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## **Harnessing the potential of epigenetic therapies for childhood AML**

Ashley A. Newcombe<sup>a</sup>, Brenda E.S Gibson<sup>b</sup> and Karen Keeshan<sup>c\*</sup>

a Beatson Institute for Cancer Research UK, Glasgow, Scotland, UK

b Department of Paediatric Haematology, Royal Hospital for Children,  
Glasgow, Scotland, UK

c Paul O'Gorman Leukaemia Research Centre, Institute of Cancer Sciences,  
University of Glasgow, Scotland, UK

\* Corresponding author

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## **Abstract**

There is a desperate need for new and effective therapeutic approaches for AML in both children and adults. Epigenetic aberrations are common in adult AML, and many novel epigenetic compounds are in clinical development that may improve patient outcomes. Mutations in epigenetic regulators occur less frequently in AML in children compared to adults. However, investigating the potential benefits of epigenetic therapy in paediatric AML is an important issue that will be discussed in this review.

## **Introduction**

Acute myeloid leukaemia (AML) results from clonal expansion of primitive myeloid cells that are incapable of differentiation. These clones have acquired genetic and epigenetic alterations that cause uncontrolled proliferation at the expense of normal haematopoiesis, leading to bone marrow exhaustion. The recent identification and characterisation of many of these alterations (Papaemmanuil et al. 2016) creates the opportunity for the development of targeted treatments. Cancer genome consortiums have identified genes commonly mutated in AML, many of which are associated with epigenetic regulation (Genovese et al. 2014; Jaiswal et al. 2014; Xie et al. 2014; The Cancer Genome Atlas Research Network 2013a), indicating that chromatin modifiers play key roles in malignant transformation and exposing novel epigenetic targets for cancer drug discovery. Whilst there are biological, molecular and clinical distinctions between adult and paediatric AML, therapies tested in adult patients in clinical trials have often been extrapolated across the age spectrum and used in paediatrics with clinical benefit. Epigenetic therapy has become an important area of research with many novel compounds being tested in adult AML; it is therefore pertinent to examine those that may be of benefit in children. This review will summarise the epigenetic landscape of adult and paediatric AML, the progress to date in epigenetic therapies for adult AML and finally their potential benefit in childhood AML.

AML is the most common acute leukaemia, affecting 3 out of every 100000 people in the UK (Cancer Research UK). The risk of developing AML is age-associated, with

the incidence rising from 7 cases per million per year in children to 170 cases per million per year in adults over 60 years (Cancer Research UK). There is a desperate and urgent need for better therapies for both children and adults with AML. Survival rates have remained low in adults (overall survival (OS) 20% at 5 years (Cancer Research UK); and although survival rates are higher in children (OS 70% at 5 years), intensive chemotherapy regimens are burdensome and relapsed/refractory AML account for half of childhood leukaemia related deaths (Moore et al. 2013). Whilst the last few decades have seen an improvement in survival of children with AML, this has been due to a combination of a more strategic use of stem cell transplantation and better supportive care reducing treatment and transplant related mortality (Kaspers et al. 2013; Perel et al. n.d.; Burnett et al. 2013; Gibson et al. 2011; Stevens et al. 1998). There have been very few new drugs that have brought benefit to patients and relapse remains the main cause of death. Epigenetic therapies may bring benefit by reducing relapse, however it is important that their toxicity profile be clarified as this may be different in children than adults.

### **Epigenetic landscape of AML**

Epigenetics describes how heritable changes to gene expression can occur in the absence of any change to the underlying DNA sequence (Egger et al. 2004). The epigenetic landscape controls gene expression through a variety of chromatin modifications, for example DNA methylation and histone post-translation modifications. Some of these modifications alter the accessibility of DNA to transcriptional machinery; others enable chromatin mediated signal transduction or the direct recruitment of DNA replication machinery. Epigenetic dysfunction is common to most cancers (Jones & Baylin n.d.; Sharma et al. 2010). Compared to other cancer types AML has few mutations (The Cancer Genome Atlas Research Network 2013a) and as mentioned, many of these mutations affect the epigenome. This finding illustrates the importance of epigenetic regulation in normal haematopoiesis, and importantly, opens up the potential for a plethora of novel targets

in cancer therapy. Mechanisms of epigenetic deregulation have been widely studied in AML and the most frequently occurring will be briefly summarised below.

### *Aberrant DNA methylation*

Disordered methylation patterns are a hallmark of AML (The Cancer Genome Atlas Research Network 2013a; Figueroa, Lugthart, et al. 2010) and have been implicated in gene silencing of tumour suppressor genes and genomic instability. More recent work has implicated both hyper- and hypo-methylation in malignant transformation (Akalin et al. 2012; Berman et al. 2011). Intriguingly, these methylation signatures have prognostic relevance and can distinguish between subtypes of AML, indicating a causal role of aberrant methylation in AML (Saied et al. 2012; Figueroa, Lugthart, et al. 2010). DNA methylation is established by DNA methyl transferases (DNMTs). DNMT3a is a *de novo* methyl transferase that regulates DNA methylation and is one of the most frequently mutated genes in adult AML (Ley et al. 2010), highlighting the importance of methylation patterns for normal haematopoiesis and AML pathogenesis. Mutations in *DNMT3a* have been found in paediatric AML at lower frequencies (1-2% versus 20-22%, respectively; see table 1). *DNMT3a* mutations in AML are predominately heterozygous R882H, (Yang et al. 2015) and are dominant-negative, inhibiting wild type DNMT3A (Russler-Germain et al. 2014). *In vivo* work suggests the mutation confers increased self renewal, impaired differentiation and a repopulation advantage over wildtype haematopoietic stem cells (HSCs) (Challen et al. 2011), although additional co-operating mutations are required to give rise to AML (Mayle et al 2015). Importantly, *DNMT3a* mutations can confer resistance to chemotherapy, giving rise to a population of cells primed for relapse (Guryanova et al. 2016; Shlush et al. 2014). Although our understanding of the relationship between *DNMT3A* mutations, DNA methylation and AML is still incomplete, targeting *DNMT3a* mutations could be a highly attractive treatment approach, particularly as this abnormality confers a poor outcome (Ley et al. 2010).

Ten-eleven translocation 2 (*TET2*) mutations represent another pathway to aberrant DNA methylation. *TET2* oxidises 5-methylcytosine (5-MC) to 5-hydroxymethylcytosine (5-HMC), leading to DNA demethylation and reversal of methylation-driven gene silencing (Li et al. 2011). *TET2* is commonly mutated in

adult AML (8-23%; see table 1) (The Cancer Genome Atlas Research Network 2013a; J. P. Patel et al. 2012; Abdel-Wahab et al. 2009), however these mutations are rare in paediatric AML (1.7%; see table 1) (Liang et al. 2013). It was hypothesised that TET2 regulated HSC self-renewal. In support of this, TET2 knock-out HSCs undergo expansion *in vivo* and outcompete wild type HSCs in serial transplantation assays (Li et al. 2011; Moran-Crusio et al. 2011). In a mouse model of AML and also in primary human patient samples, loss of *TET2* caused DNA hypermethylation at active enhancers; this was associated with down regulation of tumour suppressor genes (for example, *Mtss1*, *Las2*, *Lxn*, *Ctdsp1* and *Grap2*) and up regulation of oncogenes, including *Notch3* and *Igf1r* (Rasmussen et al. 2015). Collectively, these studies suggest that, similar to other epigenetic mutations such as *DNMT3a*, *TET2* loss leads to aberrant DNA methylation, increased HSC self-renewal and impaired differentiation, and contributes to leukaemia.

Isocitrate dehydrogenase 1/2 (*IDH1/2*) catalyse the conversion of isocitrate to alpha-ketoglutarate ( $\alpha$ -KG), a co-factor required by TET2 for conversion of 5-MC to 5-HMC and subsequent DNA demethylation (Ward et al. 2010; Dang et al. 2009). *IDH1/2* are also frequently mutated in AML (5-33%; see table 1), and are mutually exclusive with *TET2* mutations (The Cancer Genome Atlas Research Network 2013b; Marcucci et al. 2010). *IDH1/2* mutations have been found in paediatric AML at lower frequencies than adult AML (1-4%, see table 1) (Valerio et al. 2014; Liang et al. 2013; Damm et al. 2011a). Mutations in *IDH1/2* lead to both loss and gain of function; cells harbouring these mutations are unable to catalyse  $\alpha$ -KG, and instead neomorphically synthesise the oncometabolite 2-hydroxyglutarate (2-HG). Elevated 2-HG leads to aberrant DNA methylation. Work by Figueroa *et al* has shown that *IDH1/2* mutations in AML induce hypermethylation, particularly at promoter regions, and this is associated with impaired differentiation and upregulation of a HSC gene expression profile (Figueroa, Abdel-Wahab, et al. 2010).

#### *Mutations in ASXL1/2*

*Addition of sex-combs like 1/2 (ASXL1/2)* is a member of the polycomb repressor complex 2 (PRC2) and is mutated in adult and paediatric AML at various frequencies

(3-15% versus 1-9% respectively; see table 1). *ASXL1* recruits PRC2 to its target loci and is required for maintenance of H3K27me3. Knock-down of *ASXL1* *in vitro* in a human AML cell line resulted in a global loss of H3K27me3 that correlated with an increase in HOX gene expression; interestingly, this transcriptional profile was strongly enriched for a gene expression signature associated with both MLL-AF9 and NUP98-HOXA9-expressing bone marrow cells (Abdel-Wahab et al. 2012), indicating *ASXL1* mutations impair PRC function and promote myeloid malignancies by induction of HOX gene expression programs. Other gene mutations that impair PRC function have been discovered in AML, for example EZH2, SUZ12, EED, JARID2. This demonstrates the importance of this complex in haematopoiesis and myeloid malignancies (Score et al. 2012; Abdel-Wahab et al. 2011). There is a strong correlation between mutations in *ASXL1* and advanced age (>60 years) (Schnittger et al. 2012; Metzeler et al. 2011). In a paediatric cohort, 8.8% (18/204 AML patients) had *ASXL1/2* mutations, suggesting these occur less frequently than in adult populations (Shiba et al. 2016). However, the authors found a strong association between *ASXL1/2* mutations and t(8;21) AML in the paediatric cohort, (20%, similar to that of adult cohorts), highlighting the need for improved patient stratification for epigenetic therapy, particularly in paediatric patients in whom epigenetic mutations are rare yet maybe enriched and therefore targetable in certain patient subgroups.

### *Histone acetylation*

Histone acetylation is an important regulator of gene expression, governed by two opposing enzymes, lysine acetyl transferases (KATs) and histone deacetylases (HDACs). Histone acetylation weakens the interaction between DNA and histones, resulting in an open chromatin conformation, therefore KATs are gene activators. KAT rearrangements, but not mutations, have been identified in AML at exceptionally low frequencies (The Cancer Genome Atlas Research Network 2013a; Wouters & Delwel 2016; Rozman et al. 2004). HDACs remove acetyl groups from histones, leading to chromatin compaction and gene inactivation. Mutations in HDACs are extremely rare in AML (The Cancer Genome Atlas Research Network 2013a; Wouters & Delwel 2016). However, HDACs can be aberrantly recruited by leukaemia-associated fusions (such as PML-RARA, EVI1 and AML/ETO) to shut down differentiation gene expression programs and maintain the AML phenotype.

This highlights their attractiveness as therapeutic targets in AML (Grignani et al. 1998; Izutsu et al. 2001; Fazi et al. 2007; Fong et al. 2014).

### *MLL-translocations*

Mixed lineage leukaemia (MLL-leukaemia) is characterised by chromosomal translocations affecting the *MLL* gene and its binding partners, most commonly *ALL1-fused gene from chromosome 9 (AF9)* (Slany 2009). In AML, MLL fusion proteins recruit a histone methyl transferase (HMT), DOT1, resulting in aberrant methylation of H3K79 at MLL gene targets and enhanced expression of leukaemia-associated genes (Slany 2009). *MLL* translocations occur more frequently in paediatric than adult AML (30-50% versus >10%, respectively) (Chaudhury et al. 2015; Krivtsov & Armstrong 2007; Schoch 2003; Grimwade et al. 1998), and are the most common aberration in infant AML (Harrison et al. 2010). There is a strong rationale for the development of inhibitors of this complex and its enzymatic cofactors for childhood and infant AML.

### *Epigenetic readers and chromatin-mediated signal transduction*

The bromodomain and extra terminal (BET) protein family member, BRD4, mediates cross talk between chromatin organisation and gene transcription. As epigenetic readers, BET proteins bind to acetylated lysine residues on histone tails where they can initiate chromatin-mediated signal transduction, carrying out normal or cancer dependent functions (Filippakopoulos & Knapp 2014; Filippakopoulos et al. 2012). In AML, BRD4 is thought to facilitate the aberrant expression of key oncogenes, such as *c-Myc* and *BCL2*, at a high level (Dawson et al. 2011; Zuber et al. 2011). BET inhibition is thought to block aberrant transcriptional elongation of these leukaemia-relevant oncogenes, thereby preventing up regulation of leukaemic stem cell (LSC) self-renewal programmes and inducing differentiation (Zuber et al. 2011). BRD4 was identified by an RNA interference screen as a novel target in AML that is required for maintenance of *MLL*-leukaemia. BET inhibitors have demonstrated robust anti-leukaemic effects *in vitro* and *in vivo*; across a range of AML cell lines, AML patient samples and in mouse model systems (Dawson et al. 2011; Zuber et al. 2011).



Interestingly, although BRD4 has been found at many enhancers genome wide, its residence at super enhancers is associated with co-occupancy with oncogenes, for example *c-Myc*. In addition, transcriptional elongation at super enhancers is more sensitive to BET inhibition, and gene expression of BRD4 targets from these sites is more selectively impaired (Lovén et al. 2013). Using BET inhibitors to target the addiction of tumour cells to high oncogene expression (Lovén et al. 2013), malignant cells could potentially be eliminated in a therapeutic window that spares normal hematopoietic cells (Lovén et al. 2013).

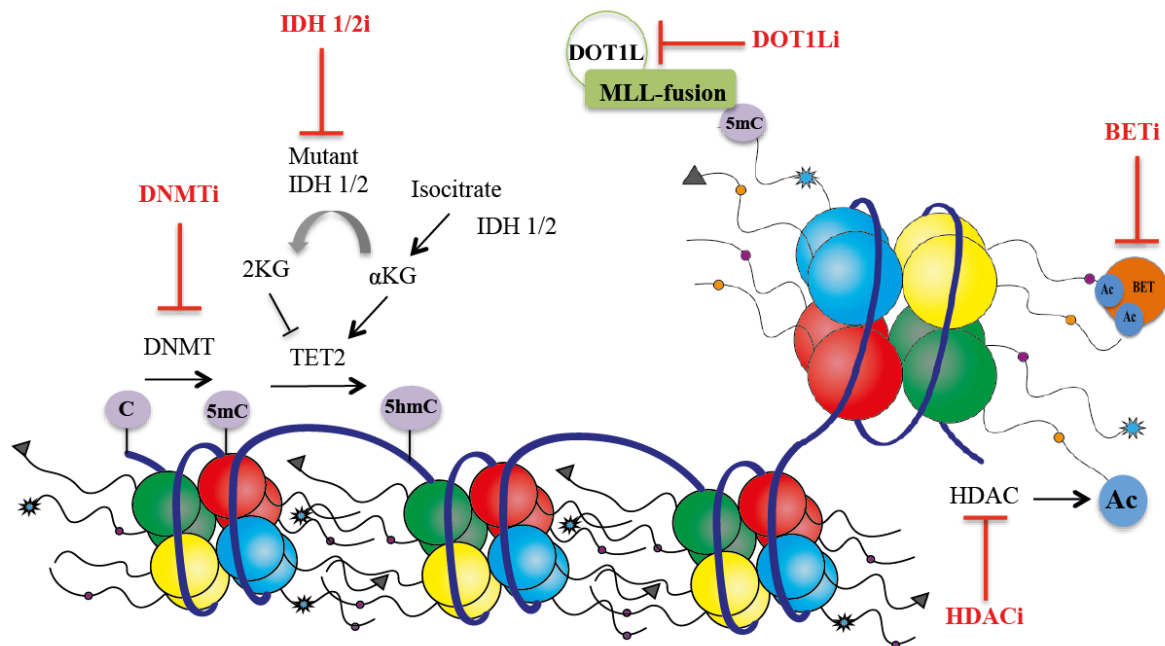
## Childhood AML

Table 1. Incidence of epigenetic gene mutations in AML by age

Gene	Mutation incidence in adult AML	Mutation incidence in paediatric AML
<i>DNMT3A</i>	20-22% (patients aged 18-81 years) (Yan et al. 2011; Ley et al. 2010)	1-2% (patients aged <18 years) (Liang et al. 2013; Thol et al. 2011; Hollink et al. 2011)
<i>TET2</i>	8-23% (patients aged 18-88 years) (Patel et al. 2012; The Cancer Genome Atlas Research Network 2013a; Metzeler, Maharry, et al. 2011)	1.7% (patients aged <18 years) (Liang et al. 2013)
<i>ASXL1</i>	3-17% (patients aged 18-100 years) (Patel et al. 2012; Boulwood et al. 2010; Metzeler, Becker, et al. 2011; Schnittger et al. 2012)	1-9% (patients aged <18 years) (Liang et al. 2013; Shiba et al. 2016).
<i>IDH1/2</i>	5-33% (patients aged 18-88 years) (The Cancer Genome Atlas Research Network 2013a; Marcucci et al. 2010; Chou et al. 2010)	1-4% (patients aged <18 years) (Valerio et al. 2014; Liang et al. 2013; Damm et al. 2011)
<i>EZH2</i>	1-5% (patients aged 18-88 years) (The Cancer Genome Atlas Research Network 2013a; X. Wang et al. 2013)	Rare (patients aged 1-20 years) (Ernst et al. 2012)

Much of the work into the epigenetics of AML has involved adult cohorts, but a small number of studies indicate epigenetic aberrations, although less common, may also play a role in paediatric AML. Table 1 summarises the frequency of epigenetic mutations in adult and childhood AML. Despite mutations in epigenetic regulators occurring less frequently in paediatric AML, an abnormal chromatin landscape appears to be a feature shared between both adult and paediatric AML; aberrant DNA methylation has been reported in paediatric patients (Rudenko et al. 2016); and hypermethylation of the GATA binding protein 4 (*GATA4*), a tumour suppressor, in primary paediatric AML samples (59/105 patients) (Tao 2015). In addition, aberrant p15 promoter methylation was reported in both adult and childhood AML (4/4 paediatric patients) (Wong et al. 2000). Some paediatric AMLs may therefore be amenable to therapeutic targeting with novel epigenetic inhibitors.

## Epigenetic therapies for adult AML and potential suitability for paediatric AML



**Figure 1. Epigenetic therapies in acute myeloid leukaemia.** DNA methylation, histone modifications and associated epigenetic regulators. Epigenetic inhibitors are highlighted in red. C (cytosine), 5mC (5-methylcytosine), 5hmC (5-hydroxymethylcytosine), 2KG (2-ketoglutarate), αKG (α-ketoglutarate), Ac (acetylation).

Epigenetic modifications, unlike those of a genetic nature, are highly susceptible to modification and/or inhibition by specifically targeted small molecules (figure 1). These therapies are rapidly entering the clinic in the hope that cancer-dependent epigenetic aberrations can be targeted (Wouters & Delwel 2016).

Table 2. Epigenetic therapies in clinical trial for AML

Epigenetic therapy	Target	Clinical trial (adult)	Clinical trial (paediatric)
Vorinostat (SAHA)	Histone deacetylases	Numerous	NCT02083250, NCT01422499•, NCT03263936
Decitabine (5-aza-2'-deoxycytidine)	DNA methyl transferases	Numerous	NCT03164057, NCT03263936, NCT03132454, NCT01177540*
Azacitidine (5-azacytidine)	DNA methyl transferases	Numerous	NCT02450877, NCT01861002•, NCT02275665, NCT03164057
Panabinstat	Histone deacetylases	Numerous	NCT02676323
Ivosidenib (AG-120)	IDH1 mutations	NCT02074839, NCT03245424	NCT03245424
FT-2102	IDH1 mutations	NCT02719574	N/A
Enasidenib (AG-221),	IDH2 mutations	NCT02577406, NCT01915498	N/A
MK-8628-005 (formerly known as OTX015)	BET-bromodomains	NCT02698189	N/A
CPI 0610	BET-bromodomains	NCT02158858	N/A
Pinometostat (EPZ 5676)	DOTL1	NCT01684150•	NCT02141828•

**\*Indicates trial has been completed**

## Targeting DNA methylation

### *DNMT inhibitors*

5-azacytidine (azacytidine) and 5-aza-2'-deoxycytidine (decitabine) are two hypomethylating agents that have been clinically approved by the Food and Drug Administration (FDA) for the treatment of myeloid malignancies, including AML. Both inhibit DNA methyl transferases and are hypothesised to have anti-cancer activity by reversing hypermethylation-associated silencing of tumour suppressors.

Recent studies have demonstrated clinical benefit with azacytidine (Dombret et al. 2015; Fenaux et al. 2009), particularly in elderly patients who are ineligible for more intensive regimens. Although a direct link between hypomethylation and gene activation following azacytidine treatment has been demonstrated (Lund et al. 2014), precisely how this elicits AML cell killing is unknown, which makes patient stratification difficult. It is not mandatory to test for aberrant methylation patterns in patients as a pre-requisite for azacytidine treatment and the effectiveness of these agents in the absence of disordered methylation (for example in some paediatric patients) is unknown.

A small retrospective analysis of 24 children with MDS reported good tolerability and clinical activity for azacytidine that included complete clinical remission in 1 patient and stable disease in 6 patients after azacytidine treatment (EWOG-MDS Cseh et al 2016). In another retrospective analysis, 8 paediatric patients with relapsed/refractory AML were treated with low-dose decitabine after failing multiple regimens and being ineligible for intensive chemotherapy (Phillips et al. 2013). 3 out of 8 patients showed a complete response with a favourable toxicity profile, suggesting promising clinical benefit in a high risk, heavily pretreated relapsed/refractory setting. Interestingly, the authors report a good response in children who subsequently received a haematopoietic stem cell transplant (HSCT); they hypothesised that demethylating treatments can relieve epigenetic silencing of tumour antigens, increasing their ‘visibility’ to the immune system and enhancing the graft-versus-tumour effect (Sigalotti 2003; Phillips et al. 2013). A large phase 2 study testing azacytidine in children with relapsed AML patients <18 years is currently on-going (NCT02450877; see table 2).

A pre-clinical study using paediatric AML cells both *in vitro* and *in vivo* demonstrated enhanced anti-leukaemic activity with decitabine and cytarabine compared with cytarabine alone (Leonard et al. 2014). However the clinical study in relapsed AML was suspended due to a lack of ‘significant clinical benefit’ (defined as response rate, duration of response, overall response, event-free survival and overall survival) (NCT01853228). A randomised phase 1 trial assessing induction chemotherapy with and without prior decitabine treatment in paediatric AML patients (aged 1- 16 years) demonstrated good tolerability and no unexpected side effects, although no difference

in complete remission rates was observed between the two treatment arms (NCT01177540). Given the small number of patients enrolled (25), further randomised trials are required to assess potential benefit of decitabine pre treatment for paediatric AML (Gore et al. 2017). Azacytidine is being tested in combination with other agents in adults with high risk or relapsed/refractory AML: Cytarabine and Mitoxantrone (NCT01839240); Mitoxantrone, Etoposide, and Cytarabine (NCT01249430) (Seval and Ozcan 2015). In the paediatric setting, a phase 1 trial testing azacytidine with fludarabine/cytarabine chemotherapy (NCT01861002) in children with relapsed or refractory AML has been completed, reporting good tolerability and clinical efficacy (complete remission achieved in 7 out of 12 (58%) AML patients) (Sun et al. 2018). Although a small number of patients were enrolled, the results of this trial are especially encouraging considering 8/12 (67%) of patients were refractory to  $\geq 2$  prior salvage treatment attempts, therefore the results of this and ongoing adult trials may encourage the use of novel combinations with azacytidine for paediatric AML.

Relapse after a HSCT is a significant cause of mortality in paediatric AML and these patients can rarely be salvaged (van den Brink et al. 2010). For patients whose minimal residual disease indicates imminent relapse after HSCT, a study by Platzbecker et al (NCT00422890) showed that azacytidine can delay relapse by 6-12 months, potentially 'opening a window' for patients until a second allogeneic HSCT is possible (Platzbecker et al. 2011).

Further investigation is required to determine the utility of hypomethylating agents in the treatment of paediatric AML. Azacytidine might represent a life-extending option for children who have failed multiple regimens and are no longer eligible for more intensive treatments. Results from adult cohorts suggest limited efficacy as a single agent (Seval and Ozcan 2015); therefore the full potential of these agents may be

reached in combination with other targeted or chemotherapeutic treatment for young patients.

### ***IDH 1/2 inhibitors***

Novel IDH inhibitors have been developed and are being tested in early phase clinical trials in adult cohorts, with promising clinical efficacy being reported. AG-120, an inhibitor of mutant *IDH1* has demonstrated good clinical efficacy in a phase 1 trial in relapsed AML patients (>18 years old), with an overall response rate of 41% (52/125 patients) (NCT02074839) (Stein & Tallman 2016). A phase 1/2 study of Enasidenib (AG-221), a selective inhibitor of mutant-IDH2 was tested in 176 relapsed/refractory AML patients harbouring *IDH1/2* mutations (aged >18 years and > 60 years in separate cohorts) (NCT01915498). 40.3% of patients achieved a haematological response to Enasidenib, including complete remissions in 34 patients (19.3%). 11% of these patients went on to receive HSCT, suggesting IDH2 inhibition could be a bridge to curative treatments - an encouraging possibility in the relapsed/ refractory setting where therapeutic options are few (Stein et al. 2017).

*IDH1/2* mutations are strongly associated with cytogenetically normal AML in adults, and co-occur with different mutations depending on the *IDH* mutation. For example, mutations in *IDH2*<sup>R140</sup>, proposed by Papaemmanuil et al. as a distinct AML subgroup, show strong co-mutation with *NPM1*, whereas *IDH2*<sup>R172</sup> are mutually exclusive with *NPM1* mutations (K. P. Patel et al. 2011; Papaemmanuil et al. 2016). Although *IDH1/2* mutations are less common in paediatric AML (see table 1), studies on paediatric patients with cytogenetically normal AML have reported higher frequencies of *IDH1/2* mutations (10.8%) than in cohorts with patients harbouring various cytogenetics (Valerio et al. 2014; Damm et al. 2011b). Given that 10-25% of paediatric AML is cytogenetically normal (Bolouri et al. n.d.; Valerio et al. 2014), further research is warranted to test the utility of IDH1/2 inhibition in specific subgroups of paediatric patients that harbour mutations.

Liang et al also found a strong association between *IDH1/2* mutations and MLL-leukaemias (Liang et al. 2013). Although the number was small, it was statistically significant and warrants further validation in larger cohorts of MLL-AML patients,

particularly given the frequency of MLL-AML in this age group (Liang et al. 2013). These leukaemias may be vulnerable to a combination strategy, targeting both *IDH1/2* mutations and the deregulated MLL-complex (with DOT1L inhibitors; see below) (Liang et al. 2013). This treatment strategy may be restricted to paediatric AML, as *MLL*-translocations and *IDH1/2* mutations do not appear to co-occur in adult AML (Papaemmanuil et al. 2016). Studies have reported an association between *IDH/2* and *FLT3* mutations in both paediatric and adult AML, indicating that these mutations cooperate in leukaemogenesis. Cells harbouring these mutations could be vulnerable to dual targeting with IDH and FLT3 inhibitors (Andersson et al. 2011; Papaemmanuil et al. 2016).

Interestingly, recent work has highlighted that *IDH1/2* mutations induce BCL-2 dependency in AML (Wouters & Delwel 2016; Chan et al. 2015), raising the possibility that dual inhibition of BCL-2 and mutant IDH1/2 may be synthetically lethal in AML. Venetoclax is a BCL-2 inhibitor currently in clinical trials for both adult and paediatric AML (NCT02203773, NCT03236857 and NCT03194932). As discussed, *IDH1/2* mutations are less frequent in childhood AML than adult, however a small subset of paediatric patients may benefit from inhibition of BCL-2 and IDH1/2.

### **Targeting histone de-acetylation (HDAC inhibitors)**

In a similar manner to DNMT inhibitors, HDAC inhibitors may activate epigenetically silenced tumour suppressor genes and elicit cancer cell killing. HDAC inhibitors (via histone re-acetylation) enable accessibility of transcriptional machinery and downstream gene expression, inducing apoptosis in leukaemic blasts (Nebbioso et al. 2004). In addition to targeting histone acetylation, HDAC inhibitors can re-acetylate other proteins with important roles in AML (such as p53), contributing to the anti-leukaemic effects of HDAC inhibition in a histone independent manner (Qi et al. 2015). HDAC inhibitors have been tested in clinical trials for adult AML patients, alone and in combination with chemotherapy and with variable efficacy (Stahl et al. 2016). For example, Kuendgen et al reported only a 5% response rate in elderly AML



patients treated with valproic acid (a class I HDAC inhibitor) in combination with all-trans retinoic acid (ATRA) (Kuendgen et al. 2006). A phase I study of vorinostat (suberoylanilide hydroxamic acid (SAHA)), a HDAC inhibitor, in 41 AML patients reported a 17 % response rate, including complete remission in 4 patients (Garcia-Manero et al. 2007). However, further studies did not support a rationale for the use of HDAC inhibitors as a monotherapy in AML, leading to examination of HDAC inhibitors in combination with chemotherapy (reviewed in (Stahl et al. 2016). The combination of HDAC inhibitors and conventional cytotoxics has been well studied: Garcia-Manero et al reported an 85% overall response rate in AML patients treated with vorinostat combined with conventional chemotherapy (idarubicin and cytarabine), although other studies have reported more modest response rates. HDAC inhibitors have been tested alongside DNMT inhibitors, but despite promising *in vitro* data, clinical studies failed to demonstrate superiority of HDAC inhibitors with azacytidine over HDAC inhibition alone (Stahl et al. 2016).

It would be important to know if paediatric AML is more vulnerable to HDAC inhibition than adult AML. Leukaemia-associated fusion proteins such as AML-ETO have been shown to block gene expression at key differentiation-gene loci via recruitment of HDACs (Wang et al. 1999; Kuendgen et al. 2006; Gelmetti et al. 1998) - importantly this repression is alleviated via HDAC inhibition, enabling differentiation of leukaemic blasts. Although clinical efficacy of HDAC inhibitors in adults has been limited, these fusions are more commonly associated with paediatric AML (Byrd 2002; Rubnitz et al. 2002; Grimwade et al. 1998), raising the possibility that paediatric patients may reap greater benefits of HDAC target therapies than adult counter-parts.

HDAC inhibitors are now being tested in paediatric AML (see table 2). A phase 1 trial of Panbinostat, a next generation HDAC inhibitor, in paediatric patients with haematological malignancies has been completed with results pending. Panobinostat is currently being tested in paediatric AML patients alongside chemotherapy (fludarabine and cytarabine) (NCT02676323). A phase I trial of Vorinostat is also underway in paediatric patients with relapsed leukaemias (NCT03263936). The

results of these trials will be the first steps in assessing utility of HDAC inhibitors for paediatric patients.

### **Targeting *MLL*-leukaemias via H3K79 methylation (DOT1L inhibitors)**

Targeting vulnerabilities of *MLL*-associated leukaemias is a highly attractive treatment strategy in paediatric AML, where *MLL* translocations occur more frequently than in adult AML (30-50% versus >10%, respectively) (Chaudhury et al. 2015; Krivtsov & Armstrong 2007; Schoch 2003; Grimwade et al. 1998). In addition, some *MLL* translocations are associated with high risk of relapse and poor prognosis in childhood leukaemias. For example, the translocation t(6;11)(q27;q23) confers a poor prognosis, with overall survival at 5 years 22%, highlighting the need for better therapies for these high-risk patient groups (Balgobind et al. 2011; Karol et al. 2014). Inhibition of DOT1L blocks *MLL* target gene expression, for example *MEIS1* and *HOXA9*, and work in model systems has demonstrated a requirement for this histone methyl transferase (HMT) in *MLL*-associated leukaemias (Bernt et al. 2011; Daigle et al. 2013) (Bernt et al. 2011; Daigle et al. 2013) .

A phase I trial using Pinometostat (EPZ-5676), a DOT1L inhibitor, was initiated in paediatric patients with relapsed/refractory AML harbouring *MLL*-gene rearrangements, but reported only transient reductions in leukaemic blast in 7/18 patients (NCT02141828) (Shukla et al. 2016) – a disappointing outcome considering promising pre-clinical work. In the adult AML setting, objective response was achieved in 6 patients, including morphologic (1 patient) and cytogenetic (1 patient) complete remission, however recruitment to this trial was higher than in the paediatric study by Shukla et al (49 versus 18, respectively), therefore may not be directly comparable to the paediatric study (NCT01684150) (Stein et al. 2015).

Whilst Pinometastat (EPZ-5676) lacks clinical benefit as a single agent, it would be interesting to test the utility of DOT1L along with conventional cytotoxic regimes for relapsed/refractory AML. Work by Liu et al (Liu et al. 2014) showed that inhibition of DOT1L sensitises *MLL*-leukaemia cells to chemotherapy through impaired DNA damage signalling, demonstrating enhanced killing of *MLL*- leukaemia cell lines and patient samples with DOT1L inhibitor compared to chemotherapy alone. This is an

encouraging finding given that some MLL-leukaemias are resistant to conventional cytotoxics, suggesting a potential window of therapeutic benefit where leukaemic blasts are sensitised to chemotherapy. Overexpression of *FLT3* has been reported in MLL-AML, and EPZ-5676 combined with sorafenib (a FLT3 receptor inhibitor) achieved synergistic toxicity compared with EPZ-5676 alone (which had a very modest effect on cell viability as a single agent) (Lonetti et al. 2016).

Given the high association of MLL gene rearrangements with paediatric AML, targeting this complex remains an attractive therapeutic strategy. Combination therapy, either with standard chemotherapy or by targeting of other components of the MLL-driven machinery, could be key for more potent disease-elimination and long-term clinical benefits. Further pre-clinical work is warranted to elucidate vulnerabilities within MLL-complexes and identify synergistically toxic combinations alongside DOT1L inhibition in MLL-rearranged leukaemias.

### **Targeting epigenetic readers (BET proteins)**

BET inhibitors are being tested in early phase clinical trials in adults with a range of solid and haematological malignancies. OTX015, a BET-bromodomain inhibitor, was associated with anti-leukaemic activity in patients with advanced AML (response rate: 5/28 patients) who had failed conventional therapies (NCT01713582) (Dombret et al. 2014). A clinical trial using CPI0610, an additional BET-bromodomain inhibitor, is underway in previously treated acute leukaemia and myelodysplastic syndrome patients (NCT02158858; see table 2). Much of the preclinical work demonstrating efficacy of BET inhibitors in AML used the MLL-AF9 model, which is thought to be extremely sensitive to BET inhibition (Dawson et al. 2011; Zuber et al. 2011). Given the association between *MLL*-leukaemia and paediatric AML, there is a rationale to use BET inhibitors for childhood AML. In addition, BET inhibitors may potentiate the effects of other targeted therapies. For example, BET inhibitors down regulate BCL-2 expression and anti-apoptotic function, and BCL-2 inhibitors (for example venetoclax) are currently in clinical trials for paediatric AML patients (NCT032368570). It could be hypothesised that drug synergy may be achieved with dual targeting of BET and BCL-2. This could be an attractive approach in AML by

targeting oncogenes (such as *c-Myc*) while simultaneously inducing apoptosis through BCL-2 inhibition.

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### **Combining epigenetic and immunotherapy in paediatric AML**

Immunotherapy, including antibodies against AML cell surface proteins and chimeric antigen receptor engineered (CAR-T) cell therapy, is a promising area of therapy for childhood AML (Tasian 2014). CD33 is a trans-membrane receptor protein which is expressed on both adult and paediatric AML blasts and is associated with poor prognosis when expressed at a high level in AML (Tasian 2014; Pollard et al. 2012; R. B. Walter et al. 2007). An anti-CD33 monoclonal antibody/calicheamicin conjugate, gemtuzumab ozogamicin (GO), was withdrawn from the market due to concerns over increased mortality, but has regained favour after a number of positive publications and has since been reintroduced (Gamis et al. 2014; Pollard et al. 2016; Castaigne et al. 2012). New dosing regimens with improved toxicity outcomes have encouraged further evaluation of GO in adult and paediatric AML (Laing et al. 2017). GO has been tested in paediatric AML as a single agent with good outcomes (remission rates of 28-54%) (Tasian 2014; Satwani et al. 2012; Zwaan et al. 2010; D. Reinhardt et al. 2004), and is currently being tested using a fractionated dosing regimen in an international phase 3 randomised trial, Myechild01, where it is given in combination with induction chemotherapy (NCT02724163). Interestingly, azacytidine treatment enhanced the anti-leukaemia activity of an alternative anti-CD33 antibody-drug conjugate, SGN-CD33, *in vivo* (Sutherland et al. 2011). Although clinical testing of SGN-CD33A has been suspended due to increased mortality in a phase 3 trial, demethylation and re-expression of CD33 and natural killer cell enzymes is the proposed mechanism of drug synergy, therefore it is predicted that this combination approach may be applicable to alternative anti-CD33 agents (and not specific to SGN-CD33). This approach may hold great promise in paediatric AML given that approximately 80% express CD33 (Tasian 2014). Similarly, demethylating agents (via a similar mechanism) may potentiate the effects of CAR-T cell therapy in paediatric AML patients. CAR-T cells are genetically engineered to target tumour antigens and therefore increased expression of tumour antigens via demethylating agents might elicit synergistic killing of AML cells (Tasian 2014). In addition, *IDH* mutations give rise to tumour-specific neoantigens that CAR-T cells could be

engineered to target; IDH1/2 inhibitors could therefore be used in combination with CAR-T therapy to target *IDH1/2* mutant AML (Wouters & Delwel 2016; Schumacher et al. 2014). Intuitively this strategy may be more appropriate for adult AML, where *IDH* mutations occur more frequently than in children (see table 1). However, given the higher incidence of *IDH* mutations in cytogenetically normal paediatric AML compared to those with cytogenetic abnormalities (10.8%) (Valerio et al. 2014), appropriate patient stratification may identify subgroups of paediatric patients who are sensitive to CAR-T therapy against *IDH* mutant AML.

Demethylating agents may also have an important role alongside immune checkpoint inhibitors in AML therapy. Immune checkpoint receptors, such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), are expressed on T cells and suppress anti-tumour immunity. Immune checkpoint inhibitors (monoclonal antibodies) targeting CTLA-4 or PD-1/PD-1 ligand (PD-1L), reverse T cell inhibition and enable AML cell killing. A number of clinical trials with checkpoint inhibitors have been initiated in AML patients and are summarised in Lichtenegger et al 2017 (Lichtenegger et al. 2017)). Azacytidine treatment has been shown to upregulate PD-1 expression in T cells of AML patients, preventing an effective immune response to the leukaemic blasts and potentially leading to therapy resistance (Ørskov et al. 2015). Trials combining immune checkpoint inhibitors with azacytidine are under way for adults with AML, including azacytidine with PD-1 inhibitor nivolumab (NCT02397720), azacytidine with or without PD-1L inhibitor durvalumab (NCT02775903) and azacytidine with or without PD-1L inhibitor atezolizumab (NCT02508870). In the phase 1 trial of azacytidine with nivolumab, an overall response rate of 35% was achieved (including 6 complete remissions), which compares favourably to historical survival outcomes with azacytidine alone (Daver et al. 2017). Nivolumab is to be tested as a single agent in paediatric AML patients. Given that azacytidine is already being tested in children with AML, promising results from trials using azacytidine and immune checkpoint inhibitors in adults may encourage clinical assessment of this therapeutic strategy in paediatric AML.

### **Clonal haematopoiesis with indeterminate potential**

Genetic mutations can be used to map the evolutionary trajectory of cancer in an

individual patient (Yates & Campbell 2012). Conversely, the presence of ancestral mutations in apparently healthy individuals can indicate an increased risk of cancer development. Clonal haematopoiesis (CH), or clonal haematopoiesis with indeterminate potential (CHIP) arises when a mutation imparts a growth advantage on a HSC, leading to expansion of the dominant clone and skewed haematopoiesis. Although not a new concept with skewed X-inactivation patterns in elderly females being reported in the 1990s (Champion et al. 1997; Busque et al. 1996), next generation sequencing has recently increased our understanding of this pre-leukaemic state. Expanded clones harbour epigenetic gene mutations commonly observed in AML, such as *DNMT3a*, *TET2* and *ASXL1*, and indeed are associated with an increased risk of haematological malignancy (Genovese et al. 2014; Jaiswal et al. 2014). Why mutations in clonal haematopoiesis are so commonly found in epigenetic regulators is not known, but this new era of epigenetic therapy brings hope that this pre-leukaemic reservoir could be eradicated before malignant transformation (Shlush et al. 2014).

It is widely accepted that clonal haematopoiesis is rare in childhood. However, sequencing efforts comparable to that done in adults are lacking in younger subjects. As mentioned, mutations in epigenetic machinery, although rare, do exist in paediatric AML. It is reasonable to hypothesise that these mutations existed as a dormant clone that later initiated the leukaemia, raising the possibility that these cancers may also be vulnerable to epigenetic targeting. In a case study of a paediatric AML patient (13 years old), a pre-leukaemic clone harbouring a *DNMT3a* mutation was identified. The mutant clone persisted during remission, indicating therapy resistance and highlighting the existence of CHIP in childhood (Göhring et al. 2016). Given the rarity of CHIP-associated epigenetic mutations in paediatric AML, they are not usually studied at diagnosis (Göhring et al. 2016); it could be speculated therefore that CHIP is more prevalent in a subgroup of paediatric patients - for example cytogenetically normal populations - than we currently appreciate.

Despite the association between CHIP and leukaemia, its precise clinical relevance to AML, once established, is unknown. Curiously, recent work suggests that CHIP is not indicative of outcome (duration of remission or overall survival), despite these mutations persisting after therapy and being detected in the relapse clone (Gaidzik et

al. 2017). It would be useful to correlate this with response to epigenetic therapies. It is important therefore to address the possibility that children with AML, although often lacking CHIP and epigenetic mutations, may also benefit from targeted epigenetic therapy. If CHIP lacks prognostic relevance, the absence of CHIP in childhood needn't be a barrier to testing epigenetic drugs and warrants further investigation. Lastly, the use of error-corrected sequencing (ECS) enables better detection of rare allelic variants than NGS (Young et al. 2015). Exploiting this technology, Young et al report a 95% incidence of clonal haematopoiesis in 50-60 year olds – much higher than was proposed in seminal papers from Genovese et al; and Jaiswal et al (Young et al. 2016). This work suggests that clonal haematopoiesis is almost ubiquitous by middle age, and progression to AML is exceptionally rare. Therefore, the significance of CHIP in leukaemia management, particularly concerning the use of epigenetic therapies, remains to be elucidated in both adult and paediatric cohorts.

## **Conclusion**

There remains an urgent need for better therapies for both adult and paediatric AML. Epigenetic therapies have demonstrated clinical efficacy in adult AML, which is associated with abnormalities to the epigenome. The low frequency of epigenetic abnormalities in paediatric AML is not a reason to limit the use of epigenetic therapies to adult AML patients; there is evidence that epigenetic therapies may benefit children with the appropriate target. Although results from some trials of conventional chemotherapy for children with relapsed AML have had more favourable outcomes (summarised in (Kaspers 2014)) than the epigenetic therapies detailed in this review, in these early stages of testing epigenetic therapy in paediatric AML it is important to consider that moving towards more 'personalised' medicine will require evaluation of targeted therapies that may only demonstrate clinical benefit after appropriate patient stratification. In addition, it is unlikely that one drug – epigenetic or otherwise – will be curative in AML due to the clonal heterogeneity which provides reservoirs for drug resistance and subsequent relapse; it is possible that dual targeting of synthetic lethal pathways can yield greater cell kill in AML and

transform the outcomes for paediatric AML patients. For these reasons, further research into drug combinations that utilise epigenetic therapies is warranted.

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