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EPIGENETICS-EPIDISEASES-EPIDRUGS

Master Thesis

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Abstract

The mechanism by which genetic expression is modified and regulated is of a main interest in the field of epigenetics. It involves various chromatin-modifying and associated proteins, often referred to as “writers”, “readers” and “erasers”, capable of establishing different modification groups (e.g. methyl, acetyl) on histone residues and consequently recruiting various transcription factors in order to further shape the gene expression. Naturally, a strict regulation of the activity of these proteins is necessary for normal cell functioning. Often it is the deregulation of writers, readers and erasers that leads to aberrant gene transcription and cancer. For that reason revealing the epigenetic mechanisms of cancer at molecular level is essential for understanding the initiation and progression of the disease. In addition, aberrant expression of histone modifying proteins can serve as a direct biomarker of certain types of cancer and an indicator of a proper diagnosis. Identifying the cause of gene deregulation also allows for further development of drugs that can target the cause and eliminate it. A novel concept in the fields of cancer research and epigenetics are the *epi*-drugs – natural or synthetic inhibitors of certain histone modifying enzymes, which are currently considered as potent candidates for anti-cancer therapeutics. The present review aims to introduce the concept of epigenetics, describe its main players, and reveal the possible application of *epi*-inhibitors in the field of cancer treatment.

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1. Epigenetics

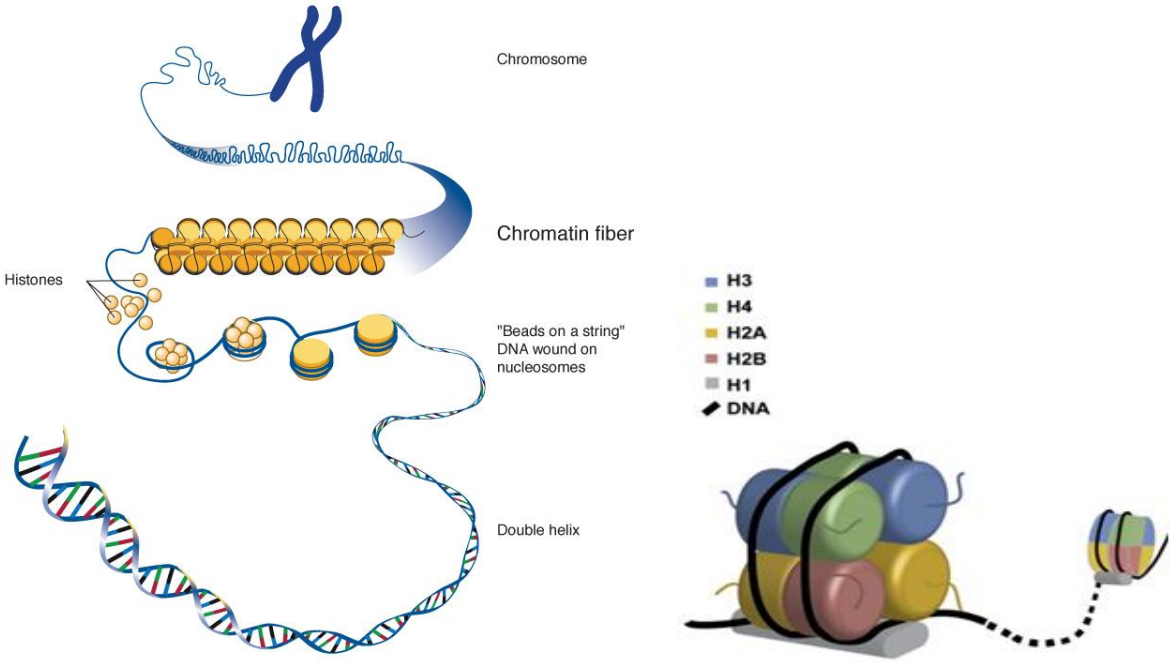
Eukaryotic DNA is densely compressed with the help of nuclear proteins, in order to be incorporated within the nucleus. This compression consists of repeated laps of chromatin around specific nuclear complexes, called *nucleosomes*, which in turn allow the formation of the structurally ordered chromosomes (Figure 1A). Nucleosomes are made of histone complexes, the core components of which are histones H2A, H2B, H3 and H4 (Luger K 1997) (for more clear impression of the nucleosomal composition and its interaction with DNA a brief overview is presented in Box1 and Figure 1B). Each of these histones contains positively charged amine groups, which directly interact with the negative backbone of the DNA and sustain its coiled shape (Alberts B 2008). The function of histone proteins is not solely limited to structural arrangement of the genetic material. They are also notorious for their ability to carry diverse covalent modification groups on various residues, which have both direct and indirect effect on the genetic expression (Allis CD 2007). The modification patterns are distributed across the whole genome and are also present on the DNA itself. They can be heritable and the field that studies extensively their impact on the individual phenotype, without altering the composition of the genome, is called *Epi*genetics (Greek: *επί*- over, above, outer).

Box 1. Nucleosome composition and histone (Figure 1B)

There are five classes of histones in the eukaryotic nucleus: H1; H2A; H2B; H3 and H4. Four of them (H2A-H4, also annotated as the *core histones*) are assembled into an octamer complex (nucleosome). Nucleosomes interact with the DNA in order to shape it into more compact form – chromatin. 146-147 bps of DNA is wrapped approximately 1.6-1.8 times around each nucleosome with about 50 bps (depending of the specie and tissue specificity) of "linker" DNA until the next nucleosome. H1 (known as linker histone) can interact with both the nucleosome and the linker DNA in order to stabilize the structure.

The core histones are assembled in an octameric complex, consisting of two copies of each core histone. The complex includes two H2A/H2B heterodimers, which sandwich a heterotetramer of two H3 and H4 copies. While a major part of the nucleosomal components is located within the C-terminal globular domain of the histones, termed "histone fold", the N-terminal extensions protrude from the main structure (Luger K 1997).

There are many external factors (*epi*-factors), such as stress, poor/unhealthy diet, climate, that alter the epigenetic mechanisms in the cells and affect the genetic expression of a given organism (Skinner MK, 2010; Faulk C, 2011; Hong X, 2012). Such transcriptional abnormalities are often correlated with cancer (Ellis L, 2009; Herceg Z, 2011). Despite the continuous research directed into understanding and treating this disease, it remains still relatively unknown what triggers the initiation, how it progresses and how it can be stopped. Taking all this into account, it now becomes clear that elucidating the regulatory mechanisms and factors of epigenetics is an essential and promising step in many clinically relevant cancer studies, and other fields of genetic research.



A B

Figure 1. A) Organization of the genome into chromosomes. Double helical DNA is wrapped around histone nucleosomes, which in turn form chromatin fibers – the structural unit of chromosome (Austin 2011). B) Schematic overview of the nucleosome. A histone octamer, consisting of two pairs of H2A/B-H3-H4 histones, wraps around it the DNA. Histone tails protrude from the structure and are actively subjected to various modifications (not shown). Figure is adapted from Hamon MA, 2008.

1.1. Histone modifications

Due to their direct impact on the gene expression, *epi*-marks, such as DNA methylation and histone modifications, are a central topic in the field of epigenetics.

DNA methylation is an epigenetic process, in which a methyl group is incorporated on the cytosine nucleotides of the DNA (Jaenisch R 2003). The methylation pattern is specific for different cell lineages with respect to their developmental stage (Holliday R, 1975; Riggs, 1975). This modification is correlated with transcriptional repression and is often localized at CpG (Cytosine-phosphate-Guanine)-rich sites (Wolffe AP 1999). The repressive ability of this *epi*-mark is due to the direct interference with the recruitment of transcription complexes and factors, which in many occasions target the CpG sequences (Jaenisch R 2003). Furthermore, the presence of methyl group on the DNA also facilitates the binding of transcription repressor complexes, which additionally contribute to the silencing effect of this modification. DNA methylation mechanisms are beyond the scope of this review; however, for further information on the role of this epigenetic mark on gene expression, cell fate and tumorigenesis there are some additional reviews recommended: (Jaenisch R 2003; Li XQ 2012).

The next most studied epigenetic modifications, and the main focus of the current review, are the **histone modifications** (Figure 2). They play an essential role in the interaction of DNA with the nucleosomes and provide a binding platform for

DNA/chromatin interacting factors, thereby affecting both directly and indirectly the gene expression.

The modifications can be located at *different residues* (e.g., lysine (K), arginine (R), serine (S), and *etc*.) on any of the four core histones, both on their *globular* (for H2A, H2B and H3) and *N-terminal tail* domains (for H3 and H4) (Berger 2007).

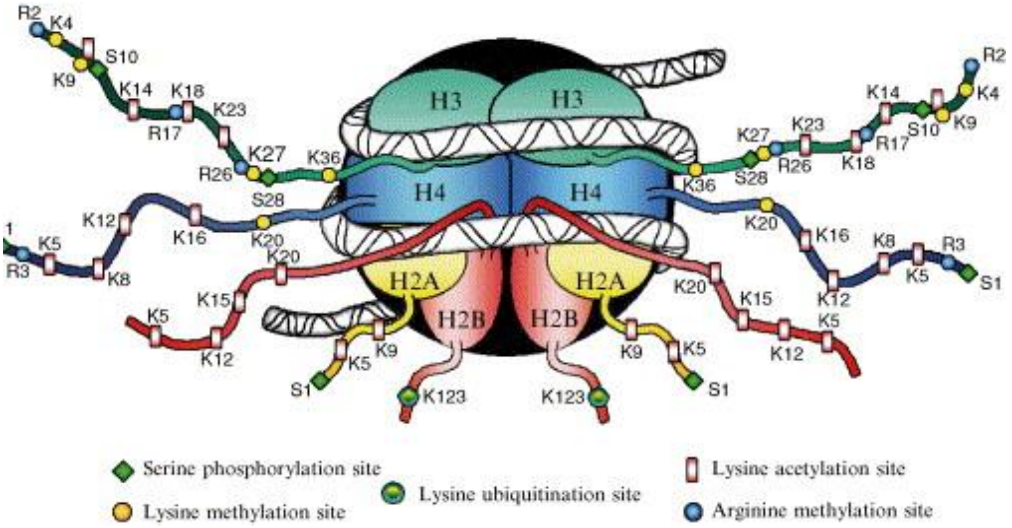


Figure 2. Nucleosome, composed of histone octamer. The histones carry various epigenetic modifications, such as methylation, acetylation, phosphorylation and ubiquitination. Histones are mainly modified on their N-terminal tails; modifications on the main body are also possible (not depicted in the figure). K – lysine; S – serine; R – arginine; (Wood A 2004).

The diversity of histone modifications is further expanded by the variety of modifying groups, which can be added to the residues. As *methylation* and *acetylation* are among to most studied histone modification and due to the fact that they are frequently correlated with cancerous abnormalities, the focus of the current review lies predominantly on them. In addition, there are other modifications, such as *phosphorylation, ubiquitination and SUMOylation*, which are also involved in cellular processes, but are less extensively described (Jenuwein T, 2001; Berger, 2007).

Histone methylation and acetylation are greatly involved in number of cellular processes, starting from cell differentiation and proliferation, to stress response mechanisms and apoptosis (Guil S 2009). Depending on the modified residue and the degree of modification, methylation can be associated with either transcriptional activation or repression. Acetylation on the other hand, is majorly linked with actively transcribed gene regions. Nevertheless, different histone modifications do not act alone. What is observed instead, is a complex combination of various methylation, acetylation (and others, including DNA methylation) marks, which are together required for proper regulation of the transcription. This multileveled interaction dependency between the epigenetic modifications gives rise to what is known as the *histone code*, described extensively in the following section.

1.2. Histone code

The concept of the histone code was first introduced by David Allis in 2000 (Strahl BD 2000). It represents a regulatory system that modulates the expression patterns of the genome (Strahl BD 2000; Chi P 2010). The code is interpreted and regulated by the cell with the help of specific proteins, often referred to as "*writers*", "*readers*" and "*erasers*" (Figure 3).

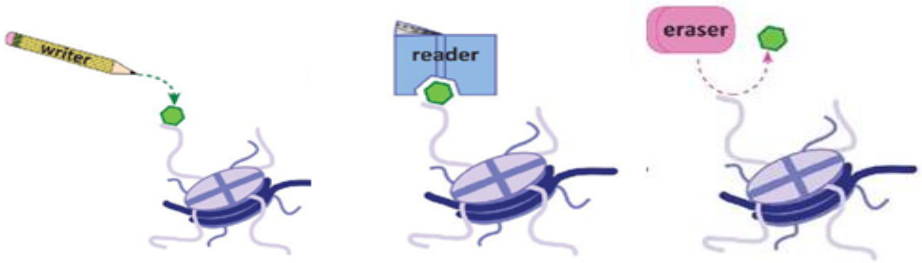


Figure 3. Artistic representation of the concept of histone modifying enzymes. Writers add modifying group to the histone residues through catalytic reaction; readers carry specific recognition sites, capable of binding certain histone modifications; easers remove the modifications through catalytic reaction as well. Adapted from (Gardner KE 2011).

The "writers" (e.g., methyltransferases, acetyltransferases) are modifying enzymes, capable of incorporating methyl, acetyl or other epigenetic groups on histones. There are numerous types of writers with specific preferences towards particular residues; e.g., Polycomb repressive complex 2 (PRC2) marks specifically lysine 27 on H3 (H3K27) (Cao R 2002); DOT1L on the other hand is a H3K79-specific methyltransferase (Feng Q 2002). This target specificity often depends on interactions with additional modifications, like in the case of DOT1L, which is targeted to H3K79 by the presence of mono-ubiquitin mark on H2BK120 (Ng HH 2002). For that reason, the dynamics of the cross-talk between histone modifications is of a crucial importance for the development and viability of the cells. As such the writing of the marks needs to be in a constant strict homeostasis, maintained by diverse feedback mechanisms.

The feedback is mainly provided by the "readers", which recognize specific modifications or combination of modifications on the histones. The readers are effector proteins containing interaction domains capable of recognizing epigenetic modifications. They come in large complexes with wide range of interaction sites and, as such, are able to recruit many additional factors. The main function of the readers, besides providing a regulatory mechanism, is to translate the histone code into a biologically applicable output – transcriptional repression or activation and/or other cellular response (Chi P 2010).

In addition, the "erasers" (e.g., demethylases, deacetylases), which through enzymatic activity remove the histone modification, can also be recruited (both by readers and the modification itself) and, as such, regulate the distribution pattern of the marks.

Due to the complexity of the cross-talk and combination of the *epi*-marks (Vermeulen M 2007; Varier R 2010) and their associated proteins, the histone code had recently been viewed as a *language*, rather than a code (Lee J-S 2010). In such way, the epigenetic marks spread around the genome are interpreted "in the context of time and space", rather than following a specific pattern and always carrying the same meaning. Understanding the grammar of histone language is an essential and indispensable step in treatment of epigenetic abnormalities, as it is the misplaced, misinterpreted or mis-erased *epi*-marks that often result in *epi*-diseases (Chi P 2010). With respect to that, the expression levels of histone modifying enzymes can serve as a direct biomarker of specific types of cancer. Moreover, in cancer accurate diagnosis at molecular level gives an opportunity to target the cause directly with various specific *epi*-drugs. The aim of the current review is to familiarize the readers with the importance of epigenetics and its direct involvement in *epi*-diseases, on the example of cancer. The epigenetic mechanisms behind some types of cancer will be discussed and the attention will be drawn to several *epi*-drugs and their possible applications.

2. Writers, Readers and Erasers

Introducing the main epigenetic players is a crucial step towards understanding the molecular background of cancer. Histone writers and erasers are of a central interest in this review. Their functions range from the control of expression or silencing of fundamental genes to fine tuning and maintenance of a particular transcriptional state. The readers of histone modifications are also largely involved in the control of gene expression. There are several classes of binding proteins, which target and bind acetyl or methyl groups (the main focus of this review). The following section will provide an extensive summary on writers, readers and erasers.

2.1. Methylation

Histone methylation is a major modification that occurs both on lysine and arginine residues. Furthermore, lysine can carry mono-, di- or trimethylation (Upadhyay AK 2011). Depending on the residue and the number of methyl groups, the modification can carry different transcriptional information.

The balance of histone methylation and the distribution pattern along the genome is a strictly regulated process maintained by histone methylation writers – histone *methyltransferases* (HMTs), and erasers – *histone demethylases* (HDMTs).

Due to their direct involvement in tumorigenesis (see *Section 3. Epi-diseases*), HMTs and HDMs can serve as primary medical targets in cancer treatment. The following section reviews the molecular mechanism of action of some main methylation players and their functions in gene expression homeostasis.

2.1.1. HMTs

As noted, histone methyltransferases can install methyl groups on both lysine and arginine residues of histones (mainly H3 and H4). As arginine histone modifications are beyond the scope of this review, arginine methylation will not be discussed here. The presence of methyl group is associated with alterations in the hydrophobicity and steric properties of the nucleosomal structure and acts as a binding platform for various "reader" complexes, containing methylation recognition sites (Upadhyay AK 2011). The marks can be also recognized by methyltransferases, as they also often come in large complexes containing many interaction sites (Upadhyay AK 2011). As such due to their diverse and complex cross-talk with additional factors, methylation marks are linked with both transcriptional repression and activation.

For the incorporation of methyl groups on lysine residues, HMTs employ co-factor S-Adenosyl methionine (SAM) as a substrate and a methyl donor for the methylation reaction (Upadhyay AK 2011; Wood A 2004). Based on their catalytic domain, the lysine

methyltransferases can be subdivided into two classes: *SET domain containing* and *non-containing*.

SET HMTs

The SET domain is a protein domain first recognized in *Drosophila* Su(var)3-9, Enhancer of Zeste and Trithorax proteins (hence – SET), known for its methyltransferase function (Dorn R 1993; Jones RS 1993; Tschiersch B 1994). SET domain is an essential part of almost all HMTs. It comprises approximately 130 amino acids and includes two additional sub-domains: pre- and post-SET, flanking the main domain on both sides. While the interactions between SET and pre-SET determine the target specificity of the methyltransferase, post-SET is mainly involved in the catalytic activity of the domain (see the reviews of Wood A 2004 and Upadhyay AK 2011 for more detailed structural description of SET domain and the molecular background of its catalytic activity). The SET-containing HMTs include large classes of proteins, such as MLL1-5, SET1A/B, SETB1/2, SETD2/3/7/8 and G9a (GLP). Overall, there are above 50 SET domain methyltransferases identified by far.

Non-SET HMTs

Up to present date only one SET non-containing HMT has been described – DOT1L. Interestingly, unlike the other HMTs, DOT1L targets the core of the nucleosome at Lys 79 of H3. The location of this residue is hidden within the globular structures of the histones and is not easily accessible for most of the proteins. The interaction of DOT1L with its target is charge-based and the specificity towards H3K79 is due to its significantly different structural orientation compared to the SET-containing HMTs. For further details, Nguyen AT *et al.* presents an excellent review of DOT1L functions and mechanism of action (2011).

Overall, no matter what their molecular background is – the involvement in gene expression, cell differentiation and tumorigenesis is a common feature of HMTs.

2.1.2. HDMs

The antagonists of histone methyltransferases are the demethylases. These are enzymes possessing the ability to remove the methyl group from histones through oxidizing reactions. Interestingly, it was long time believed that histone methylation is non-reversible and the first histone demethylase (LSD1 or Lysine Specific Demethylase 1) was discovered only few years ago (Shi Y 2004). Up to date it is clear already that the demethylation of histones is in fact a very dynamic process. After the discovery of LSD1, follow up studies identified much larger family of histone demethylases, containing Jumonji C domain (JmjC).

LSD HDMs

LSD family of demethylases includes: LSD1, specific for H3K4me1/2 (Rudolph T 2007) and H3K9me1/2 (Lan F 2007); and LSD2 demethylases, shown to be specific for H3K4me1/2 (Karytinos A 2009).

Both LSDs catalyze the demethylation reaction via a flavin-adenine dinucleotide (FAD)-dependent amine oxidation (Shi Y 2004). Interestingly, these demethylases are capable of removing only mono- and dimethyl groups. The limitation is due to the lack of free electron pair at the trimethylated lysine residues (i.e., all three available pairs are occupied by CH_3 groups), necessary for the conduction of the FAD oxidation reaction.

JmjC HDMs

The second type of demethylases belongs to the JmjC family**.** All members of this family contain JmjC domain. The domain forms a catalytic pocket and interacts through it with Fe(II) and α-ketoglutarate(KG) (Tsukada Y 2006). The interaction is required to catalyze a Fe(II)- and α-KG-dependent dioxygenase reaction, through which JmjC demethylases remove methyl groups from their targets. These structural and catalytic differences allow JmjC HDMs to demethylate trimethylated lysine as well (Klose RJ 2006).

The substrate specificity of JmjC demethylases is broader than the one of LSD, including both H3K4 and H3K9, and, in addition – H3K27, H3K36 and H4K20 (Tsukada Y 2006; Yamane K 2006; Whetstine JR 2006). The substrate specificity depends on the structure of the binding domain, which is ascribed different conformation in accordance with the targeted methyl marks. Moreover, JmjC demethylases interact with additional domains, such as PHD, TUDOR and ARID, which also contribute to the binding specificity.

More details on LSD and JmjC HDMs, their target specificity, mode of action and interaction partners, can be found in the reviews of: Klose RJ 2006, Marmorstein R 2009 and Mosammaparast N 2010.

Overall, histone modifications in various combinations, DNA methylation and additional chromatin-associated factors contribute extensively to the recruitment of various demethylases. Such diversity of interactions is ensured by the fact that histone demethylases often come in large complexes, which include many binding domains. Due to the complexity and broadness of their interactions, histone demethylases are greatly involved in chromatin remodeling and expression. As such, it is unsurprising that they are often found to be related to cell cycle and gene expression abnormalities. Targeting histone demethylases as a potential cause of tumorigenesis is a promising step in cancer treatment.

2.2. Acetylation

Histone acetylation is yet another epigenetic mark, which significantly contributes to the expression patterns of the genome. The presence of acetyl groups on the histones has a direct effect on the charge-based interactions between the nucleosomes and the DNA. Such effect is due to neutralization of the positive histone charge by the presence of acetyl groups on the Lys residues. As such the winding of the negatively-charged DNA loosens up and the chromatin is more accessible to the transcriptional machinery (Hebbes TR 1988). Furthermore, acetyl groups can also recruit various transcriptional factors (TFs) via bromodomains and, hence, stimulate gene expression. Overall, this epigenetic mark is a sign of transcriptional expression and is often found enriched in highly active genomic regions (Sterner DE 2000).

Comparably to methylation, there are two types of enzymes regulating the acetylation patterns across the genome – histone acetyltransferases (HATs) and deacetylases (HDACs). Naturally, proper acetylation of histones is essential for many nuclear processes, e.g., DNA repair, aging, transcription, differentiation, etc. (Carrozza MJ 2003; Murr R 2006; Choudhary C 2009). For that reason deregulated histone acetylation is often related to developmental and cellular abnormalities, which ultimately result in cancer. As such, similarly to the methylation enzymes, HATs and HDACs are proposed as promising targets in cancer treatment research.

2.2.1. HATs

HATs are enzymes able to transfer acetyl group on the ε-amino group of histone lysine residues by utilizing Acetyl-CoA as a co-factor. Once recruited, these complexes often attract TFs and induce gene expression. Furthermore, acetyltransferases themselves are usually found in multiprotein complexes with various subunits and interaction domains (e.g. TUDOR, chromo- and bromodomains, etc.; see Section 2.3 *"Readers of histone modifications"*). As such HATs are able to target specifically wide range of Lys residues throughout the genome. In addition to that, HATs can also come in complex with HDACs (Simone C 2004). This allows them to modify the surrounding areas and spread efficiently the desirable state of chromatin.

Eukaryotic chromatin can adopt two structurally different states: *euchromatin* and *heterochromatin*. Euchromatin is characterized as transcriptionally accessible, lightly packed DNA. Even though it is not necessarily transcribed, euchromatin is largely associated with active genomic regions. Heterochromatin is a more compacted state of the DNA and compared to euchromatin, it is more linked to transcriptional inactivation. There are two types of heterochromatin – facultative and constitutive. Whereas the latter is typical for the poorly transcribed parts of the chromosomes (e.g. centromere and telomeres), the facultative heterochromatin is not necessarily ascribed to such regions. It is a silencing marker established through epigenetic mechanisms and as such it can also be removed. Depending on the cell type and tissue, heterochromatin is used to repress certain genes, which in other case would be expressed (Figure 4).

Based on their catalytic domain there are three major groups of HATs identified by far: GNATs (Gcn5-related-N-acetyltrasferases), p300/CBPs (CREB-binding protein) and MYSTs (Lee KK 2007). Each family comprises a large number of enzymes, targeting differently histones.

The diverse substrate specificity of HATs makes them essential in many cellular processes. For that reason aberrant function of these enzymes is directly linked to tumor initiation and progression. Based on that, HATs have drawn attention as potential anti-cancerous targets in the field of cancer research.

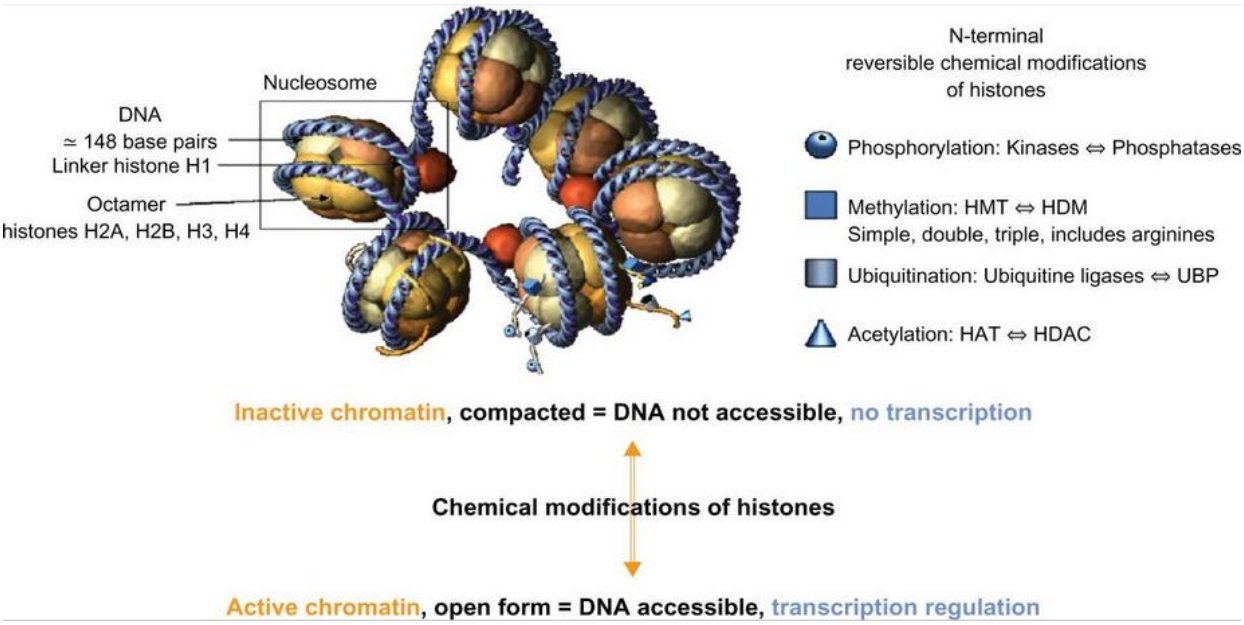


Figure 4. Structural organization of the chromatin and the nucleosomes and the effect of *epi*-marks on it. Chromatin is wrapped around the nucleosomes in a charge-based manner. Presence of epigenetic marks, like acetyl groups, on the nucleosomes interferes with the nature of this charge-interaction and loosens up the winding of the DNA. As such the DNA becomes more accessible to transcriptional machinery and is referred to as euchromatin. Heterochromatin is the compact chromatin, tightly wrapped around the nucleosomes and hard to access (Martinet N 2011).

2.2.2. HDACs

Histone deacetylases, naturally, exert the opposite effect, by removing the acetyl groups from histone Lys residues. Four distinct classes have been identified based on functional and sequence similarity (reviewed by Sun WJ 2012). Class I and II are the classical deacetylases, which include HDAC1 to 10. These enzymes are largely involved in diverse cellular processes and (in the case of Class II) can be localized even outside the nucleus, interacting with non-histone substrates. Class IV includes nuclear-specific HDAC11, which expresses sequential similarities with Class I and II (Gregoretti IV 2004; de Ruijter AJ 2003). The catalytic activity of all deacetylases, except for Class III, is Zn^{2+}-dependent. Class III (SIRT1-7), on the other hand, is NAD^{+}-dependent (Imai S 2000).

As the activity of HDACs opposes the one of HATs, it is natural that these enzymes are correlated gene repression. Due to their direct relation with tumorigenesis and also psycho-neurological disorders (Machado-Vieira R 2011), HDACs are of a major interest in clinical studies, drug innovation and medical application.

2.3. Readers of histone modifications

The modification mark, established on histones, can be recognized by various effector proteins. These proteins carry different binding sites and depending on that they can interact with specific *epi*-marks (Figure 3).

Acetyl groups are mainly known to be recognized by *bromodomains (BRDs)* (Dhalluin C 1999). Bromodomains, first described by Tamkun JW *et al.* in 1992, form a large family of proteins. In humans, there are at least 42 proteins identified, containing this domain (Schultz J 2000; a recommended review on bromodomain protein family: Sanchez R 2009). Even though the overall sequence similarity between them is not high, they all contain a specific, highly conserved, amino acid region. This region is referred to as "BRD fold" and it represents a bundle of α-helices, which are targeting acetyl groups. Interestingly, recently it had been demonstrated that *PHD fingers (Plant Homeo Domain)* can also bind acetylation (Zeng L 2010).

PHD finger is commonly known to bind to methylated histones (review: Taverna SD 2007). It can be found in over 100 proteins in human body and is often described as a part of histone modifying complexes (e.g. HATs, HMTs, HDACs and HDMs). As such it is capable of recruiting these complexes to particular modified sites of histones and trigger further modifications. Alternatively, PHD fingers can also recruit TFs, which modify the gene expression accordingly.

Besides PHD fingers, there are additional binding domains that can interact with methylated histones. These are chromodomains (Z. P. Bannister AJ 2001), TUDOR motifs and MBT (Malignant Brain Tumor) domains (Kim J 2006). Yun M *et al.* provide with an excellent review of the histone modifications readers (2011). Some additional reviews are also recommended for further familiarization with the topic: (Kouzarides 2007; Bannister AJ 2011).

An important note to make is that readers of histone modifications are often part of big complexes, which include other chromatin modifying proteins, transcription factors and repressors. Therefore, the cross-talk between histone modifications becomes even more complex and branched.

3. Epi-diseases

As it becomes clear, epigenetics is a key regulator of the gene expression in eukaryotes. For that reason, imbalance or deregulation of epigenetic mechanisms results in cellular abnormalities at genetic level. Altered gene expression, in particular of genes involved in proliferation, differentiation, tumor suppression and oncogenicity is directly linked to tumorigenesis. Because cancer is a disease, often with poor prognosis and with a considerably low rate of success in treatment, it is of a primary interest in the current review.

The role of epigenetics in cancerogenesis is an extensive topic and its full coverage is beyond the scope of the current review. For that reason, the aim is to draw attention on some clinically-challenging deregulations of histone modifying enzymes and review their effect on cancer development and progression. For further information on histone modification deregulation and cancer, there are some additional, detailed reviews recommended: (Chi P 2010; Varier RA 2011; Blair LP 2012; Sun WJ 2012).

3.1. MLL and AML

Acute myeloid leukemia (AML) is a malignancy in the hematopoietic system (see Box 2), associated with uncontrollable proliferation of leukemic cells and their accumulation in the bone marrow (Lowenberg B 1999). There are several types of AML and accurate diagnostics is crucial for proper drug selection and treatment strategy.

Box 2. Hematopoietic system and leukemia.
Hematopoietic system is involved in the formation and regulation of the blood components in the circulatory system. There are number of cell types produced by the system – all with common stem cell origins, *hematopoietic stem cells (HSC)*. HSCs give rise to different blood cells from both lymphoid and myeloid cell lineages and are also capable of self-renewing. These cells are involved daily in differentiation and proliferation at high turnover rate – approximately $10^{11\text{-}12}$ cells per day (Uribesalgo I 2011). The necessity of accurate regulation of the constantly produced new cells is of a crucial matter, as the amplification of any mutation or abnormality almost ultimately results in leukemic progression.

MLL gene

At genetic level, leukemia has been directly linked to abnormal expression and structural variations of *MLL* gene (mixed-lineage leukemia or myeloid lymphoid) (Biondi S 2000; De Braekeleer M 2005). *MLL* encodes for a H3K4 SET-domain-containing methyltransferase and it is known to have at least 50 interaction partners (Milne TA 2002). For that reason the protein is often found in multiprotein complexes, carrying additional modifying enzymes, such as HATs, HDACs and other HMTs. The protein is greatly involved in the regulation and maintenance of *HOX* genes and as such has influence in various cellular processes (Milne TA 2002). Hematopoietic mechanisms are directly regulated by *HOX* genes and, hence, by MLL, and for that reason aberrant functioning of MLL is often linked to leukemia (Smith E 2011). Such functional abnormalities are due to structural mutations, such as *chromosomal rearrangements* and *partial tandem duplications (PTDs)*.

Partial Tandem Duplications

PTDs result from an in-frame repetition of *MLL* exons in the 5'–3' direction, which ultimately leads to translatable variation of the gene (Dorrance AM 2008). PTD-*MLL* accounts for about 10% of the AML cases and is associated with aberrant expression of *HOXA* genes. Accordingly *HOXA* genes have been linked to hematopoietic mechanisms, and overexpression of certain genes, such as *HOXA5-7-9,* is strongly correlated with leukemic development (Dorrance 2006).

Chromosomal translocations

As for chromosomal translocations in *MLL*, their occurrence is more frequent compared to MLL-PTDs, which assigns them with about 60-70% of all AML cases (Muntean AG 2012). The translocations result in the formation of MLL fusions with additional proteins. Interestingly, a recent study, from the Shilatifard laboratory, demonstrated that the several of the most common leukemic fusion partners of MLL (e.g. ELL, ENL, AF4, AF9, AF10) are part of the super elongation complex (SEC), responsible for transcriptional elongation during gene expression (Smith E 2011). This observation led to the speculation that the aberrant *HOX* gene expression, observed in AML, can be a direct consequence of deregulated SEC activity and, hence, transcriptional elongation in genes which are not supposed to be transcribed (Smith E 2011).

The observed MLL fusions occur between the N-terminus of MLL and the C-terminus of the partner and directly interfere with the SET domain and methyltransferase activity of MLL. The fusion partners, on the other hand, are often seen to interact with various chromatin-modifying proteins. As a result, these fusions are targeted by MLL to its target genes and recruit along additional factors, such as Menin (Smith E 2011), other histone methyltransferases (UTX, DOT1L) (Smith E 2011) and even histone acetylases (Smith E 2011). Naturally, the SEC-associated factors are also being actively recruited. Such complex localization impairment of crucial chromatin modifying factors and complexes, associated with transcriptional activation, results in a highly mis-regulated and unwanted gene expression of developmentally significant genes, such as the *HOX* genes.

3.2. Breast cancer

JARID1B/PLU1 is an example of deregulated HDM in cancer. It is H3K4me2/3-specific demethylase, which plays an essential role in mitotic cell division, cell cycle regulation, development, differentiation, transcriptional regulation and chromatin remodeling. Recent studies show that JARID1B is overexpressed in both breast and prostate cancers (Lu PJ 1999; Yamane 2007 and Xiang Y 2007). Overexpression of JARID1B leads to excessive demethylation of H3K4, which is correlated with repression of important tumor suppressor genes such as CAV1, BRCA1 (Yamane 2007). As JARID1B/PLU1 is a strong transcriptional repressor (Tan K 2003), Barrett A *et al.* studied further which repressor factors it might be interacting with. Interestingly, they

showed that PLU1 is recruiting HDACs of class I and II (Barrett A 2007). As such, deregulated expression of one HMD leads to deregulation of HDACs as well, transcriptional repression of crucial tumor suppressor genes and ultimately – to breast and prostate cancer.

Among other histone modifying enzymes, involved in breast and prostate cancer, are EZH2 and LSD1 (Kleer CG 2003; Lim S 2010). EZH2 is a main component of the methyltransferase PRC2 complex (H3K27me3-specific) and is globally involved in gene silencing. LSD1, as H3K4-specific demethylase, is also associated with gene repression. Moreover, it was also shown to recruit HDACs and induce further transcriptional deactivation at normally active regions (Huang Y 2011). Both EZH2 and LSD1 have been reported to accumulate in breast and prostate cancer at promoters of developmental and tumor suppressor genes (Huang J 2007). As such they are proposed as possible biomarkers of breast/prostate cancer and potent *epi*-drug targets.

Overall, it becomes clear that understanding the epigenetics behind any cancerous development is essential in order to provide the patients with proper diagnosis and successful treatment.

4. Epi-drugs

It is important to understand that epigenetic malfunctions, unlike genetic ones, are reversible. With properly identified targets and successful development of compounds against them, aberrant distribution of *epi*-marks can be corrected or prevented. For that reason the essence of such treatments is to reveal the epigenetic mechanisms of diseases at the most primary levels, study accurately the language of histones and translate the obtained knowledge into promising chemical compounds with high specificity. The specificity of *epi*-drugs is by far a major drawback of this strategy. In many cases the molecular mechanisms of the inhibitors are not precisely identified and for that reason their activity is associated with number of side effects.

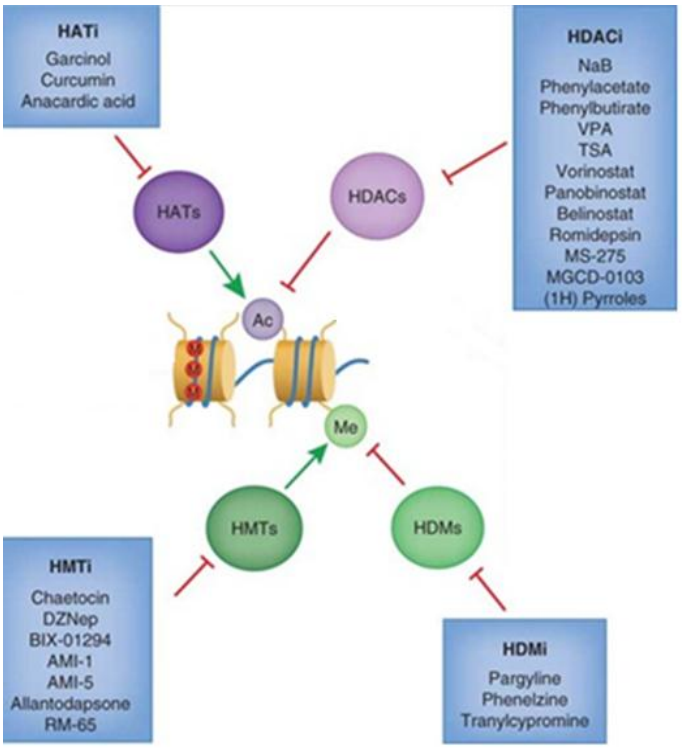


Figure 5. List of HDAC, HDM, HMT and HAT inhibitors, representing potential *epi*-drugs (Rodríguez-Paredes M 2011).

In addition, another limitation of *epi* treatments is the fact that the field in general is relatively unstudied. Due to the excessively complex multi-leveled cross-talks between diverse histone modifications, full efficacy of *epi*-drugs will be hard to achieve in many occasions. Nevertheless, it should be taken into account that epigenetics and its applications in medical research is a newborn field of study. With the current rapid development of novel high-throughput technologies it becomes easier to study the language of histones more extensively and accurately. Interaction partners, binding sites and regulatory mechanisms are described with more precision and the overall nature of interactions between histone modifications and gene expression is becoming more and more understandable. It is beyond doubt that the field of epigenetics, only in its beginning, is already giving rise to a novel, successful direction and hope in the anti-cancer treatment research. This section presents an overview of some of the most promising *epi*-drugs and their potential applications in the treatment of various cancers (Figure 5).

4.1. HMT/HDM inhibitors

Histone methyltransferase and demethylase inhibitors are the less studied *epi*-targets and their development and application is only in its beginning; it is, however, a promising beginning.

4.1.1. HMTi

Chronologically, Chaetocin was the first HMTi described (Greiner D 2005). After its discovery additional compounds were also identified as HMTi. Such are BIX01294 (GLP and G9a specific) (Kubicek S 2007); EPZ004777 (newly discovered DOT1L-specific inhibitor) (Daigle SR 2011). The following sub-sections will give a brief introduction of each one of them and redirect the readers to more detailed reviews and primary sources, if required.

a. Chaetocin is a natural secondary fungal metabolite, produced by the mold species *Chaetomium* (Figure 6). It has been shown to have antimicrobial, anti-inflammatory and, more importantly, anticancer properties (Isham CR 2007).

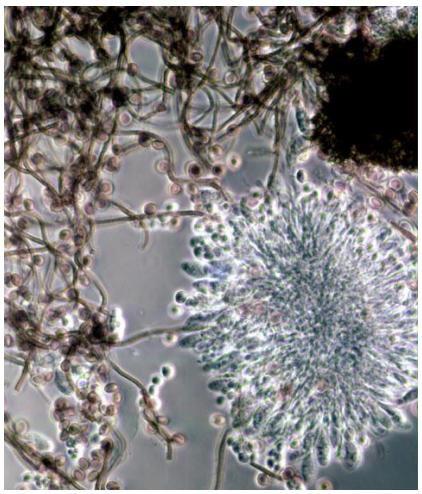


Figure 6. Spores of Chaetocin (doctor fungus 2007).

It inhibits selectively HMTs, specific for H3K9 methylations, such as SUV39H1 (predominantly), G9a, SETDB1, in a SAM-competitive manner (He Y 2012). Furthermore, Chaetocin was observed to induce strong oxidative stress in the cells, with a selective preference towards cancerous and proliferating cells (Greiner D 2005; Isham CR 2007).

It is not well defined yet what is the molecular background of such oxidative stress induction. It is speculated however, that it is due to Chaetocin competing selectively with the substrate of Theorodoxin reductase-1 (TrxR1) (Tibodeau JD 2009). TrxR1 is part of the Thioredoxin (Trx) system, largely involved in cell defence against oxidative stress (Lillig CH 2007).

As Chaetocin both increases the oxidative damage in cancerous cells and inhibits the protective mechanisms of Trx system, it is recently been seen classified as a potential anti-cancer therapeutic compound.

Furthermore, due to its selective inhibitory effect on H3K9 methyltransferases, it has been proposed as a potent *epi*-drug for cancer types with abnormal methylation patterns of H3K9, such as AML (Lakshmikuttyamma A 2010). By far it has already been successfully applied *in vitro* against myeloma cells (Isham CR 2007). Epigenetic studies report promising results, where Chaetocin has been shown to inhibit selectively SUV39H1, reduce the H3K9me3 methylation patterns in cancerous cells and re-express

important tumor suppressor genes (Lakshmikuttyamma A 2010; He Y 2012). Nevertheless, due to its non-fully-resolved mechanisms of action, Chaetocin has yet to be studied and tested, before being safely applied as an anticancer therapeutic.

b. BIX01294 is a small-molecule inhibitor, which specifically targets GLP and G9a HMTs (Kubicek S 2007; Chang Y 2009). In contrast to Chaetocin, its inhibitory effect is assigned to protein-protein interaction alteration (by occupying the histone binding pocket of the HMTs), rather than competition with SAM (Kubicek S 2007).

Importantly, this mechanism of inhibition gives space for further improvement of the compound by modifying its binding site for better interaction and more full coverage of the pocket. Such idea was already implemented in the design of a relative-compound: **UNC0224**. It is a derivative of BIX01294, with slightly different chemical composition, which includes an extension "arm" that covers larger surface area of the targeted histone binding pocket. In a series of further modifications in order to improve the specificity, potency and efficacy in *in vivo* conditions of the drug few new ones were discovered. These are **UNC0321** and the follow up **UNC0638** and **E72**, which display promising results when tested in cancerous cells (He Y 2012).

Inhibiting G9a and GLP specifically is an essential addition in the list of potentially successful *epi*-drugs. These HMTs are involved not only in the methylation of H3K9, but also in non-histone methylation of proteins such as p53 (a tumor suppressor protein). As hypermethylation of p53 has been linked to various types of cancer (Chen MW 2010), the possibility of selectively inhibiting G9a and GLP gives promising direction for anticancer therapy. However, global inhibition of these methyltransferases is also correlated with cancer (Wen B 2009); for that reason there is a need of further studies before G9a/GLP-specific HMTi can be successfully applied in the medical field.

c. EPZ004777, similarly to BIX01294 and all related to it compounds, is a small-molecule drug, which has been shown to inhibit with high selectivity H3K79 methyltransferase (a.k.a. DOT1L) (Bernt KM 2011; Daigle SR 2011). The inhibitory effect is due to the SAM-competitive properties of the drug. The drug was designed as described by Daigle *et al.*, based on the crystal structure of DOT1L active site and the chemical composition of SAM. As such, EPZ004777 was synthesized to compete with SAM for interaction with DOT1L, selectively (Daigle SR 2011). Even though the exact mechanism of action of this *epi*-drug is yet to be described, it has been shown to have promising effects in AML studies, where it selectively led to apoptosis of cancerous cells rather than normal ones (Daigle SR 2011). It is important to stress on the fact, that it is one of the first studies, where the efficacy of *epi*-drugs in anti-cancer therapy was demonstrated *in vivo*. As such, due to its high specificity and positive effects in AML experiments, EPZ004777 proves to be an auspicious *epi*-drug in the future.

4.1.2. HDMi

Identifying potent HDM inhibitors with high specificity is still a relatively new field of research. This is mainly related to the fact that histone demethylases were identified only recently and studying their mechanisms of action is still in progress.

a. Interestingly, the **LSD** class of HDMs shares high sequence similarities with monoamine oxidase A and B (MAO-A/B) and for that reason MAO inhibitors (e.g. *tranylcypromine*) have been actively tested for LSD inhibition. MAOi indeed showed some potency for LSD inhibition; however it was not selective over their original MAO targets and the efficacy was far from desirable when applied in anti-cancerous studies.

Nevertheless, up to date there are several promising candidates (labeled compound 1 to 32) for LSD inhibition. These substances represent a sequential modification of small-molecule MAO inhibitors, in order to optimize to inhibitory effect on LSD1 in *in vivo* conditions. For further pharmacological details, which are beyond the scope of this review, the readers are directed to two more recently published reviews: (Rotili D 2011; He Y 2012).

b. Similarly to LSD, selective and potent inhibitors for the second known class of HDMs – **JmjC**, are yet to be identified. Several compounds, such as 2,4-pyridine-dicarboxylate, have been suggested as potential inhibitors, due to their ability to compete with the co-factors of the demethylation reaction carried by JmjC HDMs (Rose NR 2008). 2,4-pyridine-dicarboxylate has been proposed as selective inhibitor of JARID1B/PLU-1 and, hence, as potential *epi*-drug for certain types of breast cancer. Recently, however, it specificity has been questioned, as it was seen to inhibit other HMDs as well, such as JMJD2A-C (Kristensen LH 2012). Nevertheless, it has been proposed as a scaffold for development of novel, improved HDM inhibitors with higher specificity. As such, few new compounds have already been obtained. By extending the structure of their binding sites, the potency of the compounds was significantly increased. Nevertheless, the overall specificity of HDMi, their *in vivo* viability and potency, and their direct role in anti-cancer therapy has yet to be studied and improved.

An important note to make is the fact that histone demethylases and their potential inhibitors are only in the beginning of their discovery. Further studies and research are highly recommended in this area, as LSD and JmjC demethylases are frequently related cancer development and progression (Mai A 2009; Rotili D 2011; Arrowsmith CH 2012; He Y 2012).

4.2. HAT/HDAC inhibitors

Similarly to histone methylation enzymes, acetyltransferase and deacetylase abnormalities are commonly involved in cancer (Bertrand 2010; Dell'Aversana C 2012; Sun WJ 2012). For that reason targeting specific epigenetic players includes HATs and HDACs as well.

4.2.1. HATi

By far only few natural HAT inhibitors have been identified as potentially promising *epi*-drugs. These include Anacardic acid (isolated from cashew), Garcinol (derived from Kokum) and the popular Indian spice Curcumin (see Figure 7).

Figure 7. Natural sources of HAT inhibitors.

In addition, there are a number of small molecule synthetic products, which were designed to specifically inhibit certain HATs.

The following sub-sections will give a brief introduction of the most commonly used and studied HATi and redirect the readers to more detailed reviews and primary sources, if required.

a. Anacardic acid has been shown to inhibit the activity of P300 and PCAF acetyltransferases *in vitro* (Balasubramanyam K 2003). It is a non-competitive inhibitor, which exhibits its activity by occupying the CoA binding site of its targeted HATs. The activity seems to be potent and highly selective; however a major drawback is that impermeability of the compound through the cell walls, when applied in *in vivo* assays. For that reason, additional semi-synthetic substances, which mimic the activity of Anacardic acid and are more potent *in vivo,* are currently being developed and actively studied in anti-cancer research. Such is the *long-chain alkylidenemalonate*, for instance, which is a structurally simplified version of Anacardic acid and has recently been shown to inhibit P300 *in vivo* (Sbardella G 2008; Ghizzoni M 2010).

b. Garcinol is yet another natural product, extracted from Kokum (spice derived from *Garcinia indica* fruit (Figure 7)), which had shown inhibitory properties towards HATs. Like Anacardic acid, Garcinol is selective for p300 and PCAF and inhibits their activity in a non-competitive manner. The compound binds to the HAT binding side and, hence, blocks the binding of the enzymatic substrate CoA and its targeted histone. Interestingly, the compound had proven to be efficient in both *in vitro* and *in vivo* conditions, the latter of which was performed in HeLa cells. However, it is not clear yet whether this effect is cancer-specific. Nevertheless, by far Garcinol is being investigated as a potential *epi*-drug and is further modified in order to obtain a semi-synthetic compound with less toxicity, more potency at lower concentrations and higher specificity (Balasubramanyam 2004; Arif M 2009).

c. Curcumin is a compound isolated from *Curcuma longa*, which had demonstrated promising effects in cancer-related studies. It was shown that administration of the compound in prostate cancer cells reduces the hyperacetylation, inhibits cell

proliferation and induces apoptosis (Mai A 2009). It is explicitly p300/CBP-specific inhibitor, in contrast to the two previously described compounds (which also targeted PCAF). Furthermore, again unlike Anacardic acid and Garcinol, Curcumin does not occupying any of the binding pockets of its target HATs. Instead it is speculated to induce proteasome dependent degradation of P300/CBP and as such contributes to the total reduction of acetylation in cells.

An important note with respect to Curcumin is the fact that it is highly permeable through cell membranes and has shown great potency in cancer *in vivo* studies (Milite C 2011). Similarly the Anacardic acid and Garcinol, the chemical structure of Curcumin is used as a template for the synthesis of synthetic compounds with various modifications in order to optimize their HAT-specificity, levels of toxicity, potency and efficacy.

d. Besides natural compounds, **small-molecule-HAT-inhibitors** are extensively tested and studied as promising synthetic *epi*-drugs (Mai A 2009; Milite C 2011; Piaz FD 2011). Except for the semi-synthetic products of Curcumin, Garcinol, Anacardic acid and few other potential HATi (Furdas SD 2012), additional chemical structures had shown potent effect as HAT inhibitors. Such compounds are the peptide conjugates Lys-CoA and H3-CoA-20, which were designed to target specifically P300 and PCAF, respectively (Lau OD 2000).

Overall, HATi development and application is a field that still requires further investigations. Nevertheless, due to the inevitable involvement of histone acetylation in gene transcription and the frequently observed functional abnormalities of HATs in various cancers, HAT inhibitors are considered to be a promising step in the field of cancer research.

4.2.2. HDACi

Up to date the most studied class of *epi*-drugs have been the HDACi. Like with the rest of the histone modifying enzymes, aberrant histone deacetylation is a commonly seen abnormality in various cancers and as such targeting histone deacetylases seems as a promising strategy in anti-cancer therapy. HDACi have been notably efficient and great number of these compounds was successfully applied in anti-cancerous studies. Such contrasting success of HDACi compared to HATi and even to HMT/HDMTi is perhaps due to the fact that HDACi are historically notorious for their psycho-neurological application (Gray SG 2006; Abel T 2008; Chuang DM 2009). As such they have already been extensively studied for several decades. There are many FDA-approved HDAC inhibitors (e.g. Vorinostat). HDACi have been used in combination with other chemo-therapeutical compounds in various anti-cancer treatments and in many cases have proven to be beneficial. The following sub-section summarizes the most common classes of HDACi and redirects the readers to more detailed reviews and primary sources, if required.

a. Short chain fatty acids

This class includes three main compounds: *sodium butyrate*, *sodium phenylbutyrate* and *valproic acid* (VPA). The first one is of high interest, as it is directly linked to intestinal homeostasis and colonic cancer. It is a byproduct of anaerobic intestinal bacteria and serves as energy source for epithelial cells. Furthermore, it is involved in inflammatory, carcinogenetic and ROS defensive mechanisms. Interestingly, in cancerous polyps and colon cancers, together with decreased acetylation, a systematic decrease in sodium butyrate has been observed as well (Weaver GA 1988). Because of its HDAC inhibitory activity this compound had been extensively studied as potential anti-cancer therapeutic. A major drawback however is the large concentrations of administration, required for effective results. A relative-compound, sodium phenylbutyrate, is an HDACi which has already been widely used in Phase I clinical trials of several cancer studies (Carducci MA 2001; Camacho LH 2007). However, due to its short half-life it requires further modifications. In addition, these HDACi also affect the global levels of methylation and phosphorylation of histones and for that reason optimization of their selectivity is crucial (Mai A 2009).

Valproic acid is already FDA-approved drug, used for treatment of psycho-neurological disorders. Due to its selective class I and II HDAC inhibitory properties it is currently studied as potential anti-cancer *epi*-drug. Highly potent, it has been tested in phases I and II of AML clinical trials in combination with additional drugs (Kuendgen A 2004).

The mode of action of short chain fatty acids is yet to be described in details. It is however speculated that they either block the release of acetyl group from the targeted histones or function as Zn^{2+} binding group (see section 2.2.2. for HDAC mechanism of action).

b. Hydroxamic Acids represent the most potent (high efficacy at low concentrations), and for that reason – most studied, class of HDAC inhibitors. The main representatives of these inhibitors are *Trichostatin A* (TSA) and *Vorinostat*. Notably, TSA has been shown to exert anti-proliferative and apoptotic properties towards chemotherapy-resistant hepatoma cells (Herold C 2002). Vorinostat (also known as SAHA), on the other hand, had been actively applied against wide range of cancers (Marks 2007) and had proved to be highly efficient. Correlated with cell differentiation, repressed proliferation and re-activation of silenced crucial genes, Vorinostat had already been approved by FDA for anti-cancerous application in combination with additional chemicals (Wagner JM 2010).

As the previous class of HDACi, these two compounds are also selective inhibitors of class II histone deacetylase. As mode of their activity, inhibitory binding to the catalytic pocket of the targeted HDACs has been proposed for both molecules (Song SH 2011).

c. Cyclic Peptides and Benzamides are two additional classes of HDACi, which are both knows to act as competitive binders of Zn^{2+} in the catalytic pocket of HDAC classes I and II. Both have been considered as promising therapeutic agents in anti-cancer research field, where the first one has even been granted official FDA-approval in 2009.

d. Lastly, **sirtuins** are inhibitors of HDAC class III. However, as their direct involvement in anti-cancerous treatment has not been validated yet, these compounds will not be further discussed.

4.2.3. BRDi

In addition to inhibitors of histone writers and erasers, recently a new target for *epi*-drugs has been proposed – namely bromodomains. As explained in Section 2.3, BRDs are recognition domains, specific for acetylation marks. This recognition motif is highly conserved among all BRD-carrying proteins (Mujtaba S 2007), among which are HATs (Nagy Z 2007), HMTs and chromatin-remodeling complexes (Muller S 2011). Many of these bromodomain-containing proteins are often linked to various cancerous abnormalities (Muller S 2011). As BRDs are an active and essential part of these modifying complexes, they have recently been reviewed as potential targets in anti-cancerous research. By far there are already some potent *epi*-drugs identified, targeting selectively bromodomains (Filippakopoulos P 2010), among which is **JQ1**.

JQ1 is a synthetic small-molecule inhibitor, designed to target specifically BRD4 (bromodomain-containing protein, highly involved in the development of AML (Zuber J 2011)). First tested by Filippakopoulos *et al.*, it was shown to compete selectively with BRD4 and inhibit its interaction with acetylated lysine residues (Filippakopoulos P 2010). It was already successfully used in leukemic cells, where it interfered with the active transcription of deregulated genes and prohibited leukemic self-renewal of the cells. As such the leukemic stems cells were observed to undergo terminal differentiation into myeloid both *in vitro* and *in vivo* (Zuber J 2011). It needs to be noted, however, that JQ1 has considerably short half-life (about 1 hour in rodents) and, thus, requires further optimization (Zuber J 2011). Nevertheless, because of its high target specificity, well-characterized effect profile and promising pharmacokinetic properties, JQ1 is one of the newest and most attractive compounds in the field of cancer research and *epi*-drugs.

5. Conclusion

The current review introduced of the concept of Epigenetics (in section 1 “Epigenetics”), familiarized the readers with its main enzymatic players and their mechanism of action (in section 2 “Writers, Readers and Erasers”) and emphasized on their connection with gene expression and cancer (in section 3 “*Epi*-diseases”). The strong interdependence of aberrant transcription, abnormal histone modifications and cancer, gives rise to the idea of *epi*-drugs. These compounds are novel potent drug-candidates in anti-cancer research field. Their mechanism of action and various classifications are explained and exemplified in section 4 “*Epi*-drugs”. The main aim of the review was to stress on the necessity of profound and accurate knowledge in the field of epigenetics, which is required for the optimization of cancer diagnostics, prognosis, and *epi*-drug development and application. With respect to future experiments and research, an important note to make is that *epi*genetics and *epi*-drugs are among the most promising and hopeful weapons against *epi*-diseases and further investigation is strongly recommended.

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