Review

DNA hypermethylation as a chemotherapy target

Juan Ren a,k,⁎ 1, Brahma N. Singh b,1, Qiang Huang b,c, Zongfang Li c, Ya Gao c, Prachi Mishra d, Yi L. Hwa e, Jinping Li b,f, Sean C. Dowdy f, Shi-Wen Jiang b,f,⁎⁎

a Cancer Center, First Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, 710061, China
b Department of Biological Science, Mercer University School of Medicine, 4700 Waters Avenue, Savannah, GA 31405, USA
c Second Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, 710004, China
d School of Biotechnology, Devi Ahilya University, Khandwa Road, Indore, M.P., India
e Department of Internal Medicine, Mayo College of Medicine, 2nd Street, Rochester, MN 55902, USA
f Department of Obstetrics and Gynecology, Mayo College of Medicine, 2nd Street, Rochester, MN 55902, USA

Abstract

Epigenetics refers to partially reversible, somatically inheritable, but DNA sequence-independent traits that modulate gene expression, chromatin structure, and cell functions such as cell cycle and apoptosis. DNA methylation is an example of a crucial epigenetic event; aberrant DNA methylation patterns are frequently found in human malignancies. DNA hypermethylation and the associated expression silencing of tumor suppressor genes represent a hallmark of neoplastic cells. The cancer methylome is highly disrupted, making DNA methylation an excellent target for anti-cancer therapies. Several small synthetic and natural molecules, are able to reverse the DNA hypermethylation through inhibition of DNA methyltransferase (DNMT). DNMT is the enzyme catalyzing the transfer of methyl groups to cytosines in genomic DNA. These reagents are studied intensively in cell cultures, animal models, and clinical trials for potential anti-cancer activities. It was found that accompanying DNA demethylation is a dramatic reactivation of the silenced genes and inhibition of cancer cell proliferation, promotion of cell apoptosis, or sensitization of cells to other chemotherapeutic reagents. During the last few decades, an increasing number of DNMT inhibitors (DNMTi) targeting DNA methylation have been developed to increase efficacy with reduced toxicity. This review provides an update on new findings on cancer epigenetic mechanisms, the development of new DNMTi, and their application in the clinical setting. Current challenges, potential solutions, and future directions concerning the development of DNMTi are also discussed in this review.

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Contents

1. Introduction ............................................................. 1083
2. Transcriptional down regulation due to DNA methylation ........................................ 1085
   2.1. DNA methylation and gene silencing .............................................. 1085
   2.2. Cancer epigenetics ....................................................... 1085
3. DNMT inhibitors (DNMTi). ....................................................... 1085
   3.1. Nucleoside DNMTi ....................................................... 1085
   3.1.1. Azacytidine ...................................................... 1085
   3.1.2. Zebularine ...................................................... 1086
   3.1.3. 5-Fluoro-2'-deoxycytidine ............................................... 1086
   3.2. Non-nucleoside DNMTi. ..................................................... 1087
   3.2.1. Procaine ....................................................... 1087
   3.2.2. Procainamide ..................................................... 1087
   3.2.3. Hydralazine ...................................................... 1087
   3.2.4. RG108 ........................................................ 1087
   3.2.5. Miscellaneous agents ................................................. 1087

⁎ Correspondence to: J. Ren, First Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, 710061, China.
⁎⁎ Correspondence to: S.W. Jiang, Department of Biological Science, Mercer University School of Medicine, 4700 Waters Avenue, Savannah, GA 31405, USA.
E-mail address: JIANG_SW@Mercer.edu (S.-W. Jiang).

1 These authors contributed equally.

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1. Introduction

The sequences of genomic DNA carry genetic codes that in the semiconservational replication of DNA accounts for the biological inheritance from generation to generation. An increasing body of evidence indicates that besides DNA sequences, covalent modification of the nucleotides and histone tails also regulate gene expression and cell function [1]. The “epigenetic” traits, with their characteristic inheritance pattern along the somatic lineages, effectively complement the genetic information encoded by DNA sequences, and play a critical role in the embryogenesis, tissue/organ development, maintenance of physiological function, aging and pathogenesis of diseases [1,2]. The cytosine methylation-mediated pathway is the best studied subject and its involvement in various cell processes including imprinting [3], X-chromosome inactivation [4], suppression of retrotransposon and repetitive elements, and tissue-specific genes [5]. Moreover, aberrant DNA methylation profiles have been found to be associated with a broad spectrum of disorders, including cancer, atherosclerosis, obesity, insulin resistance, kidney disease, and autoimmunity [6–8].

DNA methylation is a covalent biochemical modification, resulting in the addition of a methyl group to the 5th carbon position in the pyrimidine ring of cytosine located in the context of cytosine–phosphate–guanine (CpG) dinucleotides. Hypermethylation often limits the accessibility of transcription factors to promoters, promotes methyl-CpG binding domain (MBD) binding, which results in recruitment of additional silencing-associated proteins, and ultimately, gene silencing [9,10]. The human genome is not methylated uniformly, but contains unmethylated segments interspersed by methylated regions [11]. An estimated 60% of mammalian gene promoters contain CpG islands, many of these being housekeeping genes that are commonly unmethylated and transcriptionally active. It has been known well that CpG islands in certain genes, especially tumor suppressor genes, often become aberrantly hypermethylated during the development of cancer (Fig. 1a & b). This may give the cell a growth advantage by increased proliferation or increased resistance to apoptotic factors. Some hypermethylation events, such as the methylation of glutathione S-transferase Pi in prostate cancer, are readily detected in precancerous or early stages of cancer development [12]. These epigenetic alterations may cause or improve conditions for malignant transformation. Herman and Baylin [13] found that genes such as O6-methylguanine methyltransferase (MGMT) retinoic acid repressor β (RAR-β), the tumor suppressor p16^{INK4A}, and the DNA repair gene hMLH1 were frequently inactivated by hypermethylation in early lesions of esophageal basal cell hyperplasia.

Epigenetic changes are connected to genetic aberrations by several pathways. Epigenetic silencing of the DNA repair gene MLH1 in the hyperplastic stage can lead to genetic mutations that are found in cancers [14]. Global hypomethylation, as detected in many tumor types, is associated with genomic instability and may contribute to the development of aneuploidy, LOH and genetic amplifications [15]. It has been shown that the leukemia-promoting PML–RAR fusion protein can recruit DNMTs to target the genes and thereby induce epigenetic silencing [10]. It has also been found that in some tumor suppressor genes, one allele is genetically mutated and another allele is epigenetically silenced [16], providing an example of a cooperative mechanism by which epigenetic and genetic lesions promote tumorigenesis. Patterns of DNA methylation are established by the coordinated action of the DNA methyltransferases (DNMTs) and associated factors, such as the polycomb proteins, in the presence of S-adenosylmethionine (SAM) that serves as a methyl donor [8]. The mammalian DNMT family includes four active members: DNMT1, DNMT3A, DNMT3B, and DNMT3L [17]. Mammalian DNMTs are responsible for methylation pattern acquisition during gametogenesis, embryogenesis and somatic tissue development [18]. The DNMT1 is the major enzyme responsible for maintenance of the DNA methylation pattern. DNMT1 is located at the replication fork and methylates newly biosynthesized DNA [19]. The mammalian DNMTs are comprised of two parts: a C-terminal catalytic part and a large multi-domain N-terminal part of variable size, which has regulatory functions. The C-terminal part is composed of 500 amino acids, which is conserved between five DNMTs of eukaryotics and prokaryotics that harbor the active center of the enzyme and contains amino acids motifs characteristic of the cytosine-C5 methyltransferases. The N-terminal part contains 621 amino acids that are not essential for DNMT1 activity [20], but are required for the discrimination between hemi-methylated and unmethylated DNA. The catalytic domains of all the DNMTs share a common core structure, known as “AdoMet-dependent methyltransferase” [21]. This domain is involved, both, in cofactor binding (motifs I and X) and catalysis (motifs IV, VI and VIII) of substrate to DNMT. The non-conserved region between motifs VIII and IX, the so called target recognition domain, is involved in DNA recognition and specificity [22]. DNMT2 is the smallest mammalian DNA methyltransferase. It is composed solely of the C-terminal domain, and does not possess the regulatory N-terminal region. The structure of DNMT2 suggests that this enzyme participates in the recognition of DNA damage, DNA recombination and mutation repair [23]. However, recent studies suggest that an interaction between DNMT1 and DNMT3B may be vital for the maintenance of patterns of DNA methylation in human colon-cancer cells, particularly in repeat regions and imprinted genes [24,25]. During early embryogenesis, de novo DNA methylation is mediated by DNMT3A and DNMT3B associated with DNMT3L. The DNMT3L lacks conserved motifs of the catalytic domain but is otherwise closely related to the C-terminal domain of DNMT3A and DNMT3B [23,26]. DNMT3A and DNMT3B exhibit a high degree of primary structural homology. Upon differentiation of embryonic stem cells the activities of both enzymes are reduced and remain low in adult somatic tissues. The expression of DNMT3A is ubiquitous, while DNMT3B is expressed at very low levels in most tissues except the testis, thyroid and bone marrow [27]. It has recently been reported that DNMT3A and DNMT3B in the cell are tightly associated with nucleosomes containing methylated DNA [25]. Both the direct interaction of these proteins with the histone tails and the polymerization of DNMT3A could contribute to the stable association of these enzymes with chromatin. The levels of DNMTs, especially those of DNMT3B and DNMT3A, are often increased in various cancer tissues and cell lines, which may partially account for the hypermethylation of promoter CpG-rich regions of TSGs in a variety of malignancies [28,29]. For these reasons, DNMTs are considered valuable targets for the design of specific anti-cancer strategies.

The earliest and most successful epigenetic drug to date, DAC (also known as 5-Aza-2′-deoxycytidine zebularine), is currently
recommended as the first-line treatment of high-risk myelodysplastic syndromes. During the last few decades, an increasing number of drugs targeting DNA methylation have been developed for increased efficacy and stability, as well as reduced toxicity. These drugs have been shown to inhibit cancer cell growth, induce cancer cell apoptosis, and reduce tumor volumes in mice [30–33]. Encouraging results from preclinical and clinical studies have prompted further efforts to elucidate epigenetic alterations in cancer, and to subsequently
develop new epigenetic therapies. In this review, we will focus on the recent findings from the in vitro experiments, animal models, and clinical trials on DNMT inhibitory agents.

2. Transcriptional down regulation due to DNA methylation

2.1. DNA methylation and gene silencing

The role of DNA methylation in cancer has become one of the most extensively investigated areas. Recent genome-wide studies have demonstrated distinct patterns of DNA methylation in cancerous tissues, contrary to their normal counterparts [34–36]. These epigenetic alterations, specifically changes in the DNA methylation pattern, can be classified under two major categories: 1) Genome-wide hypomethylation which usually involves repeated DNA sequences such as long interspersed nuclear elements (LINE) [37], and 2) hypermethylation of CpG islands [38] within specific genes. Early studies that measured the global content of 5-methylcytosine found to be associated with advanced tumor stage [46]. Hypomethylation caused chromosomal destabilization and may contribute to cancer progression [42]. However, most of the studies concentrated on focal CpG island hypermethylation, often associated with tumor suppressor genes, including those for steroid receptor, cell adhesion molecules, and inhibitors of matrix metalloproteinases.

Early evidence that measured the global content of 5-methylcytosine of tumors showed that hypomethylation was a common feature in several malignancies such as metastatic hepatocellular carcinoma [39], cervical cancer [40], and prostate tumors [41]. This epigenetic change leads to the relaxation of the DNA methylation-mediated transcriptional suppression of LINE-1 and endogenous retroviral sequences, which has been shown to play an oncogenic role [14]. Hypomethylation caused chromosomal destabilization and may contribute to cancer progression [42]. However, most of the studies focused on focal CpG island hypermethylation, often associated with tumor suppressor genes, including those for steroid receptor, cell adhesion molecules, and inhibitors of matrix metalloproteinases. [43,44]. One of the most detailed studies was conducted on tissue cultures from a patient with lung cancer. More than 40 genes were found to contain some degree of alteration in DNA methylation. The most commonly hypermethylated genes include RAR, RASSF1A, CDKN2A, CHD13, and APC [45].

While there is a general correlation between increased DNMT expression levels and rates of gene hypermethylation in cancer compared to normal controls, regression analysis based on individual samples does not support a one-to-one correlation between DNMT levels and gene hypermethylation, reflecting the complex control of DNA methylation in vivo. Nevertheless, in acute leukemia over-expression of DNMTs appears to be well correlated with hypermethylation of p15 and p16. At the time of diagnosis, it was also found that p15 hypermethylation was associated with lower survival, and transformation of myelodysplastic syndromes to acute myeloid leukemia. Based on this data the authors proposed to use p15 as a marker of leukemic transformation [46,47]. Gene methylation and silencing has also been linked to clinical manifestations. In a population-based study on human bladder carcinoma, the epigenetic silencing of three tumor suppressor genes, p16<sup>INK4a</sup>, RASSF1A and PRSS3, were examined. RASSF1A and PRSS3 promoter methylation was found to be associated with advanced tumor stage [46].

2.2. Cancer epigenetics

The detailed mechanism by which a gene undergoes hyper- or hypomethylation are still unclear. Early evidence suggested that elevated DNMT levels might trigger DNA hypermethylation, which may afford a growth advantage [48]. Indeed, the most epigenetically susceptible genes are those involved in cell cycle regulation (p16<sup>INK4a</sup>, p15<sup>INK4b</sup>, P19ARF, RH1, A4ARF), DNA repair (BRCA1, MGMT), apoptosis (DAPK, TMS1), drug resistance, detoxification, differentiation, angiogenesis, and metastasis. Considering the new findings from the stem cells, Cedar and Bergman [15] proposed an alternative mechanism by which cancer cells undergo a process similar to active epigenetic reprogramming during development. This ‘epigenetic switch’ may be regulated by DNMTs and other proteins (Fig. 1b).

Accompanying DNA methylation are the posttranscriptional modifications of histones. Upon binding to methylated DNA, MBDs can recruit histone deacetylases (HDACs) to local chromatin [49]. Indeed, HDACs are often found to be over-expressed in various types of cancerous cells (Fig. 1b), resulting in histone deacetylation around the transcription start site and the formation of a more compact structure in the region of the silenced genes [50,51]. A close link between DNA methylation and histone methylation has also been observed. H3 Lys9 methylation in Neurospora and Arabidopsis appears to be a prerequisite for DNA methylation [52]. Lehner et al. [53] observed that Suv39H1/2 knockout also alters the DNA methylation pattern in the pericentric heterochromatin. Recently, Lim et al. [54] pointed out that H3K4me is selectively demethylated by histone lysine demethylase (LSD1), which is upregulated in cancer. Thus, DNA and histone methylation are likely to form a positive feedback loop resulting in long term gene silencing. From this point of view, some effects of DNMT inhibitors may be achieved through the disruption caused by the interactions between DNMTs- and HDACs-mediated pathways. This partially explains why the simultaneous targeting of the two pathways can often achieve synergistic effects.

3. DNMT inhibitors (DNMTi)

For most of the currently available DNMT inhibitors, blocking of DNA methylation was originally identified as a demethylating activity; which raises questions about their specificity as well as cytotoxicity. Nevertheless, optimization of treatment regimens combined with additional drugs have much improved their profile and inspired expectation for their clinical application. Approval of 5-Azacytidine by the FDA as an anti-tumor agent for the treatment of myelodysplastic syndrome, has invigorated efforts for the development of novel strategies to inhibit DNMTs. Despite the recent reports of several novel DNMT inhibitors, the number of available DNMTi compounds remains limited. Targeted design of DNMTi and expanded screening, using an effective reporter are considered feasible approaches that will facilitate the identification of more specific molecules [55].

3.1. Nucleoside DNMTi

3.1.1. Azacytidine

The two best studied DNMT inhibitors are Azacytidine (5-Azacytidine; 5-Aza-CR), a simple derivative of the nucleoside cytidine, and deoxyctydine (5-Aza-2’-deoxycytidine; 5-Aza-CdR), the deoxyribose analogue of 5-Azacytidine. The two pyrimidine analogues, which were first synthesized as cytotoxic agents, have been approved by the FDA in the USA for the treatment of myelodysplastic malignancies. In the 1980s, these potential drugs were found to show demethylating activity after incorporation into the DNA of actively replicating tumor cells by forming a covalent protein–DNA complex [56]. Their anti-cancer activity is mediated by two main mechanisms: 1) cytotoxicity resulting from incorporation into the RNA and/or genomic DNA, and 2) restoring normal growth and differentiation by demethylation of tumor suppressor genes [10]. Upon uptake by the concentrative nucleoside transporter 1 (hCNT1) [57], 5-Aza-CR and 5-Aza-CdR are phosphorylated sequentially by uridine–cytidine and diphosphate kinases which convert them to the active triphosphate forms 5-Aza-CTP and 5-Aza-dCTP, respectively (Fig. 1c) [58,59]. 5-Aza-CdR in genomic DNA can prevent the resolution of the covalent reaction intermediate of DNMT and DNA [60] which leads to the trapping and inactivation of DNMT. 5-Aza-CTP is incorporated into RNA which affects nuclear and cytoplasmic RNA metabolism including ribosome biogenesis and protein synthesis [61]. Therefore, 5-Aza-CR can exert cellular effects independent of demethylation [62]. These drugs appear to be most toxic during the S-phase of the cell cycle; however, the mechanism of cytotoxicity is not fully understood.
Recently, Stresemann et al. [63] pointed out that the 5-Aza-CR can also be incorporated into DNA after intracellular conversion to 5-Aza-dCTP by phosphorylation and reduction. Thus, both drugs are able to inhibit the methylation of replicating DNA by the stoichiometric binding and trapping of DNMTs for proteosomal degradation. The depletion of DNMT in combination with continued DNA replication lead to genomic DNA hypomethylation through the passive dilution of methylated cytosine [64,65] rather than active demethylation. In daughter cells the decreased levels of DNA methylation are associated with reduced H3K9me3 levels and increased H3ac and H3K4me3 modifications around gene promoter regions. Lin et al. [66] observed the formation of compact nucleosomes in the methylated and silenced MLH1 promoter. A recent genome-wide study of modifications in the epigenetic landscape after 5-Aza-CR treatment further validated this concept [67]. Interestingly, studies from Momparder et al. [62] indicated that 5-Aza-CdR may be more specific and less toxic than 5-Aza-CR. Indeed, the molecule shows a greater inhibitory effect on DNA methylation as well as higher anti-tumor activity against myelodysplastic syndrome, acute myelogenous leukemia (AML), and chronic myelogenous leukemia (CML) [68]. More recently, Qin et al. [69] observed that the demethylation functions of 5-Aza-CR and 5-Aza-CdR are potent inhibitors for DNA methylation even at low concentrations. This observation bears strong clinical implications since these reagents at higher concentrations exhibit cytotoxic effects such as myelosuppression with neutropenic fever [70] due to DNA damage and/or interference with DNA synthesis [71]. These cytotoxic effects highlight the necessity for cautious interpretation of laboratory and clinical data derived from DNMT1 studies. Based on the action of these reagents, S-phase is required for the effective incorporation of these two drugs into DNA. Therefore, rapidly proliferating cells such as malignant cells may be more sensitive than normal cells, undergoing regular cell cycle. Further exploration of this specific feature may provide opportunity for achieving an improved efficacy and at the same time, reduced side effects by these drugs in the clinical setting.

3.1.2. Zebularine

Several cytidine analogues have been developed in order to improve the stability and efficacy of 5-Aza-nucleosides. Zebularine (1-(β-D-ribofuransyl)-2-(1H)-pyrimidinone), a cytidine lacking 4-amino group of the pyrimidine ring, is the most recent addition to the list of demethylating agents in the family of nucleoside analogues [72]. This agent is described as a potent inhibitor that acts by forming a covalent complex with DNMT and cytidine deaminase when incorporated into DNA. Synthesized in 1961, it was first characterized as a potent inhibitor of cytidine deaminase with anti-tumor properties [40]. It was later described as a DNMTi [73] leading to DNA demethylation [59] and reactivation of methylation-silenced TSGs [74,75] in cancer cell lines. Zebularine appears to be less cytotoxic and more stable than 5-Aza-CTP and 5-Aza-dCTP [73,76]. Zebularine’s low toxicity allowed it to be given continuously, for long period of time, to maintain the reversal of aberrant hypermethylation [77]. Moreover, zebularine enhances tumor cell chemosensitivity and chemoradiation sensitivity [78] and has angiostatic and antimitogenic activities [79,80]. Additionally, zebularine is stable in vivo and suitable for oral administration. It has been well known that when 5-Aza-CR or 5-Aza-CdR is withdrawn, demethylated DNA targets become remethylated. However, when cancer cells are transiently treated with 5-Aza-CdR and then subjected to a continuous treatment with zebularine, remethylation is hindered and gene expression is maintained, suggesting that this treatment modality is able to achieve a sustained response [77,81,82]. However, zebularine is not without drawbacks and therefore further clinical investigations are required for better understanding of its efficiency, as well as side effects in cancer patients. For example, it is not clear why higher concentrations of zebularine are needed to obtain similar levels of demethylation in cells in comparison with 5-Aza-CdR [75,83]. Moreover, Suzuki and their colleagues [84] reported that zebularine suppresses the apoptotic potential of 5-fluorouracil against human oral squamous cell carcinoma cells, indicating that combination therapies with this drug must be carefully investigated. It also has been observed that zebularine can act as a potent mutagen in Escherichia coli [85]. Its impact on genomic DNA stability in mammalian cells remains to be examined.

3.1.3. 5-Fluoro-2′-deoxyoctidine

The cytidine analogue 5-fluoro-2′-deoxycytidine (FdCyd, NSC 48006) was previously investigated as a tumor type-selective prodrug of the more potent thymidylate synthase inhibitor 5-fluoro-2′-dUMP (FdUMP) [68,86]. FdUMP can be produced from FdCyd through deamination of FdCyd to 5-fluoro-2′-deoxyuridine (FdUr) by cytidine deaminase followed by phosphorylation to FdUMP [87]. This compound was later found to have demethylating effect in the mouse cells as well as in the human breast and lung carcinoma cells [87]. Newman and Santi [88] concluded that FdCd is not only a prodrug for FdUMP, but also has antineoplastic effects by inhibition of cytidine deaminase within the FdUr resistant S-49 mutant cell lines [89]. In FdCyd, a fluorine atom replaced one hydrogen at C5, which during the methylation reaction receives the methyl group. The FdCd can be incorporated into DNA which then exerts an inhibitory effect on the action of DNMT at the β-elimination step of the methyl transfer reaction. The presence of the fluorine atom makes it difficult for the DNMT molecule to leave the moiety [94]. The DNMT enzyme becomes trapped in an abortive covalent complex at targeted 5-fluorocytosine residues in DNA in a similar manner as with 5-Aza-CdR [91]. This drug has already entered phase-I clinical trials for the treatment of breast cancer and other solid tumors [92]. A number of clinical studies showed that combination therapy of FdCd with tetrahydrouridine (THU), a potent cytidine deaminase inhibitor, improves the stability of FdCd [89]. More recently Kratzke et al. [93] tested the combined therapy with FdCd and another stable analogue, dihydro-5-Azacytidine (DHAC), for the treatment of malignant mesothelioma. DHAC was later found to significantly inhibit DNMT1 activity, and to have demethylating activity in human breast cancer cells [94]. DHAC also competes with cytidine triphosphate for incorporation into RNA, leading to ribosomal degradation and defective protein synthesis [95]. FdCd failed to show clear clinical benefit in this study. The clinical use of FdCd is also limited by its in vivo generation of the potentially toxic 5-fluorodeoxouridine as a metabolites [96].

Efforts to develop new nucleoside analogue DNMTi with improved stability are underway [12]. One 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 that inhibits DNMTs after its activation by human carboxylesterase in a liver cancer cell line [97]. Byun et al. [97] reported that the NPEOC–DAC, compared to decitabine, was 23-fold less potent at low doses (≤10 μM) for inhibiting DNA methylation; and was also associated with a 3-day delay in its effect. However, at higher doses (≥10 μM) NPEOC–DAC was more effective in inhibiting DNMT. NPEOC–DAC is chemically distinct from decitabine with differing metabolism. Thus, changing the N4 NPEOC group of NPEOC–DAC to a smaller carbon chain may lead to a molecule much more efficient at inhibiting DNA methylation. Moreover, the activity of NPEOC–DAC is dependent upon carboxylesterases, which are not expressed in every tissue. Further studies are required to explore the use of this compound in combination therapy [97]. S110 (a 5′-AzapG-3′ dinitolacte, containing the 5′-azacytosine ring) has also been shown to improve the efficacy of 5-Aza-CdR by protecting it from deamination by blocking the activity of deaminase enzyme. The compound is well-tolerated and has shown to reduce the level of DNA methylation in the CDKN2A promoter region in xenografts [98].
3.2. Non-nucleoside DNMTi

Nucleoside analogues, though effective in inducing DNA demethylation and reactivation of hypermethylated genes, carry a considerable concern on cytotoxicity [99], which is probably associated with the drugs’ incorporation into DNA. This concern prompted the search for non-nucleoside DNMT inhibitors. Some compounds assessed for their potential to induce hypomethylation in solid tumors include procaine, L-tryptophan derivative RG108, hydralazine, MG98, procainamide, and (−)-epigallocatechin-3-gallate (EGCG), which is the main polyphenol compound in green tea [100]. Several non-nucleoside compounds are documented for anti-DNMT activity [68]: dietary polyphenols like EGCG, the bisulfide bromotyrosine derivatives psammaplins, the L-tryptophan derivative RG108, and procaine and procainamide, which were originally approved by the U.S. Food and Drug Administration as local anesthetics and for the treatment of cardiac arrhythmias, respectively. Chuang et al. [101] observed that the non-nucleoside compounds, however, induce limited epigenetic changes in living cells.

3.2.1. Procaine

The local anesthetic drug procaine acts as an inhibitor of DNMT in breast cancer cells, causing global genomic DNA demethylation and reactivation of TSGs with hypermethylated CpG islands [55]. Procaine also exerts demethylating activity in the mouse tumor xenograft [39,102]. Procaine appears to bind CpG-rich sequences and thereby block the binding of DNMTs to DNA [39]. According to Vilar-Garea et al. [102] procaine acts as an effective DNMTi at high concentrations (100–500 μM) in a cell type-dependent manner. Castellano et al. [103] prepared a series of procaine analogues and tested their inhibitory activity against DNMT1. Among those tested, the procaine derivative pyrrolidine exhibited the highest inhibiting potency on demethylation of chromosomal satellite repeats in HL60 human myeloid leukemia cells. Derivative pyrrolidine is considered a lead compound for further studies in this field. In an experiment using human hepatoma cell lines, the cell viability was significantly decreased by procaine treatment. Many genes transcriptionally suppressed by DNA hypermethylation were demethylated and reactivated following procaine treatment. Moreover, the combined treatment with TSA and procaine produced a stronger reduction in cell viability [104]. These data indicated that procaine had DNA demethylating and growth-inhibitory effects on human hepatoma cells. Recently, procaine was shown to be effective for induction of DNA demethylation and growth inhibition in the human breast cancer cell line MCF-7 [102]. These findings point to the possible application of procaine and its derivatives for epigenetic therapies.

3.2.2. Procainamide

The antiarrhythmic drug procainamide has been known as an inhibitor of DNMT in breast cancer cells. Its demethylating effect on T cells led to the over-expression of lymphocyte function-associated antigen 1 that makes T cells autoreactive [105,106]. Initially proposed as a perturbative of the interactions between DNMTs and CpG-rich sequences, procainamide was reported to specifically inhibit the maintenance methyltransferase activity of DNMT1 and to demethylate hypermethylated genes [107]. Procainamide causes global DNA hypomethylation and restores the expression of the detoxifier gene glutathione S-transferase P1 (GSTP1) [105]. The mechanism of action is thought to be mediated by its binding to GC-rich DNA sequences [108]. The nitrogen atoms of procainamide may participate in a covalent reaction intermediates[63]. RG108 is capable of inhibiting human DNMT activities and reactivate TSGs in human colon-cancer cells [119]. However, the action of RG108 is not fully understood. Direct interaction with DNMT (rather than forming a covalent enzyme trap) and/or the binding to CG-rich DNA sequences [102] were considered possible mechanisms. Brueckner et al. [118] concluded that RG108 treatment induced demethylation and reactivation of TSGs in a human colon-cancer cell line, but did not affect the methylation of centromeric satellite sequences [119]. The preferential action of RG108 for euchromatic regions might suggest that euchromatin and heterochromatic sequences are methylated by distinct pools of DNMTs. This gene- or genomic domain-specific action, if proven to be true, may be used for the development of more specific epigenetic therapies. We can take advantage of the selective activity of this drug to reactivate silenced TSG, yet at the same time conserve the methylation status of satellite DNA and therefore, the chromosome stability in treated cells [38,120]. Thus, RG108 is considered as an attractive candidate for further evaluation.

3.2.5. Miscellaneous agents

Recently, the lipophilic, quinoline-based compound SGI-1027 containing a 5-Aza-CdR moiety was demonstrated to be a novel DNMTi in vitro. Treatment of RKO cells with SGI-1027 resulted in the degradation of DNMT1, demethylation of the CDKN2A gene promoter, and reactivation of methylation-silenced genes [121]. A unique property of SGI-1027 and probably other compounds in this class is that, unlike the nucleoside compounds, it is not incorporated into DNA. Alternative strategies to inhibit DNMT1 include the use of short anti-sense oligodeoxynucleotides or microRNAs. MG98 is a 20-bp anti-sense oligonucleotide that specifically binds to the 3’ UTR of human DNMT1 mRNA thereby interfering with its translation. Despite promising results in preclinical studies, the clinical use of MG98 has not been validated [122]. Recently, Garzon et al. [123] reported that the microRNA specifically, mir29a can act as a potential DNMTi. mir29a targets DNMT3A/B directly and DNMT1 indirectly by binding...
and p16INK4a to restore the expression of the methylation-sensitive TSGs in cholangiocarcinoma tissues. It has also been shown that down-regulation of DNMT1 by miR-148a and miR-152 transfection was able to restore the expression of the methylation-sensitive TSGs RASSF1A and RASSF1C.[124].

Cytarabine (1-h-o-arabinofuranosylcytosine, AraC) is a deoxycytidine analogue that has been used either alone or in combination with other chemotherapeutic agents for the treatment of relapsed and refractory acute lymphoblastic leukemia [125], acute myeloid leuke-

Table 1
DNMTi and related in vitro and clinical trials.

<table>
<thead>
<tr>
<th>DNMTi</th>
<th>Mode of action</th>
<th>Targeting stage</th>
<th>Cancer</th>
<th>Clinical trial results</th>
<th>Comments</th>
<th>Ref.</th>
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<tr>
<td>(a) Nucleoside</td>
<td></td>
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<tr>
<td>5-Azacytidine</td>
<td>Cytotoxicity and restoring normal growth after incorporation into the DNA</td>
<td>Phase II</td>
<td>MDS</td>
<td>7% CR, 16% PR, and 37% HI</td>
<td>Delayed progression time to AML.</td>
<td>[81]</td>
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<td></td>
<td>MDS</td>
<td>17% CR, 12% PR, and 42% SD</td>
<td>Reduced OS compared to CCR.</td>
<td>[131]</td>
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<td>MDS</td>
<td>44% 45%, and 56% HI</td>
<td>RBC transfusion independence achieved.</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MDS</td>
<td>10.8% CR, 9.5% PR, and 20.3% HI</td>
<td>Survival benefits in responder group.</td>
<td>[133]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MDS</td>
<td>27% ORR</td>
<td>Reduced OS in this 5-day treatment regimen.</td>
<td>[134]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MDS/AML</td>
<td>15.6% CR, 25% HI, and 34.4% SD</td>
<td>Survival benefits in responder group.</td>
<td>[135]</td>
</tr>
<tr>
<td>5-Aza-2′-deoxycytidine</td>
<td></td>
<td>Phase II</td>
<td>SM</td>
<td>9% CR, 13% HI, and 17% ORR</td>
<td>Suppression of DNMT1 expression.</td>
<td>[136]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MDS</td>
<td>34% CR</td>
<td>RBC transfusion independence and delayed progression time toward AML.</td>
<td>[137]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RST</td>
<td>34% CR</td>
<td>Relative reduction of tumor size.</td>
<td>[29]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MDS</td>
<td>17% CR, 18% HI, and 32% ORR</td>
<td>Better response rate at low dose.</td>
<td>[139]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MDS/AML</td>
<td>26% SD</td>
<td>Significant difference in ORR compared to single-center trial.</td>
<td>[140]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MDS</td>
<td>13.4% CR, and 7.5% PR</td>
<td>No clinical benefits observed.</td>
<td>[141]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MDS/AML</td>
<td>26% SD</td>
<td>Inhibited DNMTs, and other enzymes.</td>
<td>[142]</td>
</tr>
<tr>
<td>Zebularine</td>
<td>Associated with the drugs incorporation into DNA</td>
<td>Phase II</td>
<td>SM</td>
<td>26% SD</td>
<td>Reduce global DNA methylation and reactivates CDK2B.</td>
<td>[123]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ST</td>
<td>26% SD</td>
<td>DNMTs, other enzymes.</td>
<td>[143]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Colon cancer</td>
<td>26% SD</td>
<td>DNMTs, HDACs, and other enzymes.</td>
<td>[143]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Colon cancer</td>
<td>26% SD</td>
<td>Inhibitor of DNMT.</td>
<td>[102]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cervical cancer</td>
<td>26% SD</td>
<td>Reactivate the expression of TSGs.</td>
<td>[110]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Breast cancer</td>
<td>26% SD</td>
<td>Inhibit human DNMTs activities and reactivates TSGs in human cells.</td>
<td>[119]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5-Fluoro-2’-deoxycytidine</td>
<td>26% SD</td>
<td>Inhibitory effect on the action of DNA MTases.</td>
<td>[91]</td>
</tr>
<tr>
<td>(b) Non-nucleoside</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MG08</td>
<td>Isolated from tea</td>
<td>Phase I</td>
<td>Not validate</td>
<td>Cervical cancer</td>
<td>Reduce global DNA methylation and reactivates CDK2B.</td>
<td>[123]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Breast cancer</td>
<td>26% SD</td>
<td>DNMTs, other enzymes.</td>
<td>[143]</td>
</tr>
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<td></td>
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<td>ST</td>
<td>26% SD</td>
<td>DNMTs, HDACs, and other enzymes.</td>
<td>[143]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Colon cancer</td>
<td>26% SD</td>
<td>Inhibitor of DNMT.</td>
<td>[102]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cervical cancer</td>
<td>26% SD</td>
<td>Reactivate the expression of TSGs.</td>
<td>[110]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Breast and lung carcinoma</td>
<td>26% SD</td>
<td>Inhibit human DNMTs activities and reactivates TSGs in human cells.</td>
<td>[119]</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>Azacytidine + Sodium</td>
<td>Combination uses</td>
<td>Preclinical</td>
<td>Advanced Solid Tumor</td>
<td>59.24% CR; PR 11.2%</td>
<td>No clear benefits seen in the three dosing regimens tested.</td>
<td>[145]</td>
</tr>
<tr>
<td>Phenylbutyrate</td>
<td></td>
<td></td>
<td>AML/DS</td>
<td>62.30% CR; PR 15.5%</td>
<td>Increasing therapeutic level of VPA promoted efficacy of Azacytidine.</td>
<td>[146]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MDS/AML</td>
<td>44% CR, 17% HI, and 67% ORR</td>
<td>Higher CR is associated with normal cytogenetics and lower methylation levels.</td>
<td>[147]</td>
</tr>
<tr>
<td>Azacytidine + Valproic Acid</td>
<td></td>
<td>Phase II</td>
<td>AML/DS</td>
<td>22% CR, and 42% ORR</td>
<td>The increase of histone acetylation level.</td>
<td>[149]</td>
</tr>
<tr>
<td>Azacytidine + Lenalidomide</td>
<td></td>
<td>Phase I</td>
<td>MDS/AML</td>
<td>72% ORR</td>
<td>Increased response rate and duration of response observed.</td>
<td>[150]</td>
</tr>
<tr>
<td>Azacytidine + Cytarabine</td>
<td></td>
<td>Phase I</td>
<td>MDS/AML</td>
<td>19% CR</td>
<td>Lower pretreatment CDK2B methylation level correlates with higher response rate.</td>
<td>[151]</td>
</tr>
<tr>
<td>Azacytidine + Valproic Acid</td>
<td></td>
<td>Phase I</td>
<td>MDS/AML</td>
<td>44% ORR</td>
<td>A correlation between different doses of decitabine with PBC DNA demethylation.</td>
<td>[152]</td>
</tr>
<tr>
<td>Azacytidine + Etanercept</td>
<td></td>
<td>Phase I</td>
<td>MDS/AML</td>
<td>44% ORR</td>
<td>VPA increased treatment-related toxicity; Re-expression of ER associated with clinical response.</td>
<td>[153]</td>
</tr>
<tr>
<td>Decitabine + Valproic Acid</td>
<td></td>
<td>Phase I</td>
<td>AML</td>
<td>32% CR, 4% PR, and 7% HI</td>
<td>Higher activity in patients without BCR-ABL mutations.</td>
<td>[154]</td>
</tr>
</tbody>
</table>

Abbreviations: AML, acute myeloid leukemia; CML, Chronic Myelomonocytic Leukemia; CR, complete remission; HI, hematologic improvement; MDS, myelodysplastic syndrome; PR, partial response; ORR, overall response rate; CR, conventional care regimens; PBC, peripheral-blood cells; RBC, red blood cells; and SD, stable disease.
amount of the oxidizing agent H$_2$O$_2$ [130], and the oxidation of DNMTs and other proteins might contribute to its inhibition of DNA methylation as well. The same action, however, may also cause oxidative damage and substantially increase its cytotoxicity.

4. Clinical studies on DNMTi

Accumulated in vitro data indicates that DNMTi can reverse hypermethylation DNA and restore normal gene expression profiles in the different cancer cell lines. Promising in vitro results have encouraged in vivo evaluation of these drug efficacy, adverse effects, and the best regimens and/or combination strategies that fulfill their full therapeutic potential. Some important clinical studies are summarized in Table 1.

4.1. 5-Azacytidine (Vidaza, Azacytidine)

5-Aza-CR was the first drug approved by the FDA for the treatment of myelodysplastic syndromes (MDS). The approval was based on positive results from three clinical studies conducted by the Cancer and Leukemia Group B (CALGB) [131]. Before approval of 5-Aza-CR, no specific drug was available and the mainstay therapy was supportive care, including RBC/platelet transfusions and treatment with hematopoietic growth factors. In the FDA clinical trials, treatment with 5-Aza-CR resulted in consistent responses in about 16% (11.8–18.8%) of patients. The response rate was reproducible among the three trials and is consistent with other published reports [155]. Clinical benefits regarding the decreased incidence of bleeding or infections could not be established because of the low incidences of events during the trial period [156]. The response rates in the 5-Aza-CR-treated group, and the control group receiving supportive care were 60% and 5% (P $< 0.0001$) respectively, with a median response duration of 14 months in the 5-Aza-CR treated group. These data presented the first evidence in support of Azacytidine’s efficacy in MDS patients with low-risk disease [156]. 5-Aza-CR treatment also extended the overall survival time in patients diagnosed with Refractory Anemia with Excess Blasts (RAEB) or Refractory Anemia with Excess of Blasts in Transformation (RAEB-T), by delaying the progression to AML [131], and improved the quality of life [157].

A European Aza-001 trial was conducted to further validate the efficacy of 5-Aza-CR in MDS patients, and to test the cytotoxic effects. It was found that the 5-Aza-CR treated patients exhibited a significant improvement in the median survival over the patients that received conventional care regimens (CCR) (24.5 months and 15.0 months, respectively) [158]. This drug also benefited older AML patients by prolonging the overall survival (OS) from 16.0 months to 24.5 months while reducing the adverse effects on the hemoglobin levels and neutrophil count. The same trial also indicated that low-dose cytarabine was less effective but more toxic as compared with 5-Aza-CR in MDS patients [158]. 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 for treating patients with low-risk MDS [12]. In 2009, the FDA approved a 7-day regimen that requires weekend treatment, which is inconvenient for some patients and care providers. To overcome this problem, three alternative regimens that avoid weekend treatment were designed by Lyons et al. [134]. Patients who received any one of the three regimens showed similar hematological improvement as the 7-day regimen, as well as a higher transfusion independent rate. A phase-II trial on a 5-day alternative 5-Aza-CR intravenous schedule reported partial (PR) and complete remission (CR) rates comparable with the 7-day subcutaneous regimen [135]. Further studies are required to demonstrate the survival benefits of these modified regimens. According to the International Working Group, 4 treatment cycles of treatment with 5-Aza, valproic acid and all-trans retinoic acid can achieve an overall response rate of 50%. However, most clinical trials applied longer treatment (7–10 treatment cycles). Results from these trials provided consistent results on 5-Aza-CR’s efficacy in clinical settings [137,159]. Interestingly, it was reported that the in patients with myeloid malignancies, epigenetic changes and DNA damage were not accurate indicators for clinical response in an overlapping schedule of 5-azacytidine and HDAC inhibitor entinostat [144].

Safety evaluation of 5-Aza-CR was confounded by the pathophysiology of MDS which overlaps to a great extent with the common toxicities of this drug. Serious adverse events (SAEs) occurred in about 60% of Azacytidine-treated patients and in about 36% of observation arm patients. The most common SAEs resulting in hospitalization in both arms were thrombocytopenia, febrile neutropenia, fever and pneumonia. Virtually all (99%) 5-Aza-CR-treated patients and over 96% of the observation arm patients reported adverse events. Gastrointestinal events, hematologic events, injection site events, arthralgia cough, dyspnea, headache, weakness, dizziness, and insomnia were more commonly reported by patients treated with 5-Aza-CR than by patients in the observation arm [136,137]. Most MDS patients die from bleeding or infection and from progression to AML. A recent clinical trial observed that 5-Aza-CR, when administered at high doses to patients with osteogenic sarcoma or other cancers, caused side effects such as nausea, fatigue, neutropenia, thrombocytopenia, vomiting and fevers [28]. Interestingly, the study also indicated that 5-Aza-CR could act as DNMTi with minimal impact on DNA synthesis if applied at lower dosages.

4.2. 5-Aza-CdR

5-Aza-CdR was also approved by the FDA for treatment of MDS. However, there is no clear evidence indicating that 5-Aza-CdR improves OS as observed in a recent CTCL phase-II trial. While only 30% responded, those who did not respond still benefited from relief of pruritus early in the trial [140]. Two large phase-II trials of 5-Aza-CdR in MDS were carried out in Europe. In the first of two phase-II studies, patients with intermediate and high-risk MDS received 5-Aza-CdR (135 mg/m^2 total dose per course) that resulted in response rates of 51% with the high-risk MDS and 46% with the intermediate-1 disease. In the second phase-II trial on 5-Aza-CdR, both, dose intensity and subcutaneous route of administration were tested in secondary MDS [160,161]. Overall, 32 patients (34%) achieved a CR and 69 (73%) had an objective response (OR), or hematologic improvement (HI) [161]. The authors concluded that 5-Aza-CdR produces durable clinical responses and delays the time to MDS transformation with a manageable toxicity profile.

A European phase-III study was conducted in 2007. 170 patients with MDS were randomized to receive 5-Aza-CdR dose of 15 mg/m^2 intravenously over 3 h, every 8 h, for 3 days and the regimen were repeated every 6 weeks for 6 cycles. 5-Aza-CdR resulted in a higher objective response rate (17%, CR in 9% and PR in 8%) compared to a supportive care response rate (0%). In another trial, patients treated with 5-Aza-CdR had a 17% ORR, which was significantly higher than that in the best supportive care (BSC) group (0%). However, no significant improvement (14.0 vs 14.9 months) was observed when comparing the OS between the 5-Aza-CdR and control arms, even though clinical benefits (e.g., elongation of median time to AML progression) were seen after 5-Aza-CdR treatment [162]. A similar negative outcome of OS was observed in older patients with MDS or Chronic Myelomonocytic Leukemia (CML) [139]. Grades 3 to 4 nonhematologic toxicities, possibly related to 5-Aza-CdR included hyperbilirubinemia (12%), pneumonia (10%) and constipation (1%) [162]. Based on these data, 5-Aza-CdR received approval from the FDA for therapy of MDS and chronic myelomonocytic leukemia 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. [140] showed that a 5-day intravenous schedule
with the highest dose-intensity yielded the highest CR rate (39%). In another study, the ADOPt trial reported an ORR of 32%, suggesting that this 5-day schedule was as effective as the in-patient regimen [138].

It is often difficult to determine whether the effects on gene expression and cellular function (or even on anti-tumor activity) associated with the inhibitor treatment are due to non-specific cytotoxicity, or to the demethylation of genomic DNA. Intriguingly, data from a recent phase-II study of decitabine in patients with chronic myelogenous leukemia showed that drug-induced hypomethylation of DNA in peripheral-blood cells was less pronounced in responders than in nonresponders [70]. 5-Aza-CdR may be applied to treat AML patients by several ways: 1) as a single agent in elderly patients not fit for intensive chemotherapy; 2) in combination with other agents such as HDAC inhibitors; and 3) as a maintenance therapy after the completion of consolidation therapy to reduce the potential of disease recurrence through the prevention of DNA re-hypemethylation. Decitabine alone or in combination with tyrosine kinase inhibitors should be further explored in CML patients who have developed resistance to tyrosine kinase inhibitors. Decitabine may also be beneficial for ALL patients, or patients with other hematologic disorders. Based on its clear demethylating effects in virtually all the cells examined, there is a compelling scientific rationale to explore the therapeutic application of 5-Aza-CdR for treating different types of solid tumors.

5. Combination therapy

5.1. In vitro studies

Combination therapies are expected to achieve the following goals: (1) to enhance or extend the molecular effects of the inhibitors; (2) to counter the molecular effects of inhibitors that abrogate their efficacy; or (3) to reduce the side effects through applying lower dosages of one or both drugs. Combination therapies employing DNMTi and other agents are being pursued clinically. Given the in vitro evidence for the synergy, this approach remains a topic of active study, with initial trials focusing on hematologic malignancies [163]. As discussed above, restoration of the aberrantly silenced TSG expression is coordinated by multiple epigenetic events such as promoter histone acetylation and DNA demethylation. As listed in Table 1, numerous clinical trials have been conducted to assess combined treatment with DNMTi and other reagents.

HDAC is the enzyme responsible for the removal of the acetyl group from histones as well as non-histone proteins [164]. Just like DNMT inhibitors, HDAC inhibitors such as VPA, vorinostat, MS-275, FK228, sodium phenylbutyrate, and others are small hydrophobic compounds that can easily penetrate cell membranes and reach their nuclear targets. HDAC inhibitors induce dramatic changes in chromatin structure, which is accompanied by significant alterations in gene expression patterns and cell functions such as proliferation rate and apoptosis. Since DNA methylation and histone modification represent the two most important epigenetic pathways involved in TSG silencing, combined treatment with the two drugs often generate synergistic effects. Numerous reports have demonstrated the benefit of combined use of the two drugs on both the gene reactivation and chemotherapy levels [165,166]. For example, the combination of 5-Aza–dc with HDAC inhibitors synergistically activated the silenced TSGs and an enhanced antineoplastic effects on breast and lung carcinomas [167], while DNMTi decitabine (5-Aza–dc) and the HDAC inhibitors (LBH589 or MGCD0103) synergistically reduced the proliferation in SCLC cell lines by inducing DNA damage [16]. Similarly, sequential treatment of malignant cell lines, first with 5-azacytidine nucleosides and subsequently with an HDAC inhibitor resulted in a more robust re-expression of methylated tumor suppressor genes such as MLH1, TIMP3 (TIMP3), CDKN2B (INK4B, p15) and CDKN2A in vitro than either agent alone [168]; NV-ESO-1, one of the most immunogenic cancer/testis antigens, was demonstrated to be expressed in a range of solid tumors via DNA demethylation and/or histone modification. Oi et al. [169] reported that VPA, acting as an HDACi, enhanced induction of NV-ESO-1 in a synergistic action with DNMTi by eliciting DNA demethylation, histone H3 Lys9 demethylation, and acetylation. HDAC inhibitors (FK228, TSA) and DNA hypomethylating agent (5-Aza) exhibited various growth-inhibitory effects on transitional cell carcinoma cell lines in a dose- and time-dependent manner [170]. In addition to G2/M cell cycle arrest, FK228 is more potent in inducing apoptosis than the two other single agents, and combination of both FK228 and 5-Aza further enhances this effect. The p21 induction is closely associated with FK228 or TSA but not 5-Aza, which is mediated via p53-independent pathway [170].

Functional interactions between histone acetylation and DNA methylation have been observed for more than a decade. In Neurospora it was observed that TSA was able to greatly reduce DNA methylation in several specific regions [171]. Hu et al. [172] have shown that TSA treatment induces a partial relaxation of imprinting as well as demethylation of the IGF2R gene. Cosgrove and Cox [173] found that sodium butyrate administration to several human cell lines resulted in global hypomethylation. This mechanism may contribute to the synergy observed between HDAC and DNMT inhibitors with regard to gene reactivation and anti-tumor activity [174]. Studies from this laboratory showed that TSA down-regulates DNMT3B mRNA and protein expression in human endometrial cancer cells through destabilization of DNMT3B mRNA [166]. This finding, as proved later in independent investigations by other laboratories using different cell lines [172], provided a pathway through which the remarkable synergism is achieved by DNMT and HDAC inhibitors, in different tissue/cell types.

5.2. Clinical trial

In a phase-I study, Lin and their colleagues [151] co-administered 5-Aza–CR and sodium phenylbutyrate to patients with advanced solid tumors. The three dose schedule administered in that study showed mild toxicity such as neutropenia and anemia; however, the antineoplastic effects were not significant. Braithe et al. [175] observed promising outcomes in a combination therapy of 5-Aza–CR and VPA for the therapy of breast cancer, colon cancer and other cancers at advanced stages. Recently, Soriano et al. [146] conducted a phase-I/II clinical trial using 5-Aza–CR and VPA in MDS patients. An OR of 42% indicated that this combination strategy was clinically effective. Another phase-II study with sequential subcutaneous administration of VPA and 5-Aza–CR reported a CR + PR of 30.7%. VPA concentrations were confirmed to be increased in the plasma (≥50 mg/mL vs ≤50 mg/mL) and better median survival was observed (18.7 vs 10 months) by Voso et al. [176], indicating that HDACi has the potential to increase the efficacy of DNMTi. Patients with MDS or AML who received 5-Aza–CR supplemented with HDACi vorinostat showed promising outcomes. Among them, 7 patients had hematologic improvement, 3 patients showed CR (24.2%) and 4 patients showed PR (11.2%) [145]. Reversal of promoter methylation (p15INK4B, CDH-1, DAPK-1, and SOCS-1) after therapy was observed. 5-Aza-CdR + VPA appeared to achieve a better OR (22% vs 9.5%) in patients with leukemia although, in this case, the higher level of VPA did not correlate with clinical activity [153]. A later study conducted by Blum et al. [147] confirmed these findings, demonstrating that 25 mg/kg/d of VPA with 5-Aza-CdR neither enhanced the efficacy of 5-Aza-CdR nor did it increase the re-expression of CDKN2B. A very recent study combining 5-Aza–CR with the FDA-approved MDS chemotherapy drug lenalidomide revealed an impressive 67% overall response rate, 32% CR, and 4% PR [154]. In addition, combination of 5-Aza–CdR with other agents such as Imatinib, Carboplatin, or with gemtuzumab ozogamicin were examined in various clinical settings [152,172,177].
The encouraging clinical outcomes 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 multidrug transporters.

6. Conclusion and future directions

Since DNA methylation is established and maintained by enzymatic reactions, aberrant DNA methylation patterns are potentially reversible by small DNMT inhibitors. The use of these compounds represents a promising and novel approach to cancer prevention and therapy. Following decades of development, with the approval of three epigenetic agents and additional ones in the pipeline, we have created a toolbox for manipulating the epigenome in vivo. DNMT inhibitors for chemotherapy are still in its early stage and far from reaching its full potential. Progress made in epigenetic research will lead to a better understanding of the actions of DNMTi, which will promote the translation from "bench to the bedside."

While the stability of the DNA methylation pattern is well recognized, recent studies have demonstrated the dynamic nature of epigenetic regulation. The dynamic and flexible feature of DNA methylation is best demonstrated by its reversibility through pharmacological interference. Despite their significant power in changing the DNA methylation pattern, currently available DNMT inhibitors are all nonselective, which account for most of the side effects. An ideal epigenetic therapy should be able to distinguish aberrantly methylated genes (such as TSG) from normally methylated genes. Using DNMTi as a study probe, in-depth investigation on the interactions among DNA methylation, chromatin structure and gene expression regulation will be performed in the near future. Insights on DNMT targeting and DNMTi interaction with DNMT molecules will facilitate the rational design of selective inhibitors. Also, development of more efficient screening assays will speed up the identification and validation of specific DNMT inhibitors.

TSG silencing is mediated by multiple mechanisms including the modification of histones. Consequently, the use of a single demethylation agent might not be sufficient to achieve the full reversal of epigenetic alterations in native cancer tissues. In addition, concerns about side effects are the driving force for experiments on combined drug therapy. So far most studies on combinational therapy are performed in cell culture and animal models. Although the available data appears to be optimistic, more clinical trials are required to validate its benefit. Another new development will be individualized therapy based on laboratory tests for the individual patient's sensitivity to DNMTi treatment. For example, it was reported that AML patients who best respond to demethylation therapy are those with lower methyl transfer levels in the CDKN2B promoter or higher levels of mir29 by 10-day schedule of 5-Aza-CdR [147]. Developments in this new area have the potential for substantial improvements for treating cancer patients.

References
