Targeting DNA methylation for epigenetic therapy

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Patterns of DNA methylation are established during embryonic development and faithfully copied through somatic cell divisions. Based on current understanding of DNA methylation and other interrelated epigenetic modifications, a comprehensive view of the ‘epigenetic landscape’ and cancer epigenome is evolving. The cancer methylome is highly disrupted, making DNA methylation an excellent target for anticancer therapies. During the last few decades, an increasing number of drugs targeting DNA methylation have been developed to increase efficacy and stability and to decrease toxicity. The earliest and the most successful epigenetic drug to date, 5-Azacytidine, is currently recommended as the first-line treatment of high-risk myelodysplastic syndromes (MDS). Encouraging results from clinical trials have prompted further efforts to elucidate epigenetic alterations in cancer, and to subsequently develop new epigenetic therapies. This review delineates the latest cancer epigenetic models, the recent discovery of hypomethylation agents as well as their application in the clinic.

The epigenetic landscape
Primary heritable genetic information is dictated by the DNA sequence packed into chromatin. The basic repeating unit of chromatin is a nucleosome consisting of 146–147 base pairs (bp) of DNA wrapped around a histone octamer. DNA and histone proteins can undergo various post-synthetic modifications. These modifications, along with histone variants and nucleosome remodelers, can strongly influence chromatin structure and transcriptional regulation without changing the underlying DNA sequence, a process called ‘epigenetic regulation’. Epigenetic regulation can be separated into three inter-related layers: nucleosome positioning, histone modifications, and DNA methylation [1].

DNA methylation is probably the most extensively studied epigenetic mark. It plays an important part in genomic imprinting, in X-chromosome inactivation, and in the silencing of retrotransposon, repetitive elements and tissue-specific genes. In mammalian cells, a methyl group is covalently added to cytosine in the context of cytosine-phosphate-guanine (CpG) dinucleotides in somatic cells and also CpHpG nucleotide sequences in embryonic stem cells (ES) [2]. In somatic cells, most CpG dinucleotides are methylated except those located in CpG islands [3], which are defined as regions of DNA >500 bp with a GC content >55%, and a ratio of observed and expected CpG >0.65 [4]. Nearly 60% of mammalian gene promoters are located in CpG islands [5]. DNA methylation of these CpG-rich promoters silences gene expression by changing the accessibility of DNA to transcription factors (TFs) or by recruiting additional silencing-associated proteins [6]. Nevertheless, there is also growing evidence suggesting that even in CpG-poor regions such as the Oct4 promoter DNA methylation still has a role in gene regulation [7,8].

Patterns of DNA methylation are established and maintained by DNA methyltransferases (DNMTs). During early embryogenesis, de novo DNA methylation is mediated by DNMT3A and DNMT3B associated with DNMT3L, which lacks a methyltransferase domain [9–11]. To maintain patterns of DNA methylation in daughter cells, ubiquitin-like, containing PHD and RING finger domains 1 (UHRF1) recognizes hemi-methylated DNA and directs DNMT1 to methylate the appropriate cytosine in newly synthesized DNA strands during successive replications [12,13]. Recent studies have proposed an updated model, suggesting that DNMT3A/B are also required for the maintenance of patterns of DNA methylation in somatic cells, particularly of repeat regions and imprinted genes (Figure 1a) [6,14].

In contrast to DNA methylation, the patterns of histone modifications are more labile. The N-termini of histones can undergo various post-synthetic modifications, including a diverse combination of methylation, acetylation, ubiquitylation, phosphorylation and sumoylation. In recent years, the development of modification-specific antibodies in chromatin immunoprecipitation (ChIP), coupled with microarray (ChIP-chip) or direct sequencing technology (ChIP-seq), has revolutionized assessment of the global distribution of histone modifications and their roles in gene regulation [15–17]. For instance, H3K4me3 has been found to occur near the transcription start sites (TSS) of genes or miRNA, and is positively correlated with transcription level [18–21]. Similarly, H3K9/14 acetylation is highly associated with gene transcriptional activation [22]. By contrast, H3K9me and H3K27me are enriched in the vicinity of suppressed or silenced gene promoters (Figure 1a) [15]. The enzymes that catalyze these
modifications and the crosstalk between these complexes have been summarized in several important review articles [23–26].

Other crucial components of the epigenetic landscape are histone variants which partially determine nucleosome stability and ultimately affect gene expression. H2A.Z is commonly localized in promoter regions. The differences in the amino-acid sequence of H2A.Z compared with H2A might affect the interactions of the protein with the H3/H4 tetramer, thereby altering nucleosome stability [27–28]. In mammalian cells, the role of H2A.Z in gene regulation is incompletely understood, but, there is increasing evidence suggesting that H2A.Z assists in the transcription initiation of a special group of genes such as endoplasmic reticulum (ER) target genes [29–32].

The interplay between histone modifications, histone variants, and DNA methylation constitutes a global regulation network. Extensive studies have elucidated the biological and functional interactions among these three epigenetic layers. For example, DNA methylation anticorrelates with the active mark H3K4me3, H3ac or H2A.Z [33–35]. Meanwhile, highly methylated promoter regions are bound by methylcytosine binding proteins such as MeCP2, which subsequently recruits histone deacetylase (HDAC) to deacetylate histones, thus further reinforcing the suppressive nature of promoters (Figure 1) [36–37]. The interplay between DNA methylation and histone modifications can be further observed in ES cells. Some studies have proposed that histone modifications and associated proteins (e.g., EZH2 or G9a) take part in establishing de novo patterns of DNA methylation during early development [38–41].

Cancer epigenetics

It has been documented that epigenetic alterations are involved in the initiation and progression of cancer in addition to abnormal genetic events. Early studies that measured the global content of 5-methylcytosine of tumors showed that hypomethylation was a common feature of carcinogenesis, leading to abnormal chromosomal instability and transcriptional regulation [42–44]. However, most cancer epigenetic studies subsequently concentrated on focal CpG island hypermethylation in cancer, and revealed many tumor suppressor genes, cellular functional genes, and miRNAs silenced by promoter DNA methylation [45–48]. Recent genome-wide studies have demonstrated distinct patterns of DNA methylation in cancerous tissues compared with their normal counterparts [49–52].

The detailed mechanisms by which these discrete regions undergo hyper- or hypomethylation are unclear.
Early evidence suggested that elevated DNA methyltransferase levels might trigger hypermethylation of tumor suppressor gene promoters, which would consequentially result in proliferation of cancer cells [53]. In addition to this ‘selection’ model, an alternative mechanism has been proposed that takes advantage of the current genome-wide epigenetic studies in stem cells. Investigators have suggested that the establishment of aberrant epigenetic profiles in cancer undergoes a process similar to epigenetic reprogramming during development [54]. During cancer initiation, the promoters of genes (which are repressed by histone H3K27me3 in normal differentiated cells) might become methylated and thereby set up for long-term silencing. This ‘epigenetic switch’ could be regulated by the cooperation of polycomb proteins and DNMTs [55–57] (Figure 1b).

In cooperation with DNA methylation, other epigenetic mechanisms also exhibit abnormal regulation in cancer. For example, histone deacetylases (HDACs) are often found to be overexpressed in various types of cancer, resulting in histone deacetylation around the TSS region and the formation of a more compact structure to silence genes [58–59]. In addition, H3K4me is also selectively demethylated by the histone lysine demethylase (LSD1), which is upregulated in cancer, making it a potential drug target [60–61]. In some loci, polycomb-group (PcG) proteins associated with H3K27me work independently of DNA methylation to aberrantly repress genes in cancer cells [62–65]. Nucleosome occupancy is also switched from an ‘open’ to a ‘covered’ status in gene regulation elements in neoplastic cells [66–67] (Figure 1b).

**In vitro study of DNMT inhibitors (DNMTi)**

Epigenetic modifications play a crucial part in regulating normal cells, but these processes are disrupted during tumorigenesis. The relatively reversible character of epigenetic alterations (in contrast to genetic changes) has inspired the development of therapeutic strategies targeting various epigenetic components. Among them, DNA methylation and its associated enzymes have been well-studied. The understanding of their fundamental mechanisms of action and correlation with other epigenetic modifications makes them attractive drug targets.

**Nucleoside analogs**

5-Aza-2′-deoxycytidine (5-Aza-CdR) and 5-aza-2′-deoxycytidine (5-Aza-CdR) are two of the most potent DNMTi. They have been approved by the Food and Drug Administration (FDA) in the USA for the treatment of myeloid malignancies (Figure 2a). They were first synthesized as cytotoxic agents. In the 1980s, these compounds were found to have hypomethylating activity after incorporation into the DNA of actively replicating tumor cells [68–70].

Upon transport into cells by the human concentrative nucleoside transporter 1 (hCNT1), 5-Aza-CR and 5-Aza-CdR are phosphorylated by different kinases, converting them to their active triphosphate forms, 5-Aza-CTP and 5-Aza-dCTP, respectively [71–72] (Figure 3a). 5-Aza-CR can be incorporated into RNA and DNA after the reduction of 5-Aza-CDP by ribonucleotide reductase, whereas 5-Aza-CdR incorporates into DNA after its phosphorylation to 5-Aza-dCTP [73]. The incorporated 5-azanucleoside disrupts the interaction between DNA and DNMTs through the nitrogen in the 5 position of the modified pyrimidine, and traps DNMTs for proteosomal degradation [74–75] (Figure 3b). The depletion of DNMTs results in the passive loss of cytosine methylation in the daughter cells after replication. This is associated with reduced H3K9me3, increased H3ac and H3K4me3 modifications around gene promoter regions, as well as the formation of a nucleosome-deficient region [66,76]. A recent genome-wide study of alterations in the epigenetic landscape after 5-Aza-CR treatment further validated this concept [77]. The demethylation function of 5-Aza-CR and 5-Aza-CdR is most evident at low drug concentration because the drugs exhibit greater cytotoxicity, interfere with DNA synthesis, and cause DNA damage at higher concentrations [78]. The S-phase is required for the selective and effective incorporation of these two drugs into the DNA of rapidly proliferating cells, thereby limiting unwanted hypomethylation in cells undergoing the normal cell cycle.

Besides inhibiting DNMTs, 5-Aza-CR incorporates into RNA and interrupts normal cellular processes by inducing ribosomal disassembly and preventing the translation of oncogenic proteins [73,79]. The ability of 5-Aza-CR to be incorporated into DNA and RNA increases its side effects in vitro and in vivo because it can function in resting and dividing cells [80]. However, 5-Aza-CR and 5-Aza-CdR are readily hydrolyzed in aqueous solution and subject to deamination by cytidine deaminase. The instabilities of these compounds inevitably present a challenge to their clinical applications.

To improve the stability and efficacy of 5-Azanucleosides, several other cytosine analogs have been developed (Figure 2a). For example, zebularine (a cytidine analog that lacks an amino group in the 4 position of the pyrimidine ring) can inhibit DNMTs and cytidine deaminase after oral administration [81–83]. Studies have shown that zebularine induces hypomethylation in breast cancer cell lines and reactivates silenced tumor suppressor genes [84–85]. The inefficient metabolic activation of this compound has, however, delayed its clinical use as a single agent. As an inhibitor of cytidine deaminase, its co-administration has increased the efficacy of 5-Aza-CdR [82]. The cytidine analog 5-Fluoro-2′-deoxycytidine (5-F-CdR) had also been demonstrated to have hypomethylating activity in mouse cells as well as in human breast and lung carcinoma cells [68,86]. Clinical studies further showed that co-administration of 5-F-CdR with the cytidine deaminase inhibitor tetrahydrouridine (THU) improved the stability of 5-F-CdR [87]. The therapeutic potential of another stable analog, dihydro-5-azacytidine (DHAC), was also assessed for the treatment of malignant mesothelioma, but results on this compound’s clinical efficacy have been inconsistent [88].

The effort to improve the stability of DNMTi includes the development of prodrugs of the nucleoside analogs. A preclinical study showed that NPEOC-DAC (a prodrug of 5-Aza-CdR containing a 2-(p-nitrophenyl)ethoxycarbonyl (NPEOC) group at the 4 position of the pyrimidine ring) can be incorporated into DNA and inhibit DNMTs after its activation by human carboxylesterase 1 in a liver cancer cell line. The NPEOC moiety protects 5-Aza-CdR from
deamination, but the compound itself is less potent if administered at the same concentration as 5-Aza-CdR. Moreover, the activity of NPEOC-DAC is dependent upon carboxylesterases, which are not expressed in all tissues. Further studies are required to explore the use of this compound in combination therapy [89]. Alternatively, S110 (a dinucleotide containing the 5-azacytosine ring) has also been shown to improve the efficacy of 5-Aza-CdR by protecting it from deamination. The compound is well tolerated and can reduce the level of DNA methylation in the CDKN2A promoter region in xenografts [90].

Non-nucleoside analogs
Unlike cytidine analogs, non-nucleoside DNMTis (Figure 2b) do not require incorporation into DNA, and thus might exhibit less cytotoxicity. Some of the compounds assessed for their potential to induce hypomethylation in solid tumors are hydralazine and procainamide, the widely used vasodilator and antiarrhythmic agents, respectively [91]. Hydralazine has been reported to block the activity of DNMTs by the interaction of its nitrogen atoms with the Lys-162 and Arg-240 residues of the enzyme, whereas procainamide acts similarly as a competitive inhibitor by
preferentially binding to DNMT1 [92–93]. These compounds, however, have limited DNA hypomethylation activity in living cells [94]. The small molecule RG108 also shows the potential to reactivate tumor suppressor genes in human colon cancer cells [95–96]. Recently, the lipophilic, quinoline-based compound SGI-1027 was demonstrated to be a novel DNMTi in vitro. In RKO cells, SGI-1027 causes the degradation of DNMT1 and the demethylation of the \(CDKN2A\) gene promoter as well as reactivating silenced genes [97].

An alternative strategy to inhibit DNMT1 includes the use of short-chain oligodeoxynucleotides and microRNAs. MG98 is a 20-bp antisense oligonucleotide that specifically binds to the 3' UTR of human DNMT1 mRNA to prevent its translation. Despite promising results in preclinical studies, the clinical use of MG98 has not been validated [98]. MicroRNA miR29a, which targets DNMT3A/B directly and DNMT1 indirectly in a similar way to MG98, can reduce global DNA methylation and reactivate \(CDKN2A\) [99].

Successful results encouraged the use of DNMTi to develop novel clinical regimens. We have summarized several recent important clinical trials in Table 1.

**Single-agent therapy**

**5-Azagcytidine (Vidaza, Azacitidine)** In early clinical trials, 5-Aza-CR was administered at maximum tolerated doses (MTD) to patients with osteogenic sarcoma or other cancer-related diseases, but showed unfavorable toxicity [100–101]. Increasing knowledge of the mechanism of action of 5-Aza-CR indicated that lower dosages of 5-Aza-CR could act as DNMTi with minimal effects on DNA synthesis [102]. 5-Aza-CR was approved by the FDA for the treatment of MDS based on the positive results from the GALGB9221 clinical trial [103]. A reported 60% of 5-Aza-CR-treated patients exhibited various levels of response, whereas only 5% of patients in the supportive group showed hematological improvement (HI). 5-Aza-CR also benefited patients by delaying progression time to acute myeloid leukemia (AML), improving quality of life, and prolonging overall survival in Refractory Anemia with Excess Blasts (RAEB) or Refractory Anemia with Excess of Blasts in Transformation (RAEB-T) subgroups [104–105].

To further validate the efficiencies of 5-Aza-CR in MDS patients, the European AZA-001 trial was conducted for
Table 1. Selected Recent Clinical Trials with DNMT Inhibitors

<table>
<thead>
<tr>
<th>Drug(s)</th>
<th>Indication(s)</th>
<th>Author (Year)</th>
<th>Total Patients</th>
<th>Results</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azacitidine</td>
<td>MDS</td>
<td>Silverman (2002/2006)</td>
<td>191</td>
<td>7% CR, 16% PR, 37% HI</td>
<td>Low dose treatment improved quality of life and survival rate; delayed progression time to AML (CALGB9221).</td>
<td>[102, 104]</td>
</tr>
<tr>
<td></td>
<td>MDS</td>
<td>Fenaux (2009)</td>
<td>358</td>
<td>17% CR, 12% PR, 42% SD</td>
<td>RBC transfusion independence achieved in 3 alternative dosing regimens. Response rates are comparable to CALGB 9221 and AZA-001 trials but shorter OS in this 5 days treatment regimen.</td>
<td>[105, 109]</td>
</tr>
<tr>
<td></td>
<td>MDS</td>
<td>Lyons (2009)</td>
<td>151</td>
<td>44%, 45%, 56% HI</td>
<td>Survival advantage in responder group of high-risk MDS</td>
<td>[110]</td>
</tr>
<tr>
<td></td>
<td>MDS/AML</td>
<td>Muller-Thomas (2009)</td>
<td>32</td>
<td>15.6% CR, 25% HI, 34.4% SD</td>
<td>Survival benefits in responder group.</td>
<td>[111]</td>
</tr>
<tr>
<td></td>
<td>MDS</td>
<td>Musto (2010)</td>
<td>74</td>
<td>10.8% CR, 9.5% PR, 20.3% HI</td>
<td></td>
<td>[108]</td>
</tr>
<tr>
<td>Decitabine</td>
<td>MDS</td>
<td>Kantarjian (2006)</td>
<td>170</td>
<td>9% CR, 13% HI, 17% ORR</td>
<td>RBC transfusion independence and delayed progression time toward AML (D-0007).</td>
<td>[112]</td>
</tr>
<tr>
<td></td>
<td>MDS</td>
<td>Kantarjian (2007)</td>
<td>95</td>
<td>34% CR</td>
<td>Better response rate at low dose.</td>
<td>[114]</td>
</tr>
<tr>
<td></td>
<td>MDS</td>
<td>Steensma (2009)</td>
<td>99</td>
<td>17% CR, 18% HI, 32% ORR</td>
<td>Alternative dosing and significant difference in ORR compared to single-center trial by Kantarjian et al. (ADOPT). Relative reduction of tumor size but no correlation between PBMC DNA methylation and tumor DNA methylation.</td>
<td>[115, 117]</td>
</tr>
<tr>
<td></td>
<td>Refractory Solid Tumor</td>
<td>Stewart (2009)</td>
<td>31</td>
<td>30.7% CR/PR, 15.4% HI, 38.6% SD</td>
<td>In increasing therapeutic level of VPA promoted efficacy of azacitidine.</td>
<td>[123]</td>
</tr>
<tr>
<td>MG98</td>
<td>MDS/AML</td>
<td>Klisovic (2009)</td>
<td>23</td>
<td>26% SD</td>
<td>No clinical benefits observed in administered doses and no correlation between DNMT1 downregulation with SD. Suppression of DNMT1 expression in PBMC in all doses with mild toxicity observed.</td>
<td>[119]</td>
</tr>
<tr>
<td>Azacitidine in Combination</td>
<td>AML/MDS</td>
<td>Soriano (2007)</td>
<td>53</td>
<td>22% CR, 42% ORR</td>
<td>The increase of histone acetylation level is not correlated with VPA dose level.</td>
<td>[122]</td>
</tr>
<tr>
<td>Azacitidine + Valproic Acid</td>
<td>Advanced cancer</td>
<td>Raza (2008)</td>
<td>40</td>
<td>42% HI, 14% SD</td>
<td>High expression of cellular proliferation genes was observed in non-responders.</td>
<td>[135]</td>
</tr>
<tr>
<td>Azacitidine + Valproic Acid</td>
<td>Refractory Solid Tumor</td>
<td>Braith (2008)</td>
<td>55</td>
<td>25%SD</td>
<td>Safe dosage of azacitidine up to 75mg/m² in advanced malignancies. No clear benefits seen in the three dosing regimens tested.</td>
<td>[124]</td>
</tr>
<tr>
<td>Azacitidine + Sodium Phenylbutyrate</td>
<td>MDS</td>
<td>Lin (2009)</td>
<td>27</td>
<td>42%ORR</td>
<td>Lack of association between reversal of DNA methylation and clinical response.</td>
<td>[125]</td>
</tr>
<tr>
<td>Azacitidine + Valproic Acid</td>
<td>MDS</td>
<td>Voso (2009)</td>
<td>62</td>
<td>30.7% CR/PR, 15.4% HI, 38.6% SD</td>
<td>In increasing therapeutic level of VPA promoted efficacy of azacitidine.</td>
<td>[123]</td>
</tr>
<tr>
<td>Azacitidine + Entinostat</td>
<td>MDS/AML</td>
<td>Fandy (2009)</td>
<td>30</td>
<td>42%ORR</td>
<td>Lack of association between reversal of DNA methylation and clinical response.</td>
<td>[125]</td>
</tr>
<tr>
<td>Azacitidine + Allogeneic SCT</td>
<td>MDS/AML</td>
<td>Jabbour (2010)</td>
<td>17</td>
<td>44% CR, 17% HI, 67% ORR</td>
<td>Higher CR is associated with normal cytogenetics and lower methylation levels</td>
<td>[129]</td>
</tr>
<tr>
<td>Azacitidine + Lenalidomide</td>
<td>MDS</td>
<td>Sekeres (2010)</td>
<td>18</td>
<td>42% ORR</td>
<td>Limited clinical activity in patients with relapsed, refractory AML. Increased response rate and duration of response observed.</td>
<td>[137]</td>
</tr>
<tr>
<td>Azacitidine + Cytarabine</td>
<td>MDS/AML</td>
<td>Borthakur (2010)</td>
<td>34</td>
<td>19% CR</td>
<td>Lower pretreatment CDKN2B methylation level correlates with higher response rate.</td>
<td>[127]</td>
</tr>
<tr>
<td>Azacitidine + Entinostat</td>
<td>MDS/CML</td>
<td>Scott (2010)</td>
<td>32</td>
<td>32% CR, 4% PR, 7% HI</td>
<td>No increased toxicity due to decitabine. Higher activity in patients without BCR-ABL mutations.</td>
<td>[139]</td>
</tr>
<tr>
<td>Decitabine in Combination</td>
<td>Advanced leukemia</td>
<td>Garcia-Manero (2006)</td>
<td>54</td>
<td>19% CR</td>
<td>Lower pretreatment CDKN2B methylation level correlates with higher response rate.</td>
<td>[127]</td>
</tr>
<tr>
<td>Decitabine + Carboplatin</td>
<td>Solid Tumor</td>
<td>Appleton (2007)</td>
<td>33</td>
<td>44% ORR</td>
<td>A correlation between different doses of decitabine with PBC DNA demethylation. VPA increased treatment-related toxicity; Re-expression of ER associated with clinical response.</td>
<td>[132, 128]</td>
</tr>
<tr>
<td>Decitabine + Valproic Acid</td>
<td>AML</td>
<td>Blum (2007)</td>
<td>25</td>
<td>42% CR</td>
<td>Treatment facilitated subsequent HSCT.</td>
<td>[131]</td>
</tr>
<tr>
<td>Decitabine + Gemtuzumab ozogamicin</td>
<td>AML</td>
<td>Chowdhury (2009)</td>
<td>12</td>
<td>42% CR</td>
<td>Treatment facilitated subsequent HSCT.</td>
<td>[131]</td>
</tr>
<tr>
<td>Decitabine + Allogeneic SCT</td>
<td>MDS</td>
<td>De Padua Silva (2009)</td>
<td>17</td>
<td>32% CR, 4% PR, 7% HI</td>
<td>Reexpression of immunomodulatory genes.</td>
<td>[133]</td>
</tr>
<tr>
<td>Decitabine + Imatinib Mesylate</td>
<td>CML</td>
<td>Oki (2007)</td>
<td>28</td>
<td>32% CR, 4% PR, 7% HI</td>
<td>No increased toxicity due to decitabine. Higher activity in patients without BCR-ABL mutations.</td>
<td>[139]</td>
</tr>
<tr>
<td>Decitabine + IL-2</td>
<td>Metastatic Melanoma; Renal Carcinoma</td>
<td>Gollob (2006)</td>
<td>21</td>
<td>7% CR</td>
<td>Lower pretreatment CDKN2B methylation level correlates with higher response rate.</td>
<td>[127]</td>
</tr>
</tbody>
</table>

Abbreviations: CR, complete remission; PR, partial response; HI, hematologic improvement; ORR, overall response rate; SD, stable disease. CCR, conventional care regimens; RBC, red blood cells; HSCT, hematopoietic stem cells transplantation; PBMC, peripheral blood mononuclear cell; PBC, peripheral-blood cells.
intermediate- and high-risk MDS patients. In that study, patients treated with 5-Aza-CR showed a significant improvement in median overall survival (OS) than patients who received conventional care regimens (CCR): 24.5 months vs 15.0 months. It was the first time that 5-Aza-CR treatment was demonstrated to prolong OS in high-risk MDS patients [106]. Detailed analyses of AZA-001 indicated that low-dose cytarabine (one of CCR for patients with higher-risk MDS) less efficient and more toxic as compared with 5-Aza-CR [107]. Fenaux et al. also demonstrated that 5-Aza-CR benefited older AML patients by prolonging the OS from 16.0 months to 24.5 months while reducing the prevalence of side effects [107]. Based on the outcome of the AZA-001 trial, the National Comprehensive Cancer Network (NCCN) recommended 5-Aza-CR as the preferred therapy for patients with high-risk MDS. In addition to high-risk MDS, 5-Aza-CR can also be used as a potentially effective treatment for patients with low-risk MDS [108–109].

However, the current FDA-approved, 7-day course of 5-Aza-CR treatment requires weekend treatment and is inconvenient for patients and care providers. To overcome this problem, Lyons et al. [110] designed three alternative regimens which avoided weekend treatment. Patients who received any one of the three regimens showed similar hematological improvement as the previously approved 7-day 5-Aza-CR regimen, as well as a higher transfusion-independent rate. More patients who needed transfusion of red blood cells (RBCs) at baseline became independent of RBC transfusion. Another phase-II trial administered an alternative 5-day 5-Aza-CR intravenous schedule and reported a 27% partial response (PR) + complete remission (CR) rate that was comparable with the 7-day subcutaneous regimen [111]. However, further studies are required to illuminate the survival benefit of these modified regimens. Although one group reported that a limited number of treatment cycles could achieve a overall response rate (ORR) of 50% according to new criteria set by the International Working Group, most clinical trials indicate that prolonged exposure to 5-Aza-CR will benefit patients [112].

5-aza-2’-deoxycytidine (Decitabine) 5-Aza-CdR was also approved by the FDA for MDS therapy, but there is no clear evidence indicating 5-Aza-CdR improves OS. In the USA-registered trial (D-0007), patients treated with 5-Aza-CdR had a 17% ORR, which was significantly higher than that in the best supportive care (BSC) group (0%). When comparing the OS between the 5-Aza-CdR and control arms, a statistical improvement (14.0 vs 14.9 months) was not observed, even though clinical benefits (e.g. independence from RBC transfusion or elongation of median time to AML progression) were seen after 5-Aza-CdR treatment [113]. Wijermans et al. presented a similar negative result of the OS advantage of 5-Aza-CdR for older patients with MDS or Chronic Myelomonocytic Leukemia (CMML) [114]. To increase the CR rate of patients with these diseases taking 5-Aza-CdR in the outpatient setting, several clinical trials explored alternative schedules. Kantarjian et al. [115] showed that a 5-day intravenous schedule with the highest dose-intensity yielded the highest CR rate (39%). In a follow-up study, the ADOPT trial reported an ORR of 32%, suggesting that this 5-day schedule was as effective as the approved inpatient regimen [116]. 5-Aza-CdR is currently being investigated in other cancer types to assess the best conditions for administration [117–119].

**MG98** Several phase I/II clinical trials have been conducted to find out the tumor types sensitive to MG98 and an appropriate working dosage. Some of the patients already showed decreases in DNMT1 levels, but a consistent correlation of DNMT1 level and dosage has not been observed [120–121]. New evidence indicated that 7-day continuous dosing of MG98 was well tolerated for patients with advanced solid tumors, but an objective clinical response was not reported [122].

**Combination therapy** HDAC inhibitors (HDACi) are a family of epigenetic drugs that increase acetylation of histone proteins and cytoplasmic proteins such as p53. As discussed above, reversing abnormal silencing of epigenetic genes is coordinated by increasing promoter histone acetylation levels and DNA demethylation. Based on these observations, numerous clinical trials have been conducted to study the synergistic effects of DNMTi and HDACi (Table 1).

Several research teams have combined 5-Aza-CR with the HDACi valproic acid (VPA) to treat MDS and AML. Soriano et al. [123] conducted a phase-I/II clinical trial in which patients were treated with 5-Aza-CR and VPA every day for 7 days. An ORR of 42% indicated that this combination strategy was clinically effective. Another phase-II study sequentially administered 5-Aza-CR after VPA and observed a CR+PR of 30.7%. As the plasma concentration of VPA increased, (>50 μg/mL vs <50 μg/mL), a better median survival rate was observed (18.7 vs 10 months), indicating HDACi have the potential to increase 5-Aza-CR efficacy [124]. Combination therapy of 5-Aza-CR + VPA has also been investigated in breast cancer, colon cancer and other advanced cancers [125]. Other HDACi have also been combined with 5-Aza-CR. For example, 46% of patients with MDS or AML who received 5-Aza-CR supplemented with entinostat (another potential HDACi) showed promising outcomes. Among them, 3 patients had CR, 4 patients had PR and 7 patients had hematologic improvement [126]. In a phase-I study, researchers co-administered 5-Aza-CR and the first-generation HDACi sodium phenylbutyrate to patients with refractory solid tumors. The three-dose schedule administered in that study showed mild toxicity, albeit with few benefits for the patients [127].

Similar to 5-Aza-CR, 5-Aza-CdR has also been tested for its therapeutic efficacy in combination with HDACi. 5-Aza-CdR + VPA achieved a 22% objective response in patients with leukemia although, in this case, the higher level of VPA did not correlate with clinical activity [128]. A later study confirmed this conclusion, demonstrating that 5-Aza-CdR itself had promising clinical activity in elderly patients with AML. Adding 25 mg/kg/d of VPA neither enhanced the efficacy of 5-Aza-CdR nor did it increase the re-expression of CDKN2B [129].

5-Aza-CR and 5-Aza-CdR have also been combined with other conventional therapies. For example, a recent study
combining 5-Aza-CR with the FDA-approved MDS chemotherapy drug lenalidomide revealed an impressive ORR of 67% [110]. 5-Aza-CdR has been combined with carboplatin, imatinib, or with gemtuzumab ozogamicin (monoclonal antibody) for the treatment of various cancer types [113–133]. The encouraging results from these clinical trials could broaden the application of DNMTi to various tumor types, but further randomized clinical trials with control arms are needed to demonstrate the feasibility of these approaches.

Validating epigenetic targets in clinical trials

The ability of 5-Aza-CR and 5-Aza-CdR to reduce global DNA methylation in vivo has been validated in multiple clinical trials in which they have been used as single agents or combined with other anticancer therapies [118,123,125–126,129,134–140]. The reversal of DNA methylation around individual gene promoters such as CDKN2B and MAGE1A has also been confirmed in various tumor types [126,128,133]. However, most of these studies did not show a clear association between the level of induced demethylation and clinical response. Two recent studies reported that responders to 5-Aza-CR have more significant decreases in global methylation level and methylated promoters compared with non-responders as detected by the Infinium™ methylation assay [111,130].

Conclusion and future directions

This review summarizes the recent advances and future prospects for epigenetic therapy through DNA methylation. After decades of development, DNMTi are now been used in cancer therapies. Progress made in epigenetic research will further aid translation from the 'bench to the bedside'.

A densely methylated region is strongly associated with other silencing mechanisms (including chromatin proteins), and consequently the use of a single hypomethylating agent might not be sufficient to reverse epigenetic aberrations in cancer. This concept has guided the clinical application of DNMTi and HDACi combination therapy. Further systematic exploration of the overall epigenetic changes after drug-induced DNA demethylation might lead to the discovery of other drug targets with the goal of minimizing toxicity and maximizing response. Such examples might be inhibitors to LSD1 (which removes the active H3K4me mark) or inhibitors of EZH2 (which plays an important part in gene suppression in cancer) [141,142]. In addition to targeting DNMTi, new drugs could be designed to inhibit DNA methyltransferases 3A and 3B with the accumulated understanding of their functions in maintaining methylation patterns in somatic cells. The elucidation of differences between the responses of 5-Aza-CR and 5-Aza-CdR might help in understanding the different outcomes in clinical trials, and prompt trials to compare them head-to-head [80].

Analyzing available clinical data might also provide better prognostic factors for patient survival or predict markers for patient sensitivity to DNMTi. It has been suggested that there is no positive correlation between a good response to DNMT treatment and baseline methylation in the patient [126]. In fact, patients who seem to respond to hypomethylation therapy best are those who carry markers such as lower methylation levels in the CDKN2B promoter or higher levels of miR29 [128,143]. Ultimately, efforts to create a comprehensive picture of the epigenetic landscape should help in the understanding of cancer pathology and the molecular basis of tumorigenesis. This could lay the foundation for future drug development to improve the survival and quality of life of cancer patients.

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