NOVEL EPIGENETIC THERAPIES IN HEMATOLOGICAL MALIGNANCIES: CURRENT STATUS AND BEYOND

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ABSTRACT

Over the last decade transcriptional dysregulation and altered epigenetic programs have emerged as a hallmark in the majority of hematological cancers. Several epigenetic regulators are recurrently mutated in many hematological malignancies. In addition, in those cases that lack epigenetic mutations, altered function of epigenetic regulators has been shown to play a central role in the pathobiology of many hematological neoplasms, through mechanisms that are becoming increasingly understood. This, in turn, has led to the development of small molecule inhibitors of dysregulated epigenetic pathways as novel targeted therapies for hematological malignancies. In this review, we will present the most recent advances in our understanding of the role played by dysregulated epigenetic programs in the development and maintenance of hematological neoplasms. We will describe novel therapeutics targeting altered epigenetic programs and outline their mode of action. We will then discuss their use in specific conditions, identify potential limitations and putative toxicities while also providing an update on their current clinical development. Finally, we will highlight the opportunities presented by epigenetically targeted therapies in hematological malignancies and introduce the challenges that need to be tackled by both the research and clinical communities to best translate these novel therapies into clinical practice and to improve patient outcomes.

Key words: hematological malignancies, epigenetics, targeted therapy

INTRODUCTION

Normal hematopoiesis is a finely regulated process sustained by a population of hematopoietic stem cells (HSC), located within the bone-marrow in adults, that are able to both self-renew and perpetually give rise to differentiated cells throughout the lifetime of an individual. The regulated balance between self-renewal and differentiation into phenotypically distinct mature cells ensures that blood production is maintained during the life-span of an individual [1]. Hematological malignancies arise as a result of dysregulation of this ordered process and include a wide spectrum of diseases characterized by differences in both their clinical behavior and lineage affected. In broad terms, they can be divided in two major groups, myeloid and lymphoid disorders. Within each group, several subtypes are described which differ in their phenotypic manifestations and clinical behavior. Myeloid malignancies comprise chronic conditions such as the myeloproliferative neoplasms (MPN) and myelodysplastic syndromes (MDS), characterized respectively by increased proliferation of differentiated cells and ineffective hematopoiesis with cytopenias, and acute myeloid leukemia (AML), an aggressive and often rapidly fatal disease presenting with both a block in differentiation and increased proliferation. Similarly, lymphoid tumors include both the very aggressive and poorly differentiated acute lymphoblastic leukemia (ALL) as well as tumors of more mature lymphoid cells, including chronic lymphocytic leukemia (CLL), malignant lymphomas and multiple myeloma (MM) which normally arise from more mature lymphoid cells and that can behave in both an indolent or more aggressive fashion based on a variety of factors including their cell of origin, type and number of mutations and interaction with their microenvironment [2, 3].

A practical description of epigenetics is that it relates to aspects of chromatin biology that control gene expression without altering DNA sequence, including the role of RNA-based processes and alterations, including non-coding RNAs, RNA-editing and RNA modifications[4]. Chromatin comprises both DNA and a protein scaffold. Its basic structure, the nucleosome core complex, consists of 2 copies of each of the 4 histone proteins (H2A, H2B, H3 and H4), forming an octamer with DNA wrapped around it. Linker histones such as H1 associate with the nucleosome complex and stabilize DNA wrapping around the nucleosome, promoting the formation of higher order chromatin structures. These, in turn, influence nucleosome spacing on DNA and potentially regulate gene expression although many aspects of these interactions and their consequences remain unclear[5, 6]. Accessibility of the transcriptional machinery to chromatin and alterations of gene expression is determined by many different modifications of the components of the nucleosome complex [7]. Posttranslational modifications (PTM) of histone proteins are amongst the most common covalent modifications of chromatin and several different types have been described including acetylation, methylation, phosphorylation and ubiquitination. Moreover, their role in chromatin biology and regulation of gene expression has become increasingly understood[8]. PTM can act in different ways to regulate transcription. They can modulate non-covalent interactions between DNA and histone proteins, with acetylation being a prime example that is thought to increase chromatin accessibility by altering the overall histone charge thereby weakening the DNA-histone interaction. Moreover, several histone PTM provide direct and indirect binding sites for proteins that either recruit additional epigenetic regulators or components of the transcriptional machinery or that contain catalytic function to further alter the histone modification state,

affecting transcription. These changes in chromatin structure are produced by specific enzymes (the so-called “epigenetic writers”) and removed by other enzymes, (“epigenetic erasers”). The third class of protein, those that specifically bind to the modifications are known as epigenetic “readers” [7]. Overall the role of chromatin structure in the modulation of gene expression and other DNA-templated processes, such as replication and DNA repair, is the result of a finely modulated and dynamic process carried out by different classes of protein that generate or erase specific marks, leading to alterations in chromatin accessibility and binding by epigenetic readers and other associated proteins [Figure 1]. In addition, the methylation status of DNA can also be modified and this dynamic process has been shown to regulate gene expression and potentially other DNA-templated processes and will be described in more details later [9].

Modulation of transcriptional programs has been shown to play a key role in the regulation of hematopoiesis. Evidence that hematopoietic cells fate decisions often occur through epigenetic mechanisms has, in turn, resulted in an increased understanding of the function of chromatin modifiers in hematopoiesis [10, 11]. It is thus not surprising that dysregulation of this process might contribute to the development of hematological malignancies. Corroborating this hypothesis, several genome-scale sequencing studies have demonstrated that transcriptional regulators and chromatin modifiers are recurrently mutated in hematological malignancies, further suggesting that so called “epigenetic” alterations might be central to the development of these neoplasms [12-17]. Moreover, epigenetic changes are plastic and potentially reversible and theoretically provide a more attractive therapeutic target in comparison to the “fixed” nature of genetic alterations. Indeed preclinical and clinical evidence is now gathering in several hematological malignancies that targeting these epigenetic alterations can reset altered transcriptional programs, resulting in an improved therapeutic outcome for patients. The number of epigenetic regulators reported to be mutated/dysregulated in hematological malignancies continues to grow [Table 1]. However, in this review, we will focus our attention mostly on the role of those chromatin regulators whose role in the pathogenesis and as a potential therapeutic targets in hematological malignancies is relatively well understood [18-25] and for whom therapeutic inhibitors have been developed and are currently being tested in clinical trials.

THE ROLE OF ALTERED DNA METHYLATION IN HEMATOLOGICAL MALIGNANCIES AND MUTANT IDH PROTEINS AS NOVEL THERAPEUTIC TARGETS

Regulation and maintenance of DNA methylation plays a pivotal role in embryonic development, cellular differentiation and genome stability[26]. It occurs at the Carbon-5 position in cytosine nucleotides in the context of a 5’-CpG-3’ dinucleotide. This dynamic process results from the balance between active methylation and demethylation. The two *de novo* DNA methyltransferases, DNMT3A and 3B, and the maintenance methyltransferase DNMT1 are mainly responsible for DNA methylation [9]. Conversely active cytosine demethylation is carried out mostly by the TET (ten eleven translocation) family of DNA dioxygenases that oxidize cytosine via a number of intermediates (hydroxycytosine hmC, formylcytosine fC and carboxyl-cytosine caC) through reactions utilizing oxygen, Fe(II) and α-ketoglutarate as cofactors. The base excision repair (BER) pathway, through the enzyme thymidine DNA glycosylase (TDG) +/- AID-APOBEC finally leads to the conversion of the hmC, fC and caC intermediates produced by the TET proteins back to unmethylated cytosine[27, 28]. Generally, methylated cytosine (5mC), particularly in promoter CpG islands, appears to

be associated with transcriptional repression [9] while 5hmC is linked with more accessible chromatin, with an increase in 5hmC documented at the transcriptional start sites of expressed pluripotent genes in embryonic stem (ES) cells [29]. However the correlation between global methylation changes and gene transcription remains generally poor, which might reflect a more complex regulation, contributions from other epigenetic factors and also the genomic location position of the CpG dinucleotide. Finally, in a similar fashion to histone PTM, 5mC is also bound by specific methylbinding proteins, presumably to translate the DNA methylation signal. Similarly, recent evidence suggests that 5hmC and possibly 5fC and 5caC may not just be intermediate stages in demethylation but have independent functions through the binding of specific reader proteins, although the identity and exact consequences of these interactions for DNA-templated processes is not currently understood [30].

DNMT3A

Alteration in proteins involved in DNA methylation are frequently seen in hematological malignancies. Recurrent mutations in *DNMT3A* are found in around 20% of patients with AML [12, 31], as well as T-ALL and malignant lymphomas [32, 33]. These lesions appear early in malignant transformation as "pre-leukemic" mutations [34] and have also been reported in individuals that lack hematological abnormalities as "clonal hematopoiesis" [35, 36]. The mutations are usually hemizygous and are predicted to result in loss of or an alteration of the function of the native protein, with the most frequent mutation found in humans (R882H) appearing to act as a dominant negative [37, 38]. Murine bone marrow cells carrying either deletion of *Dnmt3a* or specific loss of function mutations display increased self-renewal and the respective mouse models develop a pre-leukemic phenotype, although not overt leukemia unless they acquire cooperating mutations[39-41]. *Dnmt3a* mutant cells demonstrate a disrupted methylation profile genomewide, including at CpG rich islands in key promoters, however these changes correlate poorly with gene expression changes at the same loci [39]. Recently, other genomic regions that might play an equally important role in the initiation and/or maintenance of the malignant phenotype have been demonstrated to be affected by changes in methylation. In particular, the borders of large areas of hypomethylation, so called canyons, spanning genes involved in leukemia development have been shown to be particularly affected by loss of function in Dnmt3a which result in changes in gene expression levels for the same genes [42]. Finally the effects of DNMT3A mutations on leukemia development/establishment might involve other DNA-templated processes, as recently suggested for its effects on DNA damage sensing [41].

TET proteins

TET proteins also are frequently mutated in hematological malignancies. Mutations of *TET2* were first described in MDS and MPN and subsequently shown to be mutated in other myeloid malignancies including AML, with a higher frequency of mutations in patients with MDS and chronic myelomonocytic leukemia (CMML)[43, 44]. TET2 mutations are also seen in lymphomas, particularly of the mature T-cell variety [45] and similarly have been described as clonal hematopoietic lesions[35, 36]. *Tet2* knock-out mice display increased self-renewal in *in vitro* and *in vivo* assays, however, they also require cooperating mutations

to develop overt leukemia [46]. Mechanistically, hypermethylation was observed in the promoters of some genes involved in differentiation, such as *Gata2*, with corresponding silencing of expression [46]. Overall these findings suggest that DNA methylation plays a prominent role in leukemia development. DNA methylation also appears to be particularly important in determining the development of pre-leukemic states as indirectly confirmed by the high frequency of mutations in epigenetic modifiers that regulate DNA methylation amongst patients with clonal hematopoiesis [35, 36].

IDH mutations

Despite our improved understanding of their role in the pathogenesis of hematological malignancies, mutations in DNMT3A and TET proteins cannot, at the moment, be targeted therapeutically. However changes in DNA methylation can also indirectly occur as a consequence of mutations in the isocitrate dehydrogenases (IDHs) enzymes. IDHs carry out the oxidative decarboxylation of isocitrate to α-ketoglutarate. In humans, three different isoforms have been described. IDH3 is a NAD^+ dependent mitochondrial isoform whose main role is in aerobic energy production via the Krebs cycle[47]. IDH1 and IDH2 are very similar $NADP^+$ dependent isoforms involved in other metabolic processes, including lipid metabolism and glucose sensing (IDH1) and oxidative respiration (IDH2), and are cytoplasmic and mitochondrially localized, respectively [48]. Recurrent mutations in the genes encoding *IDH1* and *2* have been described in 10-20% of mostly cytogenetically normal AML and tend to be mutually exclusive [49-51]. They have been described in up to 45% of patients with a rare subtype of lymphoma, angioimmunoblastic T-cell lymphoma (AITL)[52, 53], and to a lesser degree in MPN and MDS patients[54, 55]. They are also common in malignant gliomas [56]. The mutations described are gain-of-function and result in neomorphic proteins that bind with increased affinity to the normal IDH product α-ketoglutarate and subsequently lead to its further reduction to the "oncometabolite" 2-hydroxyglutarate (2-HG) [57]. Subsequent accumulation of 2-HG in leukemic cells interferes with a large family of dioxygenase enzymes that require α-ketoglutarate as an enzymatic co-factor. These include the catalytic DNA dioxygenase (such as TET proteins), the Jumonji-C (JmjC) domain-containing family of histone lysine demethylases and the prolyl hydroxylases involved in the degradation of hypoxia inducible factor (HIF) proteins [58, 59]. Inhibition of these enzymes results in increases in both DNA and histone methylation and provides a mechanistic link between mutations in metabolic enzymes and dysregulated transcriptional programs in malignant cells [60, 61]. *IDH1/2* and *TET2* mutations are generally mutually exclusive and patients carrying these mutations exhibit similar DNA methylation profiles, further demonstrating their functional equivalence [49, 60]. Prognostically, *IDH1/2* mutations have been reported as being associated with contrasting outcomes and it is likely that their prognostic significance will depend on specific mutations type and the presence of other compound mutations, as well as patient variables such as age and fitness [49, 62-66].

Mouse and cellular models of *IDH1/2* mutations display defective hematopoietic differentiation and expansion of stem/progenitor cells [60, 61, 67]. Hematopoietic-specific *IDH1* mutant (R132H) conditional knock-in mouse models display marked expansion of HSC and myeloid progenitors in hematopoietic organs but do not develop frank leukemia, suggesting that secondary mutations are needed for AML development[68]. These findings would be consistent with the observation that *IDH* mutations are often encountered early in

the development of hematological malignancies[34]. This finding is corroborated by the finding that they and are commonly found in individuals with clonal hematopoiesis [35, 36]. Moreover, the DNA methylation signature within the HSC and myeloid progenitors compartments of the knock-in mice was strikingly similar to that observed in AML samples carrying *IDH1/2* mutations [68] further suggesting that aberrant epigenetic and transcriptional programmes play a causative role in the establishment of the disease. Similar findings were also shown in a retroviral transduction/transplantation mouse model [69]. These findings together with the observed occurrence of these mutations in humans suggest that IDH mutations are early pre-leukemic mutations that require additional genetic or epigenetic factors to lead to AML development.

Given the novel gain-of-function nature of the IDH mutations, the recent development of inhibitors to mutant IDH1/2 proteins represents an exemplar for therapies targeting altered transcriptional programs in AML. Several small molecule inhibitors of both IDH1 and IDH2 have been developed which dramatically reduce levels of 2-HG and lead to differentiation of leukemic cell carrying the specific IDH mutations[18, 67, 69-71]. These effects also correlate with global changes in DNA methylation/histone modification state, suggesting that the phenotypic effects are at least partially secondary to rewiring of transcriptional programs in the leukemic cells [72] [Figure 2]. Clinical grade inhibitors of both IDH1 and IDH2 have now been developed with AG-221 (Enasidenib), an oral, potent, reversible, and selective inhibitor of the mutant IDH2 protein now being the most advanced. AG-221 has been tested in phase 1/2 clinical trials for patients with AML and AITL/solid tumors carrying *IDH2* mutations (NCT01915498, NCT02273739) [Table 2]. The trial in AML has recently fully reported. Doses up to 650mg once daily were well tolerated. However a dose of 100mg once daily was shown to achieve good steady-state plasma concentration, 2-HG plasma inhibition and clinical activity and was further studied. Overall only 5% of patients discontinued drug due to toxicity. A specific side effect reported was the IDH-inhibitor associated differentiation syndrome that was managed with corticosteroids and drug interruption. In the expansion phase, efficacy was evaluated in AML patients. The overall response rate was 40% in relapsed/refractory AML patients with 19% complete remissions (and the remainder 6.8% complete remission with incomplete hematologic recovery, 6.3% partial remission and 8% morphologic leukemia-free state) with a median overall survival of 9.3 months. In addition, 11% of patients were able to proceed to bone marrow transplant following their response to AG-221. Responses appeared secondary to differentiation of leukemic blasts and were not associated with a reduction in the burden of *IDH2* mutations. Interestingly, reduction in 2-HG levels were also observed in non-responders, thus suggesting that either additional effects contribute to the efficacy of AG-221 or that reduction of 2-HG levels is not sufficient by itself to achieve a clinical response. As also demonstrated for other “epigenetic” therapies, time to response could take up to several months, thus highlighting the need to judge the efficacy of these therapies differently compared to standard chemotherapeutics due to their lack of clear and immediate cytotoxic effects[73, 74]. The promising results from this study have led to the design of a phase 3 trial incorporating IDH inhibitors in the management of AML patients with IDH mutations at disease presentation that is currently recruiting (NCT02577406).

THE EMERGING ROLE OF HISTONE METHYLASES AND DEMETHYLASES AS THERAPEUTIC TARGETS IN HEMATOLOGICAL MALIGNANCIES

Histones are methylated on either lysine or arginine residues by specific lysine methyltransferases (KMTs) and protein arginine methyltransferases (PRMTs)[75]. The intermediate metabolite, S-adenosyl methionine (SAM) acts as the universal methyl donor in these reactions. Moreover the degree of methylation of each amino acid residue can be modulated as lysine can be mono-, di- or tri- methylated while arginine can be mono-, symmetrically or asymmetrically di-methylated, with some evidence to suggest specific functions for these separate modifications. Histone methylation affects transcription and other DNA-templated process by altering the ability of reader proteins to bind to the methylated residues[76].

The functional consequences of histone methylation vary depending on the specific residue modified and/or the degree of methylation. Whereas some modifications such as H3K4, H3K36 and H3K79 are associated with active transcription, others such as H3K9, H3K27 and H4K20 are generally associated with transcriptional repression. Moreover, as already mentioned, the degree of methylation can also influence functional outcomes, with H3K9 being a prime example, as monomethylation is associated with active transcription, but trimethylation associates with transcriptional repression [77]. Mutations involving histone methyltransferases and/or their aberrant activity have been reported in several hematological malignancies and have also been shown to change gene expression programs that are central to the development and maintenance of the disease phenotype [13-15, 78-80].

MLL mutations and DOT1L

The mixed-lineage leukemia (MLL) genes encode for a family of histone methyltransferases. *MLL2 (KMT2D)* is the most common mutated gene in B-cell lymphomas with an incidence of up to 90% in Follicular lymphomas (FL) and up to 30% in Diffuse Large B-cell Lymphomas (DLBL). *MLL2* tend to acquire mostly point mutations, although indels altering the reading frame have also been described, and both of these result in loss of protein function[81, 82]. Mechanistically they have been shown to impair the enzymatic activity of MLL2 leading to a global reduction in H3K4 methylation. Conditional deletion of MLL2 during B-cell development leads to an increase in B-cell proliferation and germinal center (GC) B-cells and cooperates with known lymphoma oncogenes such as *Bcl2* to increase the incidence of GC lymphoma in mouse models. These findings confirm the role of MLL2 as a tumor suppressor whose loss early in B-cell development facilitates lymhpomagenesis via global epigenetic remodeling and transcriptional changes[83].

The *MLL1 (KMT2A)* gene, also referred to as simply *MLL*, is instead mostly mutated in AML either as a result of a partial tandem duplication or as part of a translocation leading to the formation of fusion chimeric protein. *MLL* is required for normal hematopoiesis and embryonic development, as constitutive knock-out mice models are embryonically lethal with reduced functional HSC [84, 85]. A similar requirement for adult hematopoiesis has also been shown in conditional mouse models [86, 87]. *MLL* is involved in chromosomal translocation in the majority of infantile leukemias and up to 10% of adult leukemias, where it is promiscuously rearranged with up to 70 different partners [88]. Interestingly all MLL chimeras lose their C-terminus which contains its methyltransferase activity[89]. However the vast majority of its many translocation partners are members of multi-subunit protein

complexes involved in chromatin remodeling and transcriptional elongation. In particular the most frequent MLL fusion partners (AF4, AF9, AF10, ENL, ELL) are part of either the super elongation complex (SEC) or disruptor of telomeric silencing 1-like (DOT1L) containing complex [90-92].

DOT1L is the only known histone H3 lysine 79 (H3K79) methyltransferase in mammals, where it plays an important role in the regulation of cell proliferation, as demonstrated by the effects of its genetic silencing on mitotic spindle formation and cell cycle progression [93]. Moreover, H3K79 methylation has also been shown to act as a docking site for the DNA repair machinery, thus highlighting a potential role for DOT1L and its specific modifications in other DNA-templated processes [94]. DOT1L is also known to interact with other proteins that might help to explain some of its specific functions. An example of this is its specific interaction with the phosphorylated C-terminal domain of actively transcribing RNA polymerase II (RNAPII), an interaction that is critical to ensure it targets H3K79 methylation to actively transcribed areas of the genome [95]. Whereas DOT1L is essential for embryonic erythropoiesis and development, as demonstrated by the fact that germline *Dot1l* knockout mouse are embryonically lethal [96], its role in adult hematopoiesis appears less crucial. A conditional knockout model specific for the hematopoietic compartment, using the Vav-Cre recombinase, although causing a moderate-to-severe anemia and leukopenia, did not completely eradicate multi-lineage hematopoiesis, suggesting that *Dot1l* might be at least partially dispensable for adult hematopoiesis [97]. Conversely, DOT1L has been shown to be critical for the transformation of murine BM by MLL-fusions (especially when members of the DOT1L complex such as AF9 and ENL are the fusion partner) [98-100]. These data have clinical implications, and suggest a therapeutic window between malignant and normal hematopoiesis, thus validating DOT1L as a potential therapeutic target in AML.

Mechanistically, preclinical models demonstrate that MLL-driven leukemias are particularly sensitive to disruption/inhibition of DOT1L activity and the subsequent effects on specific/direct target genes of the MLL-fusion proteins that demonstrate aberrant H3K79 methylation patterns [97, 101]. Further supporting this model, DOT1L inhibition and loss of H3K79 on target genes of MLL-fusion proteins correlated with specific reduction in the expression levels of genes critical for the transforming activity of MLL-fusions, including the *HOXA* cluster genes and orphan homeobox gene *MEIS1*[97, 100]. Some of the specific effects of DOT1L inhibitors in MLL-fusion leukemias might also derive from the direct interaction of this methyltransferase with several known partners of MLL-fusions [102-104] particularly the AF10 protein, which might lead to the aberrant recruitment of DOT1L to gene targets of MLL-fusions. Based on the collective evidence of a prominent role of DOT1L in leukemogenesis driven by MLL-fusions, several preclinical studies have been reported using DOT1L inhibitors. These inhibitors also work through competitive inhibition of SAM, the methyl donor required as a substrate for the methyltransferase activity of DOT1L [20, 21]. DOT1L inhibitors have been shown to specifically reduce H3K79 methylation marks and expression of MLL-fusions target genes in leukemic cells [Figure 3]. The effects on chromatin structure and transcription correlate with a reduction in the proliferation and viability of MLL-rearrenged leukemias both *in vitro* and *in vivo*. Moreover, recent evidence suggests that DOT1L inhibitors might be efficacious in other subtypes of AML, thus expanding their potential role in AML therapy. Preclinical models of AML associated with other leukemogenic fusion, such as NUP98-NSD1, or carrying MLL partial tandem duplication have been shown to be sensitive to small molecule inhibition with DOT1L inhibitors, suggesting

more widespread roles in AML therapy [101, 105]. Interestingly, AML carrying recurrent mutations such as *IDH1/2*, *NPM1c* and *DNMT3A* mutations have also shown to be sensitive to DOT1L inhibitors [106-108]. While the exact mechanism underlying DOT1L inhibitor sensitivity in *IDH1/2* mutant AML remains unknown, in *NPM1c* mutant leukemias DOT1L inhibition resulted in downregulation of *HOX* and *MEIS1* whose expression *NPM1c* leukemias are highly dependent on. Similarly *DNMT3A* mutant leukemias have been shown to rely on DOT1L activity to maintain H3K79 methylation marks specifically at the canyons that expand following *DNMT3A* loss of function. Since expanding canyons marked by H3K79me2 are enriched for key genes in leukemia development, such as the *HOX* cluster genes, DOT1L inhibition also results in reduced expression of *HOX* and *MEIS* genes in *DNMT3A* mutant leukemias and correlates with reduced cell proliferation, viability and increased differentiation [106]. It is also noteworthy that DOT1L chemical inhibition appears to be better tolerated than its genetic deletion, as only limited toxicity was observed on healthy mice. This might be a result of incomplete inhibition, increased sensitivity of leukemic cells and/or methyltransferase-independent functions of DOT1L that are not targeted by the inhibitors. However, regardless of the exact mechanisms, the promising results provide further support to the clinical utility and further development of these inhibitors.

EPZ-5676, the most clinically developed DOT1L inhibitor, has now been in phase 1 clinical trials (NCT01684150) in patients with advanced hematological malignancies for a few years. The adult dose escalation phase is now complete and despite its pharmacokinetic limitations, where it has to be delivered as a continuous infusions of up to 28 days, EPZ-5676 has been well tolerated with some complete responses observed [109]. An adult MLL-fusion specific expansion cohort is now enrolling patients. More recently a pediatric phase 1 study in relapsed/refractory MLL-fusion leukemias has also produced a preliminary report (NCT02141828), that has shown EPZ-5676 to be well tolerated, albeit that no objective responses were observed in this difficult group of patients[110] [Table 2]. However further clinical investigation alone or in combination with standard or novel therapies has been advocated and such studies are currently under design.

EZH2 and the PRC2 complex

Polycomb repressive complexes (PRC) are critical regulators of tissue homeostasis and cell fate decisions. Two different complexes have been described, PRC1 and PRC2, which act in concert to establish and maintain transcriptional repression through post-translational histone modifications. PRC2 contains three obligate subunits, SUZ12, EED and either EZH1/2, the catalytic components of the complex, and act as H3K27 methyltransferases. PRC1 then recognizes the H3K27me marks through its CBX subunit and subsequently ubiquitylates H2AK119, resulting in the inhibition of transcriptional elongation, chromatin compaction and transcriptional silencing[111]. PRC2 has a critical role in normal hematopoiesis, as demonstrated by the effects of loss of EED on adult hematopoiesis. However not all its components are equally important for normal hematopoiesis as EZH2 appears to be dispensable, possibly because of a degree of redundancy with EZH1 [112]. Interestingly EZH2 is also the PRC2 component most often implicated in the development of hematological malignancies. Genome sequencing studies have identified gain-of-function somatic mutations of *EZH2*, mostly occurring at residue Y641, in around 20 to 30% of GC

high grade DLBL and 10% of low-grade FL [15]. Models of gain of function EZH2 mutations result in increased di- and tri-methylation activity but reduced monomethylation activity, related to a defective recognition of unmodified H3K27 in comparison with wild-type (WT) EZH2. The residual WT EZH2 activity however appears to be important as also suggested by the fact that EZH2 Y641 mutations are hemizygous, with the mutant lymphomas retaining one copy of the WT allele[79, 113]. EZH2 is required for GC development and retroviral or transgenic overexpression of EZH2 Y641 leads to the development of both GC hyperplasia and high grade lymphoma[113-115]. Mechanistically EZH2 Y641 mutations repress transcription of genes involved in B-cell exit from the GC and terminal differentiation and cell cycle checkpoint genes such as *CDKN1a*, through an increase of H3K27 trimethylation at their promoters[114]. However mutant EZH2 also appears able to disrupt WT EZH2 function on genes normally repressed by the PRC2 complex, aberrantly inducing expression of genes such as *Hoxc4/a9* and *Meis1* that might contribute to lymphoma development. These effects appear to be secondary to a global redistribution of H3K27me3 away from focal peaks near the transcription start site, that causes activation of certain genes and towards gene bodies of other genes leading to their transcriptionally repression [113]. In conclusion gain-of-function mutations of *EZH2* in lymphomas act as a truly neomorphic mutations leading to global changes in chromatin structure and subsequent aberrant transcriptional reprogramming that facilitates the development of lymphoma.

Somewhat counter-intuitively, loss-of-function mutations of *EZH2* have also been described in some lymphoid malignancies but mostly in myeloid malignancies including MDS and MPN, where they are also associated with a poor prognosis [14, 17, 116]. In T-cell ALL, up to 25% of cases have been reported to carry loss-of-function mutations in PRC2 members (mostly *EZH2*)[13] and in early T-cell precursor (ETP) ALL, *EZH2* loss-of-function mutations are present in almost 50% of patients [78]. EZH2 therefore appears to act as a tumor suppressor in both these contexts. In ETP-ALL, *EZH2* silencing increased expression of genes involved in the development of disease, specifically stem-cell-associated transcriptional programs and growth/survival signaling genes [117]. In T-ALL, *EZH2* deletion appears mainly to potentiate the effects of NOTCH1 signaling, a prominent feature of T-ALL. NOTCH1 binding causes eviction of EZH2 from its target gene promoters and a reduction of H3K27me3 in the same region. Therefore deletion of *EZH2* further increased NOTCH1 binding to its target promoters and its silencing increased the *in vivo* tumorigenic potential of human T-ALL in a NOTCH1 dependent model. Furthermore EZH2 silencing resulted in decreased apoptosis upon treatment with gamma-secretase inhibitors of NOTCH signaling in human T-ALL cell lines [13].

The pathogenic role of *EZH2* loss in myeloid malignancies is highlighted by the observation that hematopoietic specific *Ezh2*-deficient mice develop MDS and MDS/MPN like disease [118]. Three recent reports have shown that *Ezh2* deletion in a murine MPN model driven by the highly recurrent *JAK2V617F* mutation, induces a more aggressive disease similar to myelofibrosis and increases the repopulation capacity of these cells suggesting a tumor suppressor role for *Ezh2* in this setting. Mechanistically *Ezh2* deletion led to reduction of H3K27me3 with concomitant increased H3K27 acetylation through ill-defined mechanism at promoter regions of several PRC2 target genes, including oncogenes known to play a role in the pathogenesis of myelofibrosis such as *Hmga2*[119-121]. Conversely in the *BCR-ABL* mutated MPN, chronic myeloid leukemia (CML), EZH2 has emerged as a potential

therapeutic target, as CML leukemia initiating cells appear to be particularly dependent on EZH2 activity. Either genetic deletion or chemical inhibition of EZH2 leads to significant loss of leukemia-initiating potential and disease eradication [122, 123]. In AML EZH2 is rarely mutated, however its role has been studied using different models with conflicting results. *Ezh2* deletion in an established MLL-AF9 driven mouse models of AML resulted in a marked reduction of leukemia initiating cells and increased differentiation [124, 125] and prevented the transformation to AML in a MDS mouse model via compensatory repression of key oncogenic genes by the PRC1 complex [126]. While these findings suggest that EZH2 mutation or deletion might retard AML establishment, a recent report has highlighted that reduced expression of EZH2 and H3K27 methylation in several AML models of established disease appears to increase resistance of many AML subtypes to standard chemotherapy or more targeted tyrosine kinase inhibitor treatment. These effects appear to be partly secondary to de-repression of *HOX* genes following low EZH2 activity [127].

Taken together these data suggest that EZH2 can act both as an oncogene and a tumor suppressor depending on the type of mutations, hematological malignancy and cooperating mutations. Although inhibitors of EZH2 are now entering the clinical arena in several hematological malignancies and particularly lymphomas (NCT02082977, NCT01897571, NCT02395601) [Table 2], where they have shown favorable safety and tolerability profile with some good responses[80, 128], these preclinical data highlight that pharmacological inhibition of EZH2 requires to be specifically targeted to certain type of conditions/patients and caution should be taken when considering this therapeutic approach.

Histone demethylases and LSD1

Histone methylation was long considered an irreversible process, until the discovery of histone demethylases that demonstrated that it was indeed dynamic. Two main classes of histone demethylases (KDM) have been described. One class comprises several enzymes sharing the so-called Jumonji (JmjC) domain that act as Fe^{+2} and α-ketoglutarate dependent dioxygenases. The second class of enzymes are a flavin adenine dinucleotide (FAD)-dependent family of amine oxidases[76] The latter class comprises only two members, lysine-specific demethylase (LSD) 1 and 2 [129]. The prototype LSD1 acts mostly as a demethylases for the H3K4me1/2 and H3K9me1/2 histone marks, although it can also demethylate non-histone proteins, including DNMT1 and TP53[130]. Moreover, LSD1 is a multifunctional subunit of both repressive and activating histone-modifying complexes and can therefore act as both a transcriptional repressor or activator in a context-specific manner [131]. LSD1 has been shown to alter the transcriptional programs of different neoplasms [132] and appears to play a significant role in hematological malignancies [19, 133, 134]. In AML, LSD1 was previously demonstrated to be part of an MLL super-complex associated with sites of active transcription even before its function as a demethylase had been uncovered [135]. A recent report used combined analysis of gene-expression data from 23 murine MLL-rearranged leukemias to demonstrate a significant correlation between LSD1 expression levels and clonogenic potential in methylcellulose-based plating assays (a surrogate for leukemic stem cell (LSC) frequency) [133]. In further support for its role in MLL-leukemogenesis, genetic silencing and/or chemical inhibition of LSD1 caused terminal differentiation of AML cell lines and significantly decreased the clonogenic potential of human cell lines, murine leukemias and primary samples with an MLL-

rearrangement *in vitro*. These findings were also replicated *in vivo*, where LSD1 knockdown reduced LSC numbers and function, as measured by reduced engraftment capacity in secondary recipient mice. Functionally, the effects of LSD1 inhibition appear to be related to its ability to specifically modulate the levels of H3K4me2 at genes bound by the MLL-translocation protein, suggesting that pharmacological inhibition of LSD1, could potentially be exploited therapeutically and might specifically alter the oncogenic transcriptional program of MLL-AF9 driven leukemias, whilst largely sparing overall global levels of H3K4me2. Another report demonstrated that the efficacy of LSD1 inhibition could also extend beyond MLL mutated leukemias, where it has been shown to increase the sensitivity of LSCs to the pro-differentiation effects of all-trans-retinoic acid (ATRA) treatment, irrespective of PML-RARA status. These findings suggest that LSD1 might contribute to AML pathogenesis through the inhibition of normal pro-differentiation programs in leukemic cells and proposes it as a novel therapeutic target in several subtypes of AML, especially if combined with ATRA. Mechanistically, LSD1 inhibition did not cause a generalized increase in H3K4me2, but instead specifically increased H3K4me2 levels and relative expression at myeloid-differentiation–associated genes[19]. The role of LSD1 might extend beyond AML as recent evidence suggest that LSD1 is also overexpressed in myeloproliferative neoplasms, myelodysplastic syndromes and in subsets of lymphoid malignancies, thus widening the range of hematological malignancies potentially targetable with LSD1 inhibitor therapy[134].

One caveat to the clinical development of LSD1 inhibitor is that its toxicity towards normal hematopoiesis has not been conclusively established, although normal cells appear less sensitive to LSD1 inhibition than leukemic cells[133]. A study on the role of LSD1 in normal hematopoiesis highlighted that terminal granulopoiesis, erythropoiesis and thrombopoiesis were inhibited upon LSD1 knockdown, however, these effects were reversible upon discontinuation of the inhibitor [136]. Moreover although LSD1 inhibition did not affect the overall frequency of colony forming cells in normal progenitors, it profoundly inhibited erythroid lineage differentiation resulting in severe anemia in the mouse models. These findings suggest that, despite a degree of toxicity, a therapeutic window is present for LSD1 inhibition. This has recently prompted two phase 1 studies using compound GSK2879552 (NCT02177812) and ORY-1001 (EudraCT 2013-002447-29) in patients with AML [Table 2]. The ORY-1001 trial is now complete and recently reported in preliminary form. LSD1 inhibition demonstrated low toxicity and was well tolerated. Furthermore, there was evidence of blast differentiation and partial responses in almost 40% of evaluable patients, suggesting that further investigations are warranted[137].

DYSREGULATED HISTONE ACETYLATION IN HEMATOLOGICAL MALIGNANCIES AND BROMODOMAIN CONTAINING PROTEINS AS THERAPEUTIC TARGETS

Histone acetylation is a highly dynamic process, controlled by two opposing classes of enzymes, lysine acetyltransferases (KATs) and histone deacetylases (HDACs). KATs transfer acetyl groups from the metabolic intermediate acetyl-CoA to the ε-aminogroup of lysine residues in histones and non-histone proteins. Histone lysine acetylation causes neutralization of a positive charge on histone proteins thus reducing their interaction with the negatively-charged DNA, leading to a more open chromatin state that is more permissive to the access of transcription factors and chromatin-associated protein. In addition, as reported for other DNA and histone modifications, the acetylated residues provide further docking sites for transcriptional regulators. Acetylation is considered as a modification that generally facilitates active transcription and several KATs are regarded as transcriptional co-activators. Conversely, HDACs remove acetyl groups from histones, leading to a more compacted chromatin state less permissive for transcription and are therefore generally regarded as transcriptional repressors[138]. Several different classes of both KATs and HDACs have been described and many of these have been found to be recurrently mutated in hematological malignancies [Table 1].

Histone Acetyl Transferases

Cyclic AMP-response element binding, binding protein, *CBP* (also known as *CREBBP* and *KAT3A*) and its paralogue the E1A binding protein p300, generally known as *p300* (*EP300* or *KAT3B*) are among the most commonly mutated KATs in hematological malignancies. The mutations are predominantly hemizygous and loss-of-function, mainly affect the acetyltransferase domain and its activity. They are very frequent in lymphoid malignancies, occurring in up to 40% of DLBL[139] and 60% of FL[140] but also occur with reduced frequency in B-cell ALL[141], T-cell ALL[142] and cutaneous T-cell lymphomas[143]. In myeloid malignancies *CBP* mutations are much less common and sporadic chromosomal translocation affecting *CBP* have also been described [12, 144-146]. CBP and p300 has been shown to act not only through their ability to acetylate histone (including H3K27Ac, H3K18Ac and H3K56Ac) and non-histone proteins but also through binding of other proteins [147]. They regulate several processes during homeostasis and development and the loss of *Cbp* in normal adult murine hematopoiesis causes gradual loss of phenotypic HSCs and differentiation defects that are exacerbated under replicative stress [148]. However the exact mechanism through which CBP contributes preferentially to lymphoid malignancies has only recently been investigated. *Cbp* loss, along with overexpression of *Bcl2* in murine lymphoma models leads to focal depletion of H3K27 acetylation specifically at enhancers of genes involved in B-cell signaling and immune responses and their transcriptional silencing. Moreover *Cbp* loss-of-function appears to leave the same enhancer regions under the unopposed control of the BCL6/SMRT/HDAC3 transcriptional repressor complex. This suggests HDAC3 inhibition as a specific therapeutic vulnerability of *CBP* mutated lymphomas [149]. *CBP* mutations are often early mutations in mature lymphoid malignancies [150, 151] and recent work in murine models has shown that *Cbp* loss in early hematopoietic progenitors was also conducive to the development of lymphoid malignancies through the accumulation of a premalignant population. *Cbp* deleted premalignant lymphoid progenitors were susceptible to increased DNA damage and displayed an altered DNA-damage response

which in the context of increased exposure to physiological DNA-damage during lymphoid ontogeny could explain the high incidence of *CBP* mutations in mature lymphoid malignancies[152].

The role of CBP in the generation and maintenance of myeloid malignancies has also recently been investigated. The AML-specific translocation MOZ-TIF2, which occurs as a consequence of an inversion of chromosome 8, inv(8),[153] has been shown to recruit CBP to activate self-renewal transcriptional programs necessary for leukemic development and indeed MOZ-TIF2 was unable to transform murine progenitors in the absence of CBP [154, 155]. More recently the acetyltransferase activity of p300 has also been shown to target the recurrent AML fusion protein RUNX1/RUNX1T1 (AML1-ETO) and play a key role in the self-renewal and leukemogenic capacity of this recurrent translocation [156]. These findings have prompted interest in the development of small molecule KATs inhibitors [157] and preclinical studies have been published supporting their efficacy in inducing cell-cycle arrest and apoptosis in several AML subtypes. The effects of KATs inhibitors are particularly correlated with downregulation of genes involved in cell cycle and DNA repair and concomitant reduction in the acetylation levels at the promoters of the same genes [155, 158]. Overall the above data support a model whereby CBP acetyltransferase function is important for leukemic transformation and, similarly to other epigenetic modifiers, suggest that the role of CBP in the development of hematological malignancies is cell-context dependent, as it appears to act as both a tumor suppressor and an oncogene in specific cellular contexts. Clinical grade inhibitors of CBP enzymatic activity and bromodomain function (see below) are currently being developed and should be tested in clinical trials soon.

Histone Deacetylases

HDACs comprise a large number of proteins with the ability to deacetylate both histone and non-histone proteins. However, unlike many other epigenetic modifiers, recurrent mutations in HDACs are not observed in hematological malignancies. Instead, their role in the development of leukemias appears mostly secondary to their recruitment by specific oncoproteins to support repressive malignant gene expression programs. This is exemplified in acute promyelocytic leukemia (APML) by the ability of the PML-RARα and PZLF-RARα fusion proteins to act as aberrant transcriptional repressors, in part by recruiting histone deacetylases [159, 160]. HDAC inhibitors have entered the clinical arena and their role in treatment of cutaneous T-cell lymphoma, Hodgkin disease and MM is now more established[161-163]. However, their broader application to other hematological malignancies will likely require a better understanding of their role in malignant transformation and the development of specific rather than generic inhibitors[164].

Bromodomain Proteins

Acetylated lysines are recognized by proteins that contain specific motifs called bromodomains. Amongst this large family are the Bromodomain and Extra Terminal (BET) protein family of epigenetic readers that have emerged as important players in hematological malignancies and as promising therapeutic targets. BET proteins comprise the ubiquitously expressed BRD2, BRD3, BRD4 and the testes specific BRDT [165, 166]. These

protein contain tandem N-terminal bromodomains that bind acetylated lysine residues of histone (and non-histone) proteins and mediate effects ranging from histone modifications, chromatin remodeling and transcriptional activation via recruitment of other partner proteins [167]. BET proteins play an essential role in cellular homeostasis, as knockout mouse models are embryonically lethal [165, 168-171]. Moreover their role in promoting transcriptional dysregulation in many cancer types has been recently highlighted, with BRD4 specifically identified as a key player in AML [22-25, 172-177]. In particular BRD4 is part of a protein complex including the positive transcription elongation factor b (P-TEFb) complex and the SEC and the polymerase-associated factor complex (PAFc), which are essential to the transforming ability of MLL-rearranged leukemias. As a result BRD4 appears to specifically recruit these complexes to active chromatin sites, which leads to the release of RNAPII from proximal promoter pausing and activation of oncogenic transcriptional programs. Inhibition of BRD4 binding to acetylated histone lysines via small molecule inhibitors was able to abrogate the activation of oncogenic MLL transcriptional programs and displayed remarkable efficacy *in vitro* and *in vivo* against MLL-fusion leukemias[Figure 3][22, 23]

Inhibition of BRD4 and other BET proteins has been shown to generate marked changes in gene expression that could not be fully explained by their ability to control transcriptional initiation and elongation. It has now become apparent that BRD4 and BET proteins also regulate enhancer function and in particular large clusters of enhancers, often called superenhancers, that drive the expression of developmentally important genes, including oncogenes such as *BCL-2*, *IRF8* and *c-MYC* critical for the maintenance of the leukemic phenotype [172, 175]. The efficacy of BRD4 inhibitors also appears to extend beyond MLL-rearranged leukemias, as small molecule BET inhibitors have shown promising results in *in vitro* and *in vivo* studies against nucleophosmin (*NPM1*), *FLT3-ITD* and *DNMT3A* mutated AML [174, 175, 177]. In *NPM1c* mutant AML, the cytosolic relocalization of both NPM1c and, via hetero-dimerization, WT NPM1 appears to abrogate an inhibitory interaction between WT NPM1 and BRD4, in turn allowing BRD4 to activate oncogenic transcriptional programs specific to NPM1c AML[175]. However, our understanding of specific mechanisms of action of BET inhibitors and of the requirement of BET proteins in other subtypes of AML remains poor. It is likely that beyond downregulating common transcriptional programs necessary for leukemia maintenance, BET inhibition might play a specific function in different subtypes through distinct interactions with driver mutations and their transcriptional partners. It is worth noting that the role of BET proteins and their therapeutic targeting has now been extended also to other hematological malignancies and specifically ALL, MM and non-Hodgkin's lymphoma[24, 25, 178]. Common aberrant transcriptional programs are present in these malignancies often converging on the nexus of MYC activation. Indeed the efficacy of BET inhibition across these malignancies could be at least partially explained via downregulation of common effectors such as MYC and BCL2, although it is unlikely that the effects of BET inhibitors could be solely ascribed to this mechanism [172].

Although BET inhibitors have demonstrated promising efficacy as monotherapies in preclinical models, there is growing evidence that they might be even more effective in combination with other therapies. Promising results from combination treatment studies in which BET inhibitors were used together with FLT3 tyrosine kinase inhibitors [174], conventional cytostatic compounds (e.g. ARA-C) [173]and HDAC inhibitors [176]have been reported. Combination with other epigenetic therapies has also been reported; a functional

interdependence between DOT1L and BRD4 in regulating transcription at highly expressed genes and in the proximity of superenhancers has been demonstrated. This suggests the possibility of combining DOT1L inhibitors with BRD4 inhibitors to achieve a more complete transcriptional repression in MLL rearranged leukemias [179]. However, a note of caution has been introduced by the modelling of BET inhibitor resistance *in vitro* and *in vivo*, that has suggested that the role of BRD4 in leukemia maintenance could become dispensable through activation of ancillary pathways such as WNT/βCatenin to maintain the critical gene programs and supporting the use of combined therapies to overcome this effect [180, 181].

Based on this preclinical data, inhibition of BRD4/BET proteins has emerged as an exciting therapeutic option in the future therapy of AML and other hematological malignancies and several early phase studies using different BET inhibitors are currently underway [Table 2]. The most mature of these, utilizing the OTX015 inhibitor have recently reported for both AML and lymphoma/myeloma patients. In both cohorts, dose limiting toxicity, mostly hematological, identified an optimal dose of 80mg once daily on a schedule of 14 days on and 7 days off. This has now been carried forward to phase 2 studies, as a tolerated dose with a satisfactory safety profile. Although preliminary clinical activity was a secondary endpoint in both these trials, the number of objective responses were limited to around 10% of patients in both studies, thus suggesting that BET inhibition as a monotherapy might not be particularly effective in the subgroup of highly pretreated patients [182, 183]. However further clinical evaluation of BET inhibitors, particularly in the setting of combinations and earlier phases of disease, is warranted and ongoing with results eagerly awaited.

CONCLUSIONS

The description of the mutational spectrum in hematological malignancies by high-throughput genetic analysis, combined with functional studies has highlighted epigenetic regulators as commonly mutated or dysregulated genes in a majority of hematological malignancies. Therefore altered epigenetic states have emerged as a hallmark of these blood cancers and have led to the development of targeted therapies aimed at eradicating malignant cells through the restoration of normal epigenetic and transcriptional states. Epigenetic regulators represent attractive therapeutic targets as they often have enzymatic activities or binding domains that lend themselves well to small molecule inhibition. However despite good preclinical evidence of efficacy and safety, only a few of these therapies have reached clinical development with encouraging results. It is therefore important to address the potential pitfalls that currently prevent us from taking full advantage of these rationally designed therapies.

One obvious problem is that often hematological malignancies, particularly the most advanced and aggressive such as AML, present a highly heterogeneous and complex clonal architecture which often evolves during the course of the disease and includes subclones possessing unique phenotypic and/or functional properties [34, 184, 185]. As such, a single therapy is unlikely to be able to completely target such complex diseases and the rational development of combination therapies based on sound preclinical studies is necessary to take full advantage of such therapies. Moreover resistance to epigenetic therapies has already been described in preclinical models [180, 181] and is likely to become a problem in the clinic when these agents are used as a single therapy. Another important issue is limiting

the toxicity of such therapies, particularly in combination. Epigenetic regulators are inevitably involved in normal processes within a cell and a rigorous understanding of the effects of inhibiting their function in such processes is necessary to design rational therapies capable of disrupting their malignant activity, while only minimally affecting their normal function. Perhaps particularly relevant in this respect, is a deeper understanding of the role of some epigenetic modifiers as both tumor suppressors and oncogenes in different cellular contexts, as is well demonstrated for both EZH2 and CBP. It is possible that extra caution needs to be exercised when developing these therapies in the clinic, possibly starting with very selected group of patients most likely to benefit from them. As an example, the experience thus far with IDH1/2 inhibitors, which arguably have been the most clinically successful of all epigenetic therapies, suggest that some of these therapies are likely to be beneficial only in highly specific subsets of patients, rather than across different patient subgroups, thus prompting the identification of sensitive genotypes and a more personalized approach in the development of epigenetic targeted therapies Another important area that will require better understanding is the role of epigenetic therapies on the immune system and in turn immune mediated tumor responses. Studies on patient samples and cell lines treated with demethylating agents suggest that these therapies might cause upregulation of PD-1 and other immune inhibitor ligands and receptors and that these effects might correlate with resistance to epigenetic therapies[186]. Conversely, studies on mouse models and cell lines representative of several hematological malignancies have highlighted a putative role of BRD4 inhibitors in downregulating the expression of the immune inhibitory molecule PD-L1. These effects might contribute to the efficacy of such therapies and potentially synergize with antibodies directly targeting the PD-1L/PD-1 axis[187]. The above findings further suggest that a deeper understanding of the broader effects of epigenetic therapies is required to fully take advantage of their antitumor effects and to best target their use towards a patient population most likely to benefit from them.

Some practical aspects need also to be addressed to ensure a more rapid clinical development of the many epigenetic therapies that have been developed in recent years. One obvious problem is that most clinical trials tend to use these therapies as single agents in highly pretreated populations, thus decreasing the likelihood to observe significant efficacy. This strategy runs the risk that promising agents might be overlooked for further clinical development because of a lack of single-agent efficacy. A more realistic and likely successful strategy would be to expedite their use in combination with standard therapies or other targeted therapies based on rationally designed combinations, designed on the basis of preclinical studies. Another appealing strategy would be to use them in previously untreated patients, perhaps not suitable for standard therapies, to fully ascertain their therapeutic efficacy. Moreover some of these therapies lack clear cytotoxic effects *in vitro* and *in vivo* and require prolonged treatment to achieve a therapeutically discernible effect. In particular in more aggressive diseases, therefore, they might be better suited as a maintenance therapy following a debulking treatment from standard therapies, perhaps with their efficacy monitored by effects on gene expression, epigenetic marks and rationally designed biomarkers of response.

In conclusion, despite the remarkable advances in our knowledge of the role of dysregulated epigenetics in hematological malignancies during the last decade, their translation into therapeutic advances for patients still requires significant efforts. Combining an even deeper

understanding of the epigenetic mechanisms leading to malignant transformation and the mechanisms of action of epigenetic inhibitors together with rationally designed clinical trials that aim to limit toxicity while enhancing efficacy of these therapies will be required if we are to fulfill the promise of such therapies in hematological malignancies.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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Table 1. Epigenetic regulators most recurrently mutated in hematological malignancies

EPIGENETIC REGULATOR	MUTATION TYPE	DISEASE
EPIGENETIC WRITERS		
DNA METHYLTRANSFERASE		
DNMT3A*	Point mutation	AML/MDS/MPN/TCL
	Indel	
DNA DIOXYGENASES		
TET2*	Point mutation	AML/MPN/MDS/TCL
	Indel	
HISTONE METHYLTRANSFERASE		
KMT2A (MLL1)	Translocation (MLL-X)	AML/ALL
	Partial Tandem Duplication	AML
KMT2D (MLL2)	Point mutation	BCL
KMT2C (MLL3)	Point mutation	BCL/AML
KMT6 (EZH2)	Point mutation	BCL
	Indel	MPN/MDS/AML
HISTONE ACETYLTRANSFERASE		
KAT3A (CBP)	Translocation (MLL-CBP, MOZ-CBP)	AML
	Point mutation	BCL
KAT3B (EP300)	Translocation (MOZ-EP300)	AML
	Point mutation	BCL
KAT6A (MOZ)	Translocation (MOZ-CBP, MOZ-TIF2)	AML
KAT6B (MORF)	Translocation (MORF-CBP)	AML
EPIGENETIC ERASERS		
HISTONE DEMETHYLASES		
KDM5A (JARID1A)	Translocation (NUP98-JARID1A)	AML
KDM6A (UTX)	Point mutation	TCL/AML
METABOLIC ENZYMES		
ISOCITRATE DEHYDROGENASE		
IDH1*	Point mutation	AML/MDS/MPN/TCL
IDH2*	Point mutation	AML/MDS/MPN/TCL
MEMBERS OF EPIGENETIC REGULATORS PROTEIN COMPLEXES		
ASXL1*	Point mutation	AML/MDS/MPN

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; BCL, B-cell lymphomas; MPN, myeloproliferative neoplasms; MDS, myelodysplastic syndromes; TCL, T-cell lymphoma/leukemia;

CBP, CREB binding protein; EZH2, enhancer of zest homolog 2; IDH, isocitrate dehydrogenase; JARID1A, Jumonji AT-rich interactive domain 1A; MLL, mixed lineage leukemia; MORF, MOZ-related factors; MOZ, monocytic leukemia zinc finger; NSD, nuclear receptor binding SET-Domain ; NUP98, nucleoporin 98kDa; TET, ten-eleven translocation; TIF (NCOA2), nuclear receptor coactivator 2; UTX, ubiquitously transcribed tetratricopeptide repeat, X chromosome; ASXL1, additional sex comb-like 1

* These mutations are also encountered recurrently in clonal hematopoiesis

Table 2. Main clinical trials of novel epigenetic therapies in hematological malignancies as of May 2017*

EPIGENETIC TARGET	TRIAL NUMBER	DRUG	PHASE	DISEASE	STATUS
Histone Demethylase inhibitors					
LSD1	NCT02177812	GSK2879552	1	AML	Recruiting
	EudraCT 2013-002447-29	ORY-1001	1	AML/ALL	Completed
Histone Methylase inhibitors					
DOT1L	NCT01684150	EPZ-5676	1	AML/ALL	Recruiting[1]
	NCT02141828	EPZ-5676	1	AML/ALL-PEDS	Completed
EZH2	NCT02395601	CPI-1205	1	BCL	Recruiting
	NCT02082977	GSK2816126	1/2	BCL/MM	Recruiting
	NCT01897571	Tazemetostat	1/2	BCL	Recruiting
Bromodomain inhibitors					
BET proteins	NCT02158858	CPI-0610	1	MNP	Recruiting
	NCT01943851	GSK525762	1/2	HN	Recruiting
	NCT01713582	OTX015	1	HN	Completed/ Reported
	NCT02308761	TEN-010	1/2	MNP	Recruiting
Metabolic enyzmes inhibitors					
IDH1	NCT02074839	AG-120	1	$IDH1^{mut}$ HN	Recruiting
IDH2	NCT01915498	AG-221	1/2	$IDH2^{mut}$ HN	Ongoing/ Reported
	NCT02577406	AG-221	3	$IDH2^{mut}$ AML	Recruiting
	NCT02273739	AG-221	1/2	$IDH2^{mut}$ TCL	Completed

*only trials involving the targets discussed in the review are presented; [1] only recruiting for extension phase

BET, bromodomain and extra-terminal domain family; DOT1L, disruptor of telomeric silencing 1-like; IDH, isocitrate dehydrogenase; LSD, lysine specific demethylase; EZH2, Enhancer of zeste homolog 2; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; PEDS, pediatric; BCL, B-cell lymphomas; MM, Multiple myeloma; MNP, myeloid neoplasms; HN, hematological neoplasms; TCL, T-cell lymphoma;

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Conflict of Interest Policy

Article Title: EPIGENETIC THERAPIES IN HEMATOLOGICAL MALIGNANCIES, CURRENT STATUS AND BEYOND	Author name: PAOLO GALLUZZI

Declarations

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NONE

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Article Title:	Author name:
Epigenetic therapies in haematological malignancies: Current status and beyond	BRIAN HUNTLY

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BRIAN HUNTLY.

Figure Legends

Figure 1. Diagram of different types of epigenetic regulators

Schematic of epigenetic regulators involved in histone methylation and acetylation are respectively shown in panel A and B. The epigenetic machinery consists of three classes of proteins, writers, readers and erasers. Epigenetic writers are enzymes catalyzing covalent histones modifications such as methylation (Me) or acetylation (Ac) of specific lysine residues. Examples of such enzymes are the mehyltransferases, EZH2 and the acetyltransferases, CBP. Conversely epigenetic erasers, such as the demethylase LSD1 and multiple histone deacetylases (HDAC), catalytically remove these histone modifications. Epigenetic readers are proteins containing highly specialized domains, such as plant homeodomain (PHD) fingers and bromodomains (BRD) which recognize and specifically bind to unique histone modifications, in this case lysine methylation and acetylation respectively. Manipulating these regulatory processes forms the basis of therapeutic targeting the epigenome.

Figure 2. Mechanism of action of IDH1/2 inhibitors

(A) Isocitrate dehydrogenase (IDH) 1 and 2 mutations give rise to neomorphic proteins that bind with increased affinity to the normal IDH product α-ketoglutarate and subsequently lead to its further reduction to 2-hydroxyglutarate (2-HG). The accumulation of 2-HG in leukemic cells inhibits dioxygenase enzymes that require α-ketoglutarate, including the TET family of DNA dioxygenase and the Jumonji-C (JmjC) domain-containing family of histone lysine demethylases. Inhibition of these enzymes results in aberrant DNA and histone methylation patterns that direct altered transcriptional programs, causing a block in cellular differentiation. (B) The highly specific IDH1 and 2 inhibitors (AG120 and AG221, shown) block the neomorphic activity of the mutant proteins specifically, restoring normal levels of 2-HG and DNA and histone methylation patterns within the cell. This in turn leads to reactivation of transcriptional programs leading to normal cellular differentiation

Figure 3. Model for the mechanism of action of DOT1L and BET proteins inhibitors in MLL fusion leukemias

(A) The MLL fusion protein are composed by the aminoterminal domain of the MLL protein and several translocation partners that are usually members of multi-subunit protein complexes involved in chromatin remodelling and transcriptional elongation. The aminoterminal domain of MLL proteins, which is uniformly preserved in MLL fusions, physically interacts with the polymerase-associated factor complex (PAFc) while its most frequent fusion partners (AF4, AF9, AF10, ENL, ELL) are part of either the super elongation complex (SEC) or disruptor of telomeric silencing 1-like (DOT1L)-containing complex. The SEC also includes the positive transcription elongation factor-b (PTEFb), composed of CDK9 and cyclin T1 or T2, and this complex phosphorylates (P) RNA polymerase II (RNA POL II) facilitating transcriptional elongation. BRD4 is a BET protein known to physically interact with both PAF

and SEC-PTEFb and to bind acetyl lysine residues (Ac) on multiple histones. As a result BRD4 specifically recruits the SEC-PTEFb complex to active chromatin, leading to transcription of MLL fusion target genes and activation of an oncogenic transcriptional program. The lysine methylase DOT1L is also known to interact with several fusion partners of MLL translocations, such as AF4, AF9, AF10 and ENL. The interaction of DOT1L with AF9 leads to its aberrant recruitment to MLL-AF9 fusion target genes. At these target sites, DOT1L methylates lysine 79 of histone 3 (H3K79) using S-(5`-adenosyl)-l-methionine (SAM) as a donor of methyl (Me) groups. H3K79 methylation is known to be an activating mark which facilitates transcription of MLL target genes. (B) EPZ-5676 is a SAM mimetic which inhibits the methylase activity of DOT1L, reduces H3K79 methylation at MLL-AF9 target genes and leads to transcriptional suppression of the MLL-AF9 oncogenic program. Similarly, bromodomain inhibitors (BRD INH) inhibit the interaction of BRD4 with acetyl-lysines and displace the SEC-PTEFb complex from active chromatin, thus inhibiting the transcription of MLL fusions target genes and suppressing its oncogenic programs.