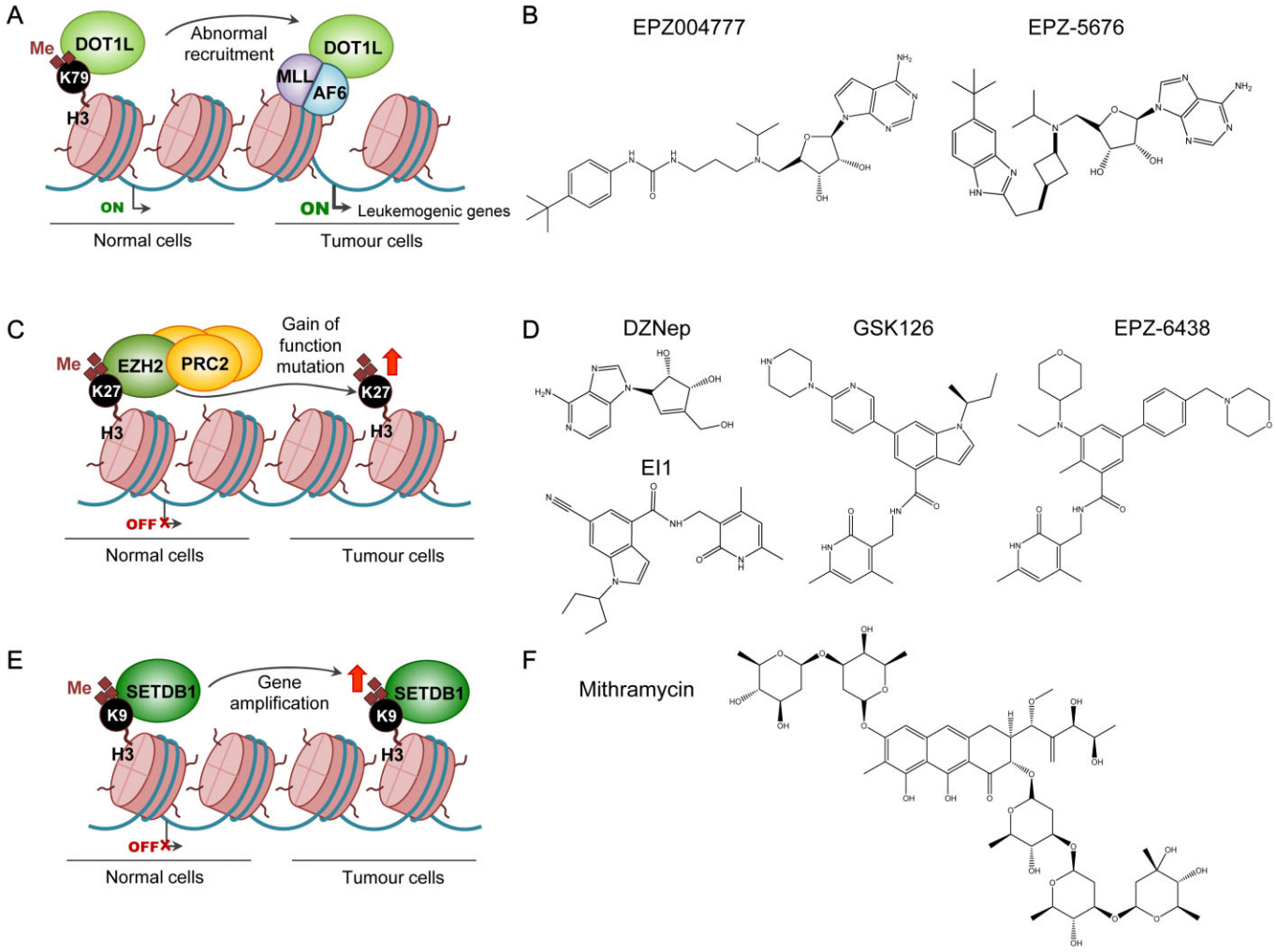

Figure 2

Inhibition of HMTs. (A) DOT1L is a HMT that specifically methylates the lysine H3K79, a histone modification that is associated with actively transcribed genes. Different subtypes of haematological malignancies involve MLL-fusion proteins (e.g. MLL-AF6) that recruit DOT1L to unusual localizations for the induction of leukaemogenic gene expression. (B) Reported DOT1L inhibitors are EPZ004777 and EPZ-5676. (C) EZH2, a protein of the PRC2, catalyses dimethylation and trimethylation of H3K27 to maintain transcription repression of target genes. EZH2, which is up-regulated in several tumours, induces cell migration, colony formation and genomic instability. (D) 3-deazaneplanocin (DZNeP), GSK126, EPZ-6438 and EI1 are specific inhibitors of EZH2. (E) SETDB1 is an HMT responsible for the methylation of H3K9, a mark associated with gene repression. This protein has been found amplified in cancer. (F) Mithramycin is a clinically approved antibiotic that represses SETDB1 function.

gene fusion and preserves the non-rearranged cell lines. Preclinical experiments in mice show good EPZ004777 tolerance and efficacy (Daigle *et al.*, 2011). Although this DOT1L inhibitor presents such attractive features, it still has poor pharmacokinetic properties, such as a short plasma half-life that entails continuous infusions. An attempt is being made to solve some of these deficiencies using second-generation DOT1L inhibitors like EPZ-5676, which is already undergoing clinical trials (Daigle *et al.*, 2013; ClincalTrials.gov identifier: NCT01684150).

EZH2

The catalytic component of the polycomb repressive complex 2 (PRC2) is the enzyme EZH2, responsible for the methylation of H3K27. EZH2 and the whole PRC2 are critical for silencing a large number of genes involved in development and differentiation processes (Morey and Helin, 2010). This HMT is overexpressed in prostate, breast, kidney and lung cancers, solid tumours in which EZH2 up-regulation induces cell migration, colony formation and genomic instability (Varambally *et al.*, 2002; Kleer *et al.*, 2003; Wagener *et al.*, 2010; Takawa *et al.*, 2011). Additionally, somatic gain-of-function mutations in the SET domain of EZH2 have been discovered in follicular and diffuse large B-cell lymphomas (Morin *et al.*, 2010; Pasqualucci *et al.*, 2011). However, EZH2 loss-of-function mutations have also been identified in MDS (Nikoloski *et al.*, 2010). The presence of both activating and inactivating mutations of EZH2 in cancer suggests a context-dependent role for these HMTs and both sides of the coin should be taken into account in the development of Polycomb-targeted therapies.

Table 1

Inhibitors of epigenetic writers

Category	Compound	Phase	Tumour type	References
HMT inhibitors				
DOT1L inhibitors	EPZ00477	Preclinical	MLL-rearranged leukaemia	Daigle *et al.*, 2011
	EPZ-5676	Clinical trials	Haematological malignancies	Daigle *et al.*, 2013; NCT01684150
	SGC0946	Preclinical	Leukaemia	Yu *et al.*, 2012
EZH2 inhibitors	DZNeP	Preclinical	Breast, colon, prostate	Tan *et al.*, 2007
	GSK126	Preclinical	EZH2 mutant lymphomas	McCabe *et al.*, 2012
	GSK343	Preclinical	Epithelial ovarian cancer	Amatangelo *et al.*, 2013
	EPZ005687	Preclinical	EZH2 mutant lymphomas	Knutson *et al.*, 2012
	EI1	Preclinical	Diffuse large B-cell lymphoma	Qi *et al.*, 2012
	EPZ-6438	Clinical trials	Advanced solid tumours, B-cell lymphoma	Knutson *et al.*, 2013; NCT01897571
	UNC1999	Preclinical	Diffuse large B-cell lymphoma	Konze *et al.*, 2013
SUV39H1 inhibitors	Chaetocin	Preclinical	AML	Chaib *et al.*, 2012
G9A inhibitors	BIX-01294	Preclinical	Neuroblastoma	Lu *et al.*, 2013
	UNC0321	Preclinical	(Enzymic inhibition of the target protein)	Liu *et al.*, 2010
	UNC0638	Preclinical	Breast	Vedadi *et al.*, 2011
	BRD4770	Preclinical	Pancreas	Yuan *et al.*, 2012
SETDB1 inhibitors	Mithramycin	Preclinical	Non-small cell lung cancer	Rodriguez-Paredes *et al.*, 2013
HAT inhibitors				
Bisubstrate inhibitors	Acetyl-CoA derivatives	Preclinical	(Enzymic inhibition of the target protein)	Wu *et al.*, 2009
Natural products	Anacardic acid	Preclinical	Breast	Hemshekhar *et al.*, 2012
	Garcinol	Preclinical	Hepatocellular carcinoma	Sethi *et al.*, 2014
	LTK14, LTK15	Preclinical	(Enzymatic inhibition of the target protein)	Mantelingu *et al.*, 2007
	Curcumin	Preclinical	Head and neck, lung	Kumar *et al.*, 2014; Malhotra *et al.*, 2014
Low MW compounds	α-methylene-γ-butyrolactone 3 (MB-3)	Preclinical	Acute lymphoblastic leukaemia	Holmlund *et al.*, 2013
	Isothiazolone	Preclinical	Colon	Stimson *et al.*, 2005
	C646	Preclinical	Prostate	Santer *et al.*, 2011

The high frequency of genetic changes affecting H3K27 has prompted the development of several histone methylation inhibitors. 3-deazaneplanocin is a SAM-derived molecule that leads to a decrease of H3K27 methylation together with apoptosis of cancer cells (Tan *et al.*, 2007). However, in some cells, this compound decreases the methylation of other histone residues and does not seem to be solely a selective inhibitor of the repressive marks but also of the active histone methylation marks (Miranda *et al.*, 2009). It is therefore useful to continue looking for more specific inhibitors of histone methylation, which could directly target the EZH2 enzyme.

More recently, several non-SAM-derived inhibitors of the catalytic activity of EZH2 have been discovered. All of them are highly potent selective inhibitors with *in vivo* anti-tumour activity. Some examples are GSK126 and EPZ005687, inhibitors effective against EZH2 mutant lymphomas, and EI1, a low MW inhibitor that blocks diffuse large B-cell lymphoma proliferation (Knutson *et al.*, 2012; McCabe *et al.*, 2012; Qi *et al.*, 2012). Another EZH2 selective inhibitor is EPZ-6438, which has already entered clinical trials for the treatment of patients with B-cell lymphoma (ClincalTrials.gov identifier: NCT01897571). EPZ-6438 is the first EZH2 inhibitor with activity in solid tumours such as pediatric malignant rhabdoid cancer (Table 1; Knutson *et al.*, 2013).

H3K9 methyltransferases

The di- or trimethylation of Lys^9 on histone H3 (H3K9me2 and H3K9me3) are histone marks generally associated with a compact, closed chromatin state (heterochromatin) and gene repression (Barski *et al.*, 2007). Several HMTs responsible for the deposition of these methyl groups are altered in cancer and some inhibitors of these enzymes have been developed.

Chaetocin is a fungal mycotoxin capable of inhibiting SUV39H1 methyltransferase *in vivo* (Greiner *et al.*, 2005) and exhibits anti-tumour effects in leukaemia cell lines *in vitro* and primary AML cells *ex vivo* (Chaib *et al.*, 2012). However, the specificity of this compound for inhibiting the SUV39H1 enzyme has already been questioned (Cherblanc *et al.*, 2013). The low MW compound BIX-01294 is also an example of an H3K9 methyltransferase inhibitor. It blocks G9A (EHMT2) and leads to a decrease of proliferation and induced apoptosis of neuroblastoma cells (Kubicek *et al.*, 2007; Chang *et al.*, 2009; Lu *et al.*, 2013). A new generation analogue based on the BIX-01294 structure is 3-deazaneplanocin, a GLP and G9a inhibitor with higher *in vitro* potency and better cell membrane permeability than its precursors (Vedadi *et al.*, 2011). The *SETDB1* gene, which encodes another HMT for H3K9, has been found to be amplified in melanoma and lung cancer and its expression can be diminished by a clinically approved anti-tumour antibiotic, mithramycin, which binds to the *SETDB1* promoter and inhibits the binding of Sp transcription factors (Figure 2; Ryu *et al.*, 2006; Ceol *et al.*, 2011; Rodriguez-Paredes *et al.*, 2013).

Histone acetyltransferases (HATs)

Acetyltransferases mediate the transfer of an acetyl group from acetyl coenzyme A (acetyl-CoA) to the ε-amino group of lysine residues in histones and other proteins. Upon acetylation, the positive charge of lysines is neutralized and, in the case of histones, the interaction with the negatively charged DNA backbone is diminished, producing an open chromatin status. HATs have been classified into type A HATs, which are nuclear proteins that acetylate chromatin-associated proteins and histones, and type B HATs, which are located both in the nucleus and the cytoplasm and acetylate newly synthesized cytoplasmic histones to promote their nuclear localization and deposition onto nascent DNA chains (Ruiz-Carrillo *et al.*, 1975). While type A HATs comprise three families of enzymes (GNATs, P300/CBP and MYST), KAT1 is the only HAT in the type B group. Despite showing little sequence homology, the catalytic domains of all HATs are organized around a recognizable acetyl-CoA binding site. This pocket that contains the acetyl-CoA cofactor seems to be a putative chemically tractable domain (Liu *et al.*, 2008).

Knowing that the catalytic activity of HAT enzymes requires the presence of acetyl-CoA, HAT inhibitors are conjugates, derivatives or compounds related to this coenzyme (Figure 3). These compounds can be classified into bisubstrate HAT inhibitors, natural product HAT inhibitors and low MW HAT inhibitors. The first HAT inhibitor class to be identified was a bisubstrate inhibitor, whose mechanism of action is to imitate the acetyl-CoA-lysine intermediate complex in HAT reactions. The major limitation of this class of HAT inhibitors is that they do not have drug-like properties because of their lack of cell permeability (Lau *et al.*, 2000). Unfortunately, most of the natural product HAT inhibitors also have a similar limitation. Anacardic acid, a bioactive phytochemical found in the shell of nuts from *Anacardium occidentale*, is a non-competitive inhibitor of p300, PCAF and Tip60 (Hemshekhar *et al.*, 2012). Chemically, anacardic acid is a mixture of organic compounds structurally related to salicylic acid, each substituted with different alkyl groups of different length, from C15 to C17, and degrees of unsaturation, up to three double bonds (Paul and Yeddanapalli, 1956; Figure 3B). Although it has HAT inhibitor activity, this molecule has poor cell permeability, which limits its practical applications (Eliseeva *et al.*, 2007). In contrast, garcinol, a compound extracted from the rinds of the *Garcinia indica* fruit, is a highly permeable but non-specific HAT inhibitor. Its non-specific nature makes it highly cytotoxic (Balasubramanyam *et al.*, 2004), which inspired the synthesis of LTK14 and LTK15, two garcinol derivatives that are more specific and less cytotoxic (Mantelingu *et al.*, 2007). One of the best characterized natural HAT inhibitors is curcumin, which is extracted from the rhizome of *Curcuma longa* and presents high efficacy in the prevention and treatment of several tumour types, such as those of head and neck and lung cancer (Kumar *et al.*, 2014; Malhotra *et al.*, 2014). The last group of HAT inhibitors include several low MW compounds with better cell permeability properties, like α-methylene-γ-butyrolactone 3, quinoline, isothiazolone and their derivatives, which have been linked to reduced cell proliferation in human colon cancer cell lines (Stimson *et al.*, 2005). One of the most recently described low MW inhibitors is C646, a selective, potent and drug-like HAT inhibitor that, after binding to EP300, acts as a cofactor competitor inducing apoptosis in prostate cancer cells (Table 1; Bowers *et al.*, 2010; Santer *et al.*, 2011).

The lack of powerful HAT inhibitors reported to date suggests that many of the functions and interactions of these enzymes are yet to be fully investigated. There are several factors complicating the analysis of HAT functions. For example, it is noteworthy that HATs are part of large multi-protein complexes and the study of the whole reconstituted complexes may be necessary if we are to discover new inhibitors.

Targeting epigenetic erasers

Epigenetic modifications of the genome that are deposited by epigenetic writers also need to be removed by specific epigenetic erasers in order to regulate gene expression. Epigenetic erasers are classified in several groups of enzymes that target histones; these include, among others, histone lysine and arginine demethylases and HDACs.

Histone demethylases

Until a decade ago, histone methylation was considered a stable chemical modification that, together with DNA methylation, defined epigenetic programmes, but this view changed with the discovery of lysine-specific demethylase 1 (LSD1; Shi *et al.*, 2004) and the identification of the JMJC-domain-containing lysine demethylase family (Tsukada *et al.*, 2006; Whetstine *et al.*, 2006). Therefore, histone lysine methylation is not a fixed modification and can be dynamically regulated at particular gene loci through the recruitment of methyltransferases and demethylases, as with acetylation (Barth and Imhof, 2010; Zee *et al.*, 2010).

Several members of the histone demethylase family appear to be genetically amplified and overexpressed in some human tumours. Moreover, the active site of these epigenetic enzymes is well studied. All these characteristics have prompted the assessment of HMTs as putative drug targets and the development of selective and high-affinity low MW inhibitors of tumour growth.

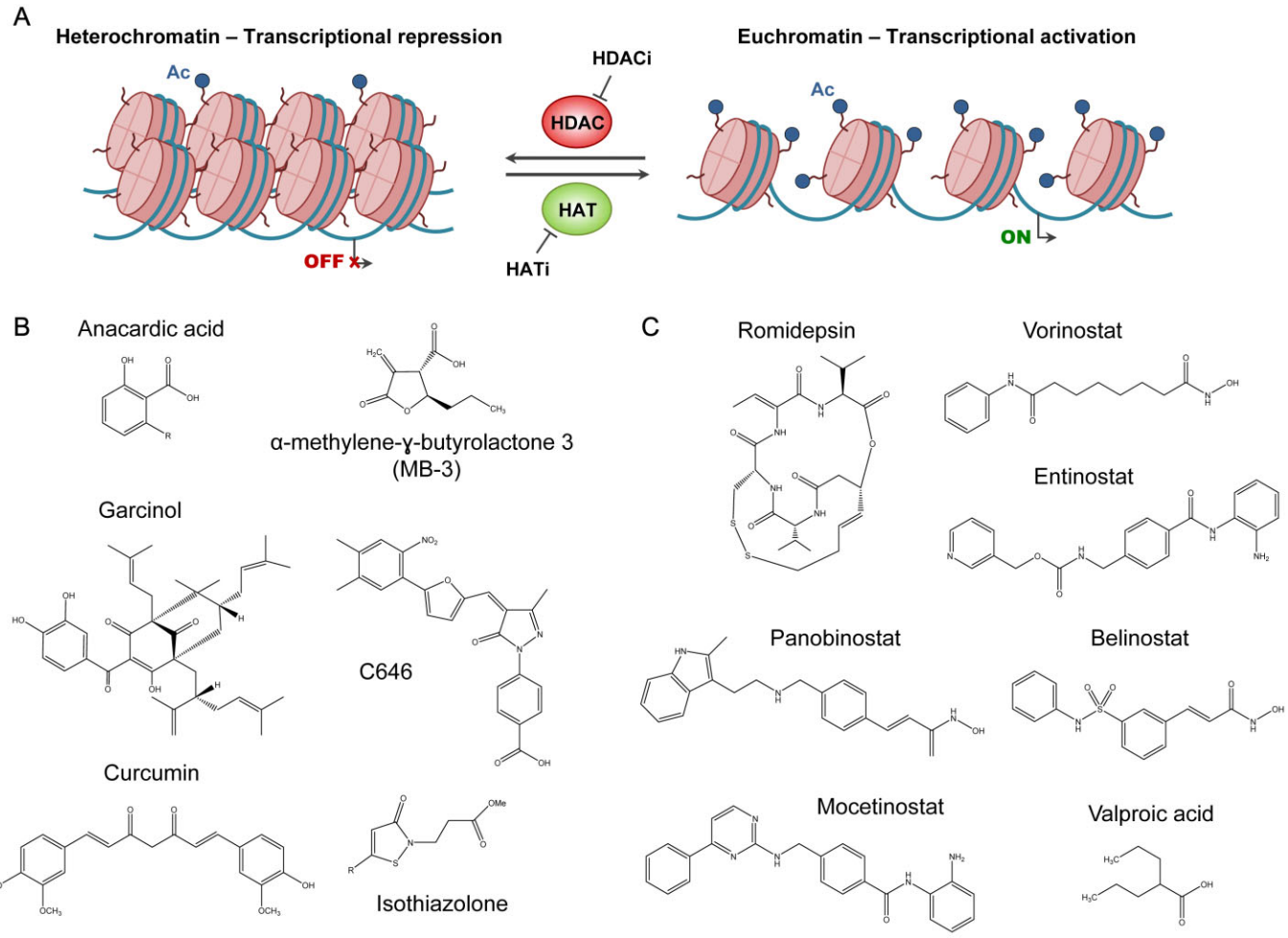

Figure 3

Inhibition of HATs and HDACs. (A) HATs are responsible for the acetylation of certain lysine residues of histone tails, inducing a looser open state of chromatin structure (euchromatin) and, therefore, transcriptional activation of certain genes. A reverse process occurs when HDACs remove the acetyl group of lysine residues, the DNA–histone interaction is intensified and, thus, chromatin acquires a more condensed state (heterochromatin). Several compounds have been designed for the inhibition of HATs and HDACs (HATi and HDACi). (B) HAT inhibitors include natural products like anacardic acid, garcinol and curcumin, and small molecules like α-methylene-γ-butyrolactone 3 (MB-3), C646 and isothiazolone. (C) Romidepsin, vorinostat, panobinostat, belinostat, entinostat and valproic acid are some of the reported HDACis.

LSD family

The LSD family comprises only two members: the histone demethylases LSD1 (KDM1A) and LSD2 (KDM1B). The oxidase-like domain of these enzymes is responsible for the catalytic activity and the removal of the methyl group from histone lysines is performed through an oxidation mechanism that depends on the cofactor FAD (Fitzpatrick, 2010). The LSD enzymes catalyse the demethylation of mono- and dimethylated lysines but not trimethylated lysines, in contrast to JMJC-domain histone demethylases. LSD1 protein presents high-level expression in prostate, oestrogen-negative breast, bladder and colorectal cancers (Kahl *et al.*, 2006; Hayami *et al.*, 2011; Kauffman *et al.*, 2011), among others. Therefore, LSD1 could be a tumour biomarker to consider as a valuable therapeutic target.

As the sequence of the catalytic domain of the LSD proteins is highly homologous with that of the MAO A and MAO B enzymes, some existing MAO inhibitors, like tranylcypromine, can also inhibit LSD1 (Lee *et al.*, 2006; Schenk *et al.*, 2012). Given that non-selective amine oxidase inhibitors have several side effects, derivatives of tranylcypromine are being developed as more potent and selective LSD1 inhibitors. The biotech company Oryzon Genomics has already examined ORY-1001 in clinical trials for the treatment of relapsed or refractory acute leukaemia (EudraCT Number: 2013–002447-29; Table 2). Although tranylcypromine derivatives are the most promising compounds described so far, other molecules have been identified as LSD1 inhibitors, such as polyamines with no target selectivity and clear potential side effects (Huang *et al.*, 2007; 2009). Other studies have investigated a weak but selective LSD1 inhibitor which has *in vitro* and *in vivo* activity (Willmann *et al.*, 2012), and an inhibitor that was designed to mimic peptide-based inhibitors (Wang *et al.*, 2011).

Table 2

Inhibitors of epigenetic erasers

Category	Compound	Phase	Tumour type	References
Histone demethylase inhibitors				
LSD1 inhibitors	Tranylcypromine	Preclinical	AML	Schenk *et al.*, 2012
	ORY-1001	Clinical trials	Relapsed or refractory acute leukaemia	2013-002447-29
JMJC domain-containing inhibitors	8-hydroxyquinolines	Preclinical	(Enzymatic inhibition of the target protein)	King *et al.*, 2010
	Pyridine hydrazones	Preclinical	(Enzymatic inhibition of the target protein)	Chang *et al.*, 2011
	GSK-J1	Preclinical	(Enzymatic inhibition of the target protein)	Kruidenier *et al.*, 2012
HDAC inhibitors				
Cyclic peptides	Romidepsin	Approved	Refractory cutaneous T-cell lymphoma	Whittaker *et al.*, 2010
Hydroxamic acids	TCA	Preclinical	Lung	Chan *et al.*, 2013
	Vorinostat	Approved	Cutaneous T-cell lymphoma, lymphoid malignancies	Mann *et al.*, 2007; NCT00691210
	Panobinostat	Clinical trials	Haematological malignancies, solid tumours	Cassier *et al.*, 2014; NCT01321346
	Belinostat	Clinical trials	Ovary, colon, haematological malignancies	Plumb *et al.*, 2003; NCT01686165
	Pracinostat	Clinical trials	Colorectal, MDS	Novotny-Diermayr *et al.*, 2010; NCT01873703
	Quisinostat	Clinical trials	Advanced solid tumours, cutaneous T-cell lymphoma	Venugopal *et al.*, 2013; NCT01486277
	CHR-3996	Clinical trials	Refractory solid tumours	Banerji *et al.*, 2012; NCT00697879
Short-chain fatty acids	Phenylacetate	Clinical trials	Brain	NCT00003241
	Phenylbutirate	Clinical trials	Advanced colorectal cancer	NCT00002796
	Valproic acid	Clinical trials	Leukaemia, solid tumours	Fredly *et al.*, 2013; Coronel *et al.*, 2011
Benzamides	Entinostat	Clinical trials	Refractory advanced non-small cell lung cancer	Juergens *et al.*, 2011
	Mocetinostat	Clinical trials	Haematological malignancies, advanced solid tumours	Younes *et al.*, 2011; Siu *et al.*, 2008
	CS055	Clinical trials	Advanced non-small cell lung cancer	NCT01836679

JMJC domain-containing demethylases

The catalytic JMJC domain is characteristic of the second family of histone lysine demethylases. This demethylation reaction occurs through an oxidative mechanism that requires two cofactors, 2-oxoglutarate and iron, and it concludes with the hydroxylation of the methyl group (McDonough *et al.*, 2010; Kooistra and Helin, 2012). Through this mechanism, JMJC proteins are able to remove methyl groups from mono-, di- and trimethylated lysines. Thus, the JMJC demethylases can demethylate trimethylated lysines, in contrast to the LSD demethylases (Cloos *et al.*, 2006; Whetstine *et al.*, 2006). Several results have associated this histone lysine demethylase family with cancer. For example, members of the JMJD2 subfamily are overexpressed by genomic amplification in breast cancer, squamous cell carcinoma and medulloblastoma (Yang *et al.*, 2000; Ehrbrecht *et al.*, 2006; Liu *et al.*, 2009). Moreover, members of the JARID1 family are overexpressed in breast and bladder cancers (Lu *et al.*, 1999; Hayami *et al.*, 2010), and FBXL10 is also up-regulated in leukaemia (He *et al.*, 2011). Given its strong association with cancer development, targeting the enzymic activity of JMJC proteins could have therapeutic potential.

Most of the current inhibitors of JMJC domain-containing demethylases are methyl chelators that bind to the catalytic pocket containing iron and compete with the 2-oxoglutarate cofactor for its binding (Lohse *et al.*, 2011). This is the case of 8-hydroxyquinolines and pyridine hydrazones, which are potent and selective inhibitors with good

drug-like properties, such as cell permeability (King *et al.*, 2010; Chang *et al.*, 2011). Another encouraging compound is GSK-J1, which is an inhibitor of the JMJD3 subfamily and works by binding competitively to the 2-oxoglutarate cofactor and chelating the metal in the active site (Table 2; Kruidenier *et al.*, 2012). A recently discovered therapeutically promising compound is a highly specific and potent inhibitor of the JARID1 family developed by the biotech company EpiTherapeutics. Xenograft mouse models have already been treated with this drug and a reduction of the proliferation of cancer cells has been shown (L.-O. Gerlach, pers. comm.).

HDACs

HDACs are enzymes responsible for the removal of the acetyl group of lysine residues in histones. Upon histone deacetylation, the positive charge of lysines is restored and the intensified interaction with the DNA backbone brings about a transcriptional repression state within heterochromatin, a more condensed form of chromatin. HDACs are divided into five classes based on their phylogenetic comparison with yeast enzymes. Class I comprises HDAC1, HDAC2, HDAC3 and HDAC8; class IIa consists of HDAC4, HDAC5, HDAC7 and HDAC9; class IIb includes HDAC6 and HDAC10; class III comprises the sirtuins from SIRT1 to SIRT7; and class IV contains only HDAC11. Enzymes from classes I, II and IV require a zinc ion for catalysis, whereas sirtuins are NAD^+-dependent enzymes with protein deacetylase and ADP-ribosylase activity (Sauve, 2010).

Many proteins other than histones have been recognized as substrates for HDACs (Choudhary *et al.*, 2009). For example, HDAC6 is involved in the deacetylation of microtubules and the hsp90 chaperone (Hubbert *et al.*, 2002; Kovacs *et al.*, 2005). Other non-histone proteins like the tumour suppressor p53 are deacetylated by class I HDACs (Luo *et al.*, 2000). HDACs are sometimes referred to as lysine deacetylases rather than HDACs because of the many non-histone targets.

More than 10 years ago, Lin *et al.* (2001) discovered that deregulation of HDAC activity in association with chromosomal translocation was involved in the stimulation of leukemogenesis. To date, several studies have provided evidence of aberrant acetylation and altered expression of HDACs in cancer cells and tumour tissues (Ropero and Esteller, 2007; West and Johnstone, 2014). Therefore, using HDAC inhibition to reverse epigenetic aberrancies in cancer cells is a powerful approach for the treatment of several tumour types (Figure 3).

HDAC inhibitors are compounds that bind to the catalytic pocket of HDACs and prevent substrate binding to the enzyme, leading to re-expression of particular genes. These inhibitors affect several biological processes: they cause cell cycle arrest in G1 and/or G2 phase, leading to inhibition of cell growth (Bolden *et al.*, 2006); they induce cell differentiation and promote apoptosis (Nebbioso *et al.*, 2005); they inhibit angiogenesis and even enhance sensitivity to chemotherapy (Geng *et al.*, 2006; Qian *et al.*, 2006). HDACi have been classified into four major structural classes with different HDAC subtype selectivity profiles: cyclic peptides, hydroxamates, short-chain fatty acids and benzamides (Table 2).

Romidepsin (Istodax®) is a member of the cyclic peptide group. This class I HDAC-selective inhibitor is a prodrug isolated from *Chromobacterium violaceum*, which is activated in the cell after a reduction reaction (Furumai *et al.*, 2002). Romidepsin induces cell cycle arrest and apoptosis in several human cancer cells and it was approved by the US FDA in 2009 for the treatment of refractory cutaneous T-cell lymphoma (Piekarz *et al.*, 2009; Whittaker *et al.*, 2010) and in 2011 for the treatment of peripheral T-cell lymphoma (Piekarz *et al.*, 2011; Coiffier *et al.*, 2012).

The high potency of romidepsin, like most of the HDAC inhibitors, is due to a crucial functional group that binds strongly to the Zn^{2+} ion in the active site of the enzyme. Nevertheless, this feature of its inhibition seems also to be responsible for undesirable off-target interactions with other metalloenzymes. In order to diminish these consequences, cyclic peptides that exhibit HDAC inhibition in the nM range and lack the Zn^{2+} binding moiety have been described recently. They are active against human cancer cell lines *in vitro* and, thus, could be precursors for the development of new drugs (Vickers *et al.*, 2012).

Another important structural group is the hydroxamic acids, which include the first compound found to inhibit HDACs: the natural product, trichostatin A (TSA; Yoshida *et al.*, 1990). Although TSA has a wide range of anti-cancer effects (Hu and Colburn, 2005; Chan *et al.*, 2013), it has negative side effects that have excluded this molecule from clinical trial. However, a new generation of compounds like vorinostat, panobinostat, belinostat, givinostat and pracinostat (SB939) are already under clinical investigation. Vorinostat (Zolinza®), which inhibits HDAC1, HDAC2, HDAC3 and HDAC6, can induce differentiation, cell growth arrest and apoptosis in various cancer cell lines at μM concentrations, and inhibits tumour growth with low toxicity in murine xenograft models (Marks, 2007). In 2006, vorinostat was approved by the FDA for the treatment of cutaneous T-cell lymphoma in patients with persistent, progressive or recurrent disease (Mann *et al.*, 2007). Moreover, there is an ongoing clinical trial combining vorinostat with two other anti-cancer drugs for the treatment of lymphoid malignancies (Amengual *et al.*, 2013; ClincalTrials.gov identifier: NCT00691210). Panobinostat, a non-selective HDAC inhibitor, exhibits cytotoxic and anti-proliferative activity in several cancer cell lines and induces apoptosis in human tumour xenografts (Vilas-Zornoza *et al.*, 2012). This compound is currently undergoing clinical trials for the treatment not only of several myeloproliferative disorders like multiple myeloma, chronic myeloid leukaemia and Hodgkin's lymphoma (Younes *et al.*, 2012), but also of solid tumours like prostate cancer and ovarian sex-cord tumours (Cassier *et al.*, 2014). Belinostat is a potent HDAC inhibitor that exerts growth-inhibitory and proapoptotic effects in human cancer cell lines and xenografts at nM concentrations (Plumb *et al.*, 2003). Pracinostat (SB939) is a novel HDACi with improved pharmacokinetic properties: it has higher bioavailability and a longer plasma half-life than vorinostat (Novotny-Diermayr *et al.*, 2010). Quisinostat (JNJ-26481585) is a hydroxamic acid-containing inhibitor with potent anti-tumoural activity and encouraging pharmacodynamic properties that are currently investigated in clinical trial (Arts *et al.*, 2009; Venugopal *et al.*, 2013). CHR-3996 is a potent and promising class I HDAC inhibitor with good oral bioavailability and the ability to completely inhibit human tumour xenografts

(Moffat *et al.*, 2010). This compound is also already being used in the clinical setting (Banerji *et al.*, 2012).

A third category of HDAC inhibitor is the short-chain fatty acids, including sodium phenylacetate, phenylbutyrate and valproic acid. These three compounds have weaker inhibitory effects than romidepsin or vorinostat, but they are already used clinically for other reasons (acute hyperammonemia, urea cycle disorders and epilepsy respectively). The well-characterized kinetic properties and side effects of these molecules has led to some of them, such as valproic acid, being investigated to determine their value as anti-leukaemic agents in combination with other drugs (Fredly *et al.*, 2013). In addition, the only HDAC inhibitor that has achieved a phase III clinical trial in solid tumours is valproic acid. These trials shown a significant improvement in progression-free survival of cervical cancer patients treated with valproic acid added to a current standard combination chemotherapy (Coronel *et al.*, 2011).

Benzamides like entinostat (MS-275), mocetinostat (MGCD-0103) and CS055 are another class of HDAC inhibitors. These compunds have an amino anilide group which is responsible for enzyme inhibition and the selectivity for class I HDACs (Bressi *et al.*, 2010). Entinostat administered orally inhibits cell proliferation and growth in human cancer cell lines implanted into nude mice (Saito *et al.*, 1999), and induces apoptosis of B-lymphocytic leukaemia cells (Lucas *et al.*, 2004). It has already undergone clinical trials in combination with other existing anti-tumour drugs (Juergens *et al.*, 2011). Mocetinostat, developed by the biotech company MethylGene, induces apoptosis and exhibits antiproliferative activities against human cancer cell lines and xenografts (Fournel *et al.*, 2008). It is currently in phase II clinical trials for the treatment of haematological malignancies (Younes *et al.*, 2011) and in phase I/II trials with solid tumours (Siu *et al.*, 2008). CS055, a compound structurally similar to entinostat, is a class I selective inhibitor with lower toxicity and better tolerance than other benzamides, and which induces growth arrest, apoptosis and differentiation of leukaemia cells (Ke *et al.*, 2012).

Although HDAC inhibitors were initially included in clinical trials as single therapeutic agents, the tendency to use them in combination with other anti-cancer drugs is increasing. In haematological malignancies, for example, HDACs are aberrantly recruited to nuclear protein complexes and, for this reason, the current DNA-methyltransferase inhibitor therapy is now combined with HDAC inhibtion (Khan and La Thangue, 2012). As HDAC6 has a role in protein degradation, another combined regimen that can enhance the effects of HDAC inhibition involves proteasome inhibitors (Jagannath *et al.*, 2010). Therefore, the synergistic effects achieved by the combination of HDAC inhibition and classical chemotherapy represent a further step forward in cancer therapy research.

Targeting epigenetic readers

Many epigenetic regulators contain specialized domains that allow them to 'read' the chromatin by recognizing specific covalent histone modifications, interpreting them and imposing structural changes (Taverna *et al.*, 2007). Chromatin readers are able to identify different modified amino acids and also different modification states of the same amino acid. For instance, as mentioned above, lysine residues can be affected by several covalent modifications, such as methylation, acetylation, sumoylation and ubiquitination. Another level of complexity is achieved when each lysine residue can undergo several degrees of methylation: unmethylation, monomethylation, dimethylation or even trimethylation. Chromatin readers contain several types of methyl-lysine-recognition motifs like tudor domains, chromodomains, PWWP domains and plant homeodomain fingers (PHD). Each type of domain within a family of proteins can have variants that alter their preferred binding substrate. For example, the PHD finger of the protein ING2 binds to di- and trimethylated lysines (Pena *et al.*, 2006; Shi *et al.*, 2006), while the PHD fingers of DNMT3L and BHC80 prefer binding to unmethylated residues (Lan *et al.*, 2007; Ooi *et al.*, 2007). On the other hand, if a lysine residue undergoes acetylation instead of methylation, a different docking site is generated and it immediately recruits proteins with acetyl-lysine binding motifs, like bromodomains (Jacobson *et al.*, 2000). Finally, adding a new layer of difficulty, many chromatin regulators include several types of reader domains in their structure and the binding at specific chromatin sites depends on the surrounding histone modification map (Ruthenburg *et al.*, 2007).

Many epigenetic readers have been found to be disrupted in a variety of diseases, including cancer (Chi *et al.*, 2010). These chromatin reader domains are novel targets for the development of new therapies against this malignancy. For example, low MW compounds that specifically inhibit the bromodomain family proteins are being studied (Table 3; Dawson *et al.*, 2011).

Bromodomain proteins

Bromodomains are highly conserved motifs which, after identifying and binding to acetylated lysines on histone tails, form a scaffold for the assembly of multi-protein macromolecular complexes that facilitate DNA-templated processes. More than 50 bromodomain proteins are encoded by the human genome and they can be clustered in nine subfamilies according to sequence homology (Filippakopoulos *et al.*, 2012). Bromodomain proteins are physiologically important because experimental knockout of particular proteins of this family in mice results in embryonic lethality (Gyuris *et al.*, 2009).

The bromodomain and extraterminal (BET) subfamily includes four protein members (BRD2, BRD3, BRD4 and BRDT), which contain a tandem bromodomain at the N-terminal. These proteins play a decisive role in the regulation of transcription and cell growth (Figure 4). For instance, BRD4 is associated with a coactivator complex of transcription (Malik and Roeder, 2010) and promotes transcriptional elongation by increasing the processivity of RNA polymerase II, leading to expression of growth-promoting genes (Jang *et al.*, 2005; Yang *et al.*, 2005). BET proteins are usually part of large nuclear complexes that are involved not only in transcription processes, but also in chromatin remodelling, replication and DNA damage (Dawson *et al.*, 2011). Hence, dysregulation of BET proteins has been reported in several diseases such as cancer. BRD2, for example, is overexpressed in lymphocytes of B-cell lymphoma patients (Greenwald

Table 3

Inhibitors of epigenetic readers

Category	Compound	Phase	Tumour type	References
Bromodomain inhibitors				
BET inhibitors	JQ1	Preclinical	NUT midline carcinoma, MLL-rearranged leukaemia, multiple myeloma	Filippakopoulos *et al.*, 2010; Dawson *et al.*, 2011; Delmore *et al.*, 2011
	I-BET151	Preclinical	Multiple myeloma	Chaidos *et al.*, 2014
	I-BET762	Clinical trials	Haematological malignancies, NUT midline carcinoma, solid tumours	NCT01943851; NCT01587703
BAZ2B inhibitors	GSK2801	Preclinical	(Enzymic inhibition of the target protein)	http://www.thesgc.org/chemical-probes/GSK2801
Chromodomain inhibitors				
L3MBTL1 inhibitors	UNC669	Preclinical	(Enzymic inhibition of the target protein)	Herold *et al.*, 2011
L3MBTL3 inhibitors	UNC1215	Preclinical	(Enzymic inhibition of the target protein)	James *et al.*, 2013

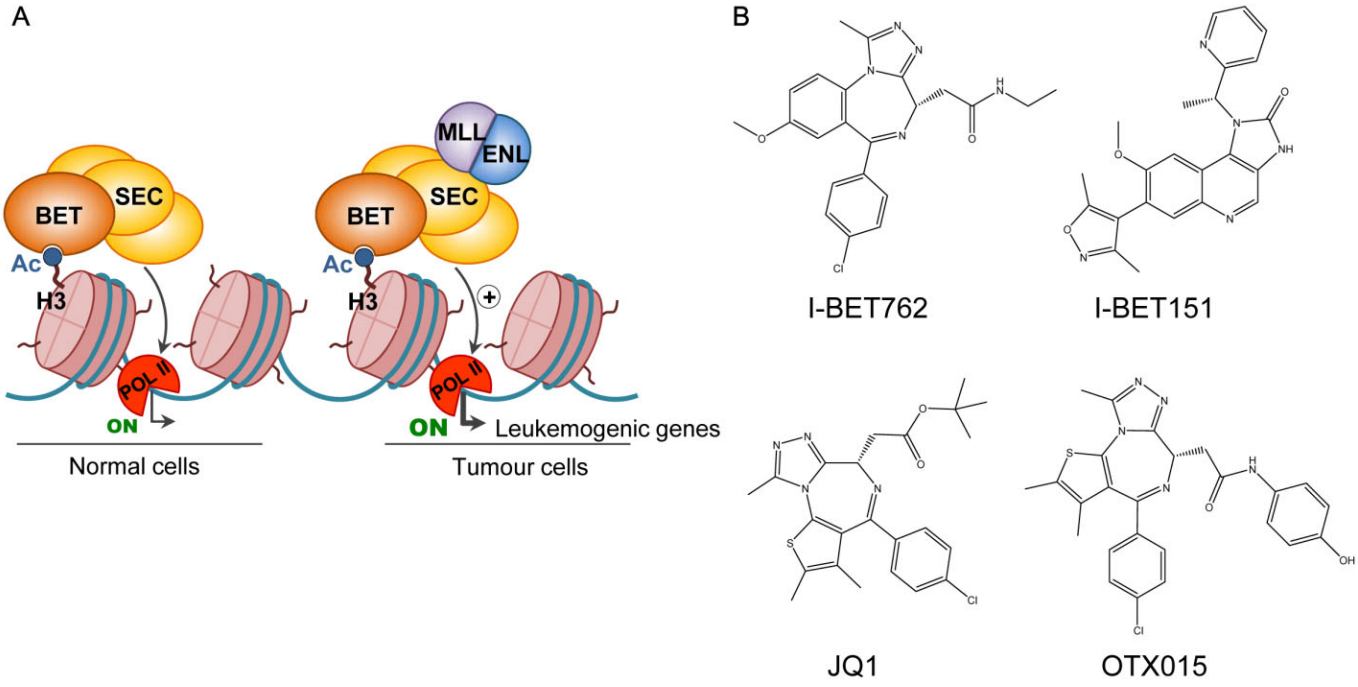

Figure 4

Inhibition of bromodomain proteins. (A) Bromodomains can recognize and bind to acetylated lysine residues on histone tails, recruiting macromolecular complexes that facilitate DNA-templated processes. For example, BET proteins are associated with the superelongation complex (SEC) for the regulation of gene transcription. However, in MLL-translocated leukaemia, MLL-fusion proteins are responsible for an abnormal activity of this complex, which leads to aberrant transcriptional programmes that culminate in disease. (B) The chemical structures of the BET inhibitors JQ1, I-BET762, I-BET151 and OTX015 are shown.

et al., 2004) and recurrent translocations of both BRD3 and BRD4 are drivers of proliferation in the lethal malignancy NUT midline carcinoma (French, 2010). Moreover, BRD4 has been discovered as a therapeutic target in AML (Zuber *et al.*, 2011). All these discoveries have increased current interest in the therapeutic targeting of BET proteins.

BET inhibitors are a new class of molecules designed to block the assembly of a functional protein complex at a particular gene locus by obstructing the interaction of the bromodomain with the acetylated residue (Table 3). To date, some BET inhibitors (JQ1, I-BET151, I-BET762, OTX015, TEN-010 and CPI-0610) have been developed for the treatment of haematopoietic malignancies and the rare NUT midline carcinoma disease (French, 2010; Dawson *et al.*, 2011; Filippakopoulos and Knapp, 2014). The BET bromodomain inhibitor JQ1 induces differentiation and stops the prolifera-

tion of NUT midline carcinoma cell lines and murine xenografts because it is able to displace BRD4-NUT, one of the aberrant fusion proteins responsible for this disease (Filippakopoulos *et al.*, 2010). JQ1 is also highly effective *in vitro* and *in vivo* against AML with MLL translocations, a scenario in which the inhibition of BET proteins reduces the transcriptional activity exerted by the leukaemic MLL fusions and, thus, the transcription of genes that are essential for the maintenance of leukaemia (Dawson *et al.*, 2011). Additionally, JQ1 has a dramatic effect on multiple myeloma cell lines, preventing the binding of BRD4 in the upstream region of the MYC promoter and diminishing the transcription and expression of this potent oncogene (Delmore *et al.*, 2011; Mertz *et al.*, 2011). The BET bromodomain inhibitor I-BET151 is also responsible for reducing the expression of crucial oncogenes such as MYC in multiple myeloma (Chaidos *et al.*, 2014) and the BET inhibitor I-BET762 is undergoing clinical trials for the treatment of haematological malignancies, NUT midline carcinoma and other solid tumours such as N-MYC-amplified lung and colorectal cancers (ClincalTrials.gov identifiers: NCT01943851 and NCT01587703).

OTX015 targets three of the four members of the BET subfamily: BRD2, 3 and 4. In preliminary results from an ongoing phase I trial, the drug shows tolerability and promising clinical responses in some patients with acute leukaemia and other haematological malignancies (ClinicalTrials.gov identifier: NCT01713582). TEN-010 is another BET inhibitor that is already in clinical trials for the treatment of NUT midline carcinoma patients and for the treatment of those cases with advanced solid tumours that do not respond to approved therapies (ClinicalTrials.gov identifier: NCT01987362). The BET inhibitor CPI-0610 is also undergoing clinical trials for the treatment of AML, MDS, multiple myeloma and lymphoma (ClinicalTrials.gov identifiers: NCT01949883, NCT02158858 and NCT02157636).

Concluding remarks

The ongoing research into cancer epigenetics is increasing general knowledge about the molecular bases of this disease and it is now definitely established as an important source for drug development. The epigenetic proteins described in this review represent several targets for the discovery of new active drugs. In fact, the scientific community already has at its disposal inhibitors of reading, writing or erasing of the histone code that have been discovered through different approaches. On the one hand, many studies have shown that amplifications, translocations and somatic mutations in genes that encode for chromatin-related proteins appear to be frequent in cancer, and the discovery of compounds that target the active domain of these epigenetic regulators has been fruitful. On the other hand, targeting protein–protein interactions that confine chromatin elements in particular locations has enabled the discovery of other novel anticancer drugs.

Although some of the existing inhibitors are already in clinical trials for the treatment of various tumour types, there is still a long way to go. Most of the current clinical trials have been based on genetic aberrations of the targeted protein in a specific cancer type but, in some tumours, the epigenetic therapeutic targets are not necessarily mutated. Thus, simple mutational screenings are not enough to enable responses to be predicted. They should be combined with drug sensitivity studies in which specific inhibitors are tested in large well-characterized cell line panels (Barretina *et al.*, 2012; Garnett *et al.*, 2012).

Chromatin proteins are mostly components of larger complexes in the cell, implying that the activity of inhibitors against individual proteins, outside their cellular context, could differ considerably from their activity inside the natural multifunctional complexes. Additionally, these multicomponent functional units are linked with several genes and specific locations along the genome. Depending on the tissue type, the genetic scenery of each cell and the biological circumstances, the same chromatin protein could act as an oncogene or a tumour suppressor, thereby increasing the level of complexity. A full understanding of the biological functions of the target proteins and also a more detailed mechanism of action of chromatin protein inhibitors is still a challenge. In fact, the generation of new active chemical molecules with higher specificity will be decisive in revealing the biological function of new chromatin-associated proteins and discovering other pathways that could also be crucial for tumour development.

Currently, the best anti-tumour therapies responses are achieved by targeting several oncogenic pathways simultaneously. Epigenetic drugs are already used in combination with established cancer chemotherapy and, in some cases, targeting the epigenome could directly reverse transcriptional resistance mechanisms (Stronach *et al.*, 2011). In fact, the regulation of gene expression at the transcriptional level is also the mechanism of the recently discovered bromodomain inhibitors for the silencing of crucial oncogenes in several tumour types. Although more detailed knowledge about how these BET inhibitors work is required, they are very attractive compounds for introduction into the clinical setting. Despite all the well-acknowledged limitations, current epigenetic-targeting molecules are already proving successful in cancer therapy and this is a good basis to motivate basic research scientists, clinicians and also the pharmaceutical industry to look for new weapons in the present and future fight against cancer.

Author contributions

Both authors contributed equally to this work.

Conflict of interest

The authors declare no conflict of interest.

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