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Nucleosidic DNA demethylating epigenetic drugs – A comprehensive review from discovery to clinic

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ABSTRACT

DNA methylation plays a pivotal role in the etiology of cancer by mediating epigenetic silencing of cancer-related genes. Since the relationship between aberrant DNA methylation and cancer has been understood, there has been an explosion of research at developing anti-cancer therapies that work by inhibiting DNA methylation. From the discovery of first DNA hypomethylating drugs in the 1980s to recently discovered second generation pro-drugs, exceedingly large number of studies have been published that describe the DNA hypomethylation-based anti-neoplastic action of these drugs in various stages of the pre-clinical investigation and advanced stages of clinical development. This review is a comprehensive report of the literature published in past 40 years, on so far discovered nucleosidic DNA methylation inhibitors in chronological order. The review will provide a complete insight to the readers about the mechanisms of action, efficacy to demethylate and re-express various cancer-related genes, anti-tumor activity, cytotoxicity profile, stability, and bioavailability of these drugs. The review further presents the far known mechanisms of primary and secondary resistance to azanucleoside drugs. Finally, the review highlights the ubiquitous role of DNA hypomethylating epi-drugs as chemosensitizers and/or priming agents, and recapitulate the combinatorial cancer preventive effects of these drugs with other epigenetic agents, conventional chemo-drugs, or immunotherapies. This comprehensive review analyzes the beneficial characteristics and drawbacks of nucleosidic DNA methylation inhibitors, which will assist the pre-clinical and clinical researchers in the design of future experiments to improve the therapeutic efficacy of these drugs and circumvent the challenges in the path of successful epigenetic therapy.

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Abbreviations: ALL, Acute lymphoblastic leukemia; AML, Acute myeloid leukemia; AUC, Area under the curve; AZA, 5-azacytidine; 5-aza-CdR, 2′-deoxy-5-azacytidine; 5-aza-CR, 5-azacytidine; AZN, Azanucleosides; BED, Biologically effective dose; BSC, Best supportive care; CCR, Conventional care regimen; CDA, Cytidine deaminase; Cmax, Maximum observed plasma concentration; CMML, Chronic myelomonocytic leukemia; CR, Complete remission; DAC, 2′-deoxy-5-azacytidine; DCK, Deoxycytidine kinase; DFS, Disease-free survival; DLT, Dose-limiting toxicities; DNMT, DNA methyltransferase; DOR, Duration of response; EFS, Event-free survival; MDS, Myelodysplastic syndrome; MTD, Maximum tolerated dose; ORR, Overall response rate; OS, Overall survival; PD, Pharmacodynamics; PFS, Progression-free survival; PK, Pharmacokinetics; RFS, Relapse-free survival; SAE, Serious adverse events; T1/2, Terminal phase half-life; Tmax, Time to maximum plasma concentration; TSG, Tumor suppressor gene; TTP, Time to progression.

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1. Epigenetics in cancer

Epigenetics is an emerging frontier in science, especially due to the fact that unlike genetic changes such as point mutations, gene deletions, and rearrangements which occur in DNA sequence, epigenetic changes impart temporal and spatial control on gene expression without changing the underlying DNA sequence (Melki & Clark, 2002). These epigenetic changes in gene expression are mainly established during cellular differentiation and are heritable through multiple cell division cycles, thus imparting distinct identity to the cells while possessing the same genetic information (Sharma, Kelly, & Jones, 2010). The fact that epigenetic marks are reversible offers increased opportunities to ameliorate the disease phenotype. The potential utility of epigenetics in cancer research has long been established, and it is being widely accepted that cancer is as much a disease of dysregulated epigenetic alterations as it is a genetic disease (Lambert & Herceg, 2008). Indeed, recent advances in epigenetics substantiate the fact that epigenetic alterations are the key causes in origin of some cancer types (Feinberg, Ohlsson, & Henikoff, 2006). Within cancer cells there are three fundamental epigenetic mechanisms that operate along the common pathway, associated with improper chromatin activation or repression ultimately resulting in activation or inhibition of different cell signaling pathways associated with cancer. These epigenomic modifications include: methylation of cytosine bases in DNA referred to as DNA methylation, post-translational modifications of histone proteins, and mutations in chromatin remodeling complexes (Grant, 2009). Since four decades of discovery, DNA methylation is the most widely studied lesion of the malignant cell and remains to be a major hallmark in most cancer types.

2. DNA hypermethylation – A key instructor of epigenomic silencing

Aberrant DNA methylation-mediated epigenetic gene silencing has constantly engaged the researchers since years after it was first linked to cancer. Traditionally, in normal mammalian cell 70% of CpG-enriched sequences are methylated, however, tumor cells display the state of global DNA hypomethylation accompanied by specific hypermethylation of CpG dinucleotides, near promoter and proximal coding regions of genes where transcription is initiated, and are otherwise unmethylated (Melki & Clark, 2002). As a rule, DNA methylation occurs by covalent addition of a methyl group to the 5′ carbon of the cytosine ring, resulting in 5-methylcytosine. The addition of methyl group to the cytosine residues is catalyzed by DNA methyltransferases (DNMTs), Fig. 1. The mammalian DNMT family includes four active enzymes: DNMT1, DNMT3A, DNMT3B, and DNMT3L (and DNMT2 which potentially methylate RNA instead of DNA). DNMT1 is the proposed maintenance methyltransferase responsible for copying DNA methylation patterns to newly biosynthesized DNA during replication. DNMT3 consists of two related proteins, DNMT3A and DNMT3B which function as de novo methyltransferase and set up DNA methylation patterns during early development. DNMT3L is homologous to DNMT3s but does not possess catalytic activity (Subramaniam, Thombre, Dhar, & Anant, 2014). After the establishment of methylation by DNMTs, methylated DNA then interacts with various proteins including methyl-CpG binding domain proteins which drive the recruitment of chromatin-remodeling proteins responsible for transcriptional repression (Bogdanovic & Veenstra, 2009). Modifications of core histone proteins (particularly the N-terminal "tails") such as acetylation and phosphorylation further play the role in recognition of chromatin by multiprotein complexes which either facilitates chromatin relaxation and genes "switched on" or chromatin compaction and genes "switched off" (Dario, Rosa, Mariela, Roberto, & Caterina, 2008), Fig. 1. Overall, modifications of the epigenome due to DNA hypermethylation events at CpG islands have frequently demonstrated transcriptional silencing of many genes involved in cell cycle regulation, tumor cell invasion, DNA repair, and other critical growth regulators that suppress malignancy (Malik & Brown, 2000; Subramaniam et al., 2014). Consequently, the substantial role of DNA methylation in etiology of cancer creates the need for effective therapeutic options that target DNMTs, major enzymes involved in regulation of DNA methylation machinery.

3. DNA methyltransferase inhibitors – A promising anti-cancer drug class

DNA methylation-mediated epigenetic silencing of cancer-related genes has greatly emphasized on the development of anti-cancer therapies that work by inhibiting DNA methylation and restore normal epigenetic landscape by reprogramming of genes involved in disease mechanisms. DNMT inhibitors (DNMTIs) are a promising class of anticancer therapeutics which modulate the epigenome by reversing the DNA hypermethylation patterns, leading to renewed transcription of previously silenced tumor suppressor genes (TSGs). These DNMT targeting drugs are classified as nucleoside analog inhibitors which incorporate into DNA during replication and sequester DNMTs by mediating their proteasomal degradation, and non-nucleoside analog classes which directly bind to the catalytic region of DNMTs and render the enzyme inactive, without covalent enzyme trapping. At present, two prototypal nucleosidic DNMTIs, 5-azacytidine (azacytidine, 5-aza-CR, AZA) and 2′-deoxy-5-azacytidine (decitabine, 5-aza-CdR, DAC) have received regulatory approval for the treatment of hematologic malignancies (Mack, 2010), and are now gaining rapid interest as priming agents in the treatment of solid tumors (Cowan, Talwar, & Yang, 2010). Apart from these established therapies, the cohort of many DNMT targeting drugs is currently in clinical trial phases or in pre-clinical development for blood-related malignancies as well as various solid tumors.

This review extensively summarizes the available literature on the far discovered nucleosidic DNMTIs in various stages of the pre-clinical investigation and advanced stages of clinical development, with particular emphasis on their role in epigenetic cancer therapy. The review also discusses the so far known mechanisms of primary and secondary resistance to hypomethylating agents, and mutations in epigenetically regulated genes as molecular determinants of azanucleosides (AZN) response, and identifies the unmet requirements towards the success of AZN based epigenetic cancer therapy. Apart from the single agent activity, the review further highlights the effective potential of these hypomethylating agents as chemo-sensitizers and/or priming agents in hematologic as well as various solid tumors and puts together the combinatorial cancer preventive effects of these drugs with other epigenetic agents, conventional chemo-drugs or immunotherapies.

4. First generation FDA approved DNMTIs

The prototypal epigenetic drugs, azacytidine and decitabine, synthesized in 1964 and originally developed as conventional cancerostatics for use at higher doses (Sorm, Piskala, Cihak, & Vesely, 1964) were first linked with DNA methylation in 1980s when cellular differentiation induced by these AZN was associated with changes in DNA methylation (Jones & Taylor, 1980, 1981). Consequently, the anti-tumor activity of these AZN analogs were determined to be due to dual mechanisms of action (i) at high doses, azacytidine induce pronounced cytotoxicity via incorporation into RNA and DNA, and decitabine inhibit cell proliferation via incorporation into DNA, and (ii) at low doses, these drugs

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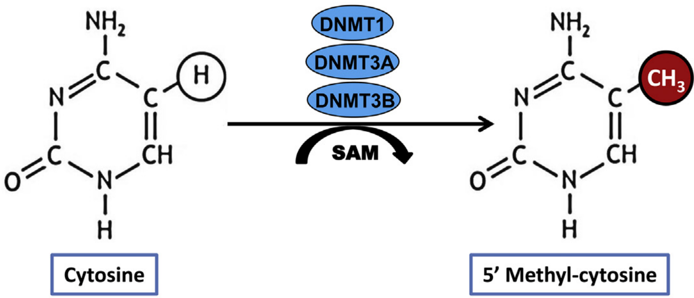

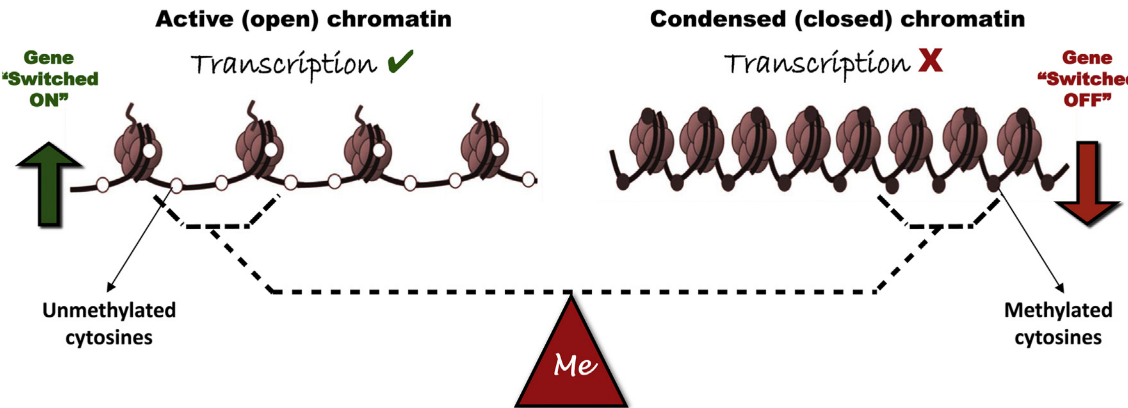

Fig. 1. Interplay between DNA methylation, gene transcription, and chromatin structure The process of DNA methylation involves the transfer of methyl group from S-adenosylmethionine (SAM) to the C-5 position of cytosine, catalyzed by DNA methyltransferases (DNMTs). DNA methylation is an "epigenetic switch" that regulates the balance between "open" and "closed" form of chromatin by changing the interactions between DNA and protein. The rate of DNA methylation is inversely proportional to transcription. The increase in the amount of methyl group accompanied by modifications of core histone proteins (such as acetylation and phosphorylation) results in alteration of the chromatin structure from open to closed conformation, in which case DNA is less accessible for transcriptional machinery, and hence transcription is impeded, ultimately resulting in gene silencing.

induce DNA hypomethylation by inhibiting DNMTs, causing reactivation of silenced genes and affecting the processes of cell differentiation and tumor suppression, Fig. 2 (Gnyszka, Jastrzebski, & Flis, 2013).

The molecular action of these AZN drugs is completed in three main steps which include cellular uptake, intracellular metabolism, and incorporation into nucleic acids (Fig. 2). Decitabine is incorporated into newly synthesized DNA, whereas, 80–90% of azacytidine is incorporated into RNA, and only 10–20% is incorporated into DNA after multistep conversion by the enzyme ribonucleotide reductase (Stresemann & Lyko, 2008). After incorporation into DNA, azacytosines substitute for cytosine forming azacytosine-guanine dinucleotides which are recognized by DNMTs as natural substrate (DNMT1 at low doses and DNMT3A/3B only at high doses). Consequently, the covalent bond formation between azacytosine-containing DNA and DNMTs results in irreversible trapping of DNMTs, eventually resulting in depletion of these enzymes and loss of methylation marks during replication, ultimately leading to reactivation of silenced TSGs (Stresemann & Lyko, 2008).

In addition, covalent DNMT-azacytosine DNA adducts also trigger DNA damage ATM/ATR response pathways resulting in growth inhibition, G2 cell cycle arrest, and apoptosis (Palii, Van Emburgh, Sankpal, Brown, & Robertson, 2008). Besides, as azacytidine is mostly incorporated into RNA, its partial efficacy is due to RNA-dependent (cell-cycle-independent) effects. Azacytidine upon incorporation into RNA inhibits methylation of tRNA at DNMT2 target sites (Schaefer, Hagemann, Hanna, & Lyko, 2009) and further disrupts rRNA processing ultimately leading to inhibition of protein synthesis and induction of apoptosis (Lee & Karon, 1976). Furthermore, a recent study has shown that azacytidine incorporation into RNA inhibits ribonucleotide reductase and interferes with the conversion of ribonucleotides to deoxyribonucleotides leading to inhibition of DNA synthesis and repair (Aimiuwu et al., 2012).

While 5′ modified cytosine analogs exert anti-cancer effects via targeting DNMT-dependent DNA methylation (at low doses), and by downstream effects of DNMT-trapping resulting in induction of nucleosidic cytotoxicity (at high doses), the usage of 5′ modified AZN at high doses (which masks DNA demethylation effects of these drugs) was largely abandoned after rejection by USA Food and Drug Administration (FDA), owing to high toxicity observed during clinical trials (Issa & Kantarjian, 2009). These hypomethylating AZN regained attention only with renewed interest in DNA methylation, to be used as epigenetic modifiers at relatively low to moderate doses, to treat older patients ineligible for intensive chemotherapy (Lubbert et al., 2011; Silverman et al., 2002). Relevantly, the in vitro studies clearly mark that at optimal low doses, sufficient to induce DNA hypomethylation and reactivate silenced gene expressions, AZN show anti-cancer effects by affecting multiple pathways regulating cell survival and death, such as induction of senescence via *p16* activation, apoptosis via pro-apoptotic genes reactivation, differentiation via responsiveness to retinoic acid, inhibition of angiogenesis via reactivating angiogenesis inhibitor *THBS1*, immune recognition via activation of cancer testis antigens, and interestingly down-regulation of oncogenes such as *BCL6, CDK6* and various other growth promoters via reactivating DNA methylation-silenced microRNAs (Issa & Kantarjian, 2009). The anti-cancer effects of these epigenetic modifiers via targeting DNMT-dependent DNA methylation is further evident from DNMT knockdown studies which showed analogous effects. Knockdown of DNMTs in A549 lung cancer cells mediated apoptosis via induction of *RASSF1A* and *p21*, as well as caspases-9 and -10 (Beaulieu et al., 2002). The selective depletion of DNMT1 using antisense or siRNA markedly augmented the ability of decitabine to reactivate silenced TSGs in HCT116 colorectal cancer cells, thereby confirming DNMT inhibition as key factor in decitabine-induced gene-reactivation (Robert et al., 2003).

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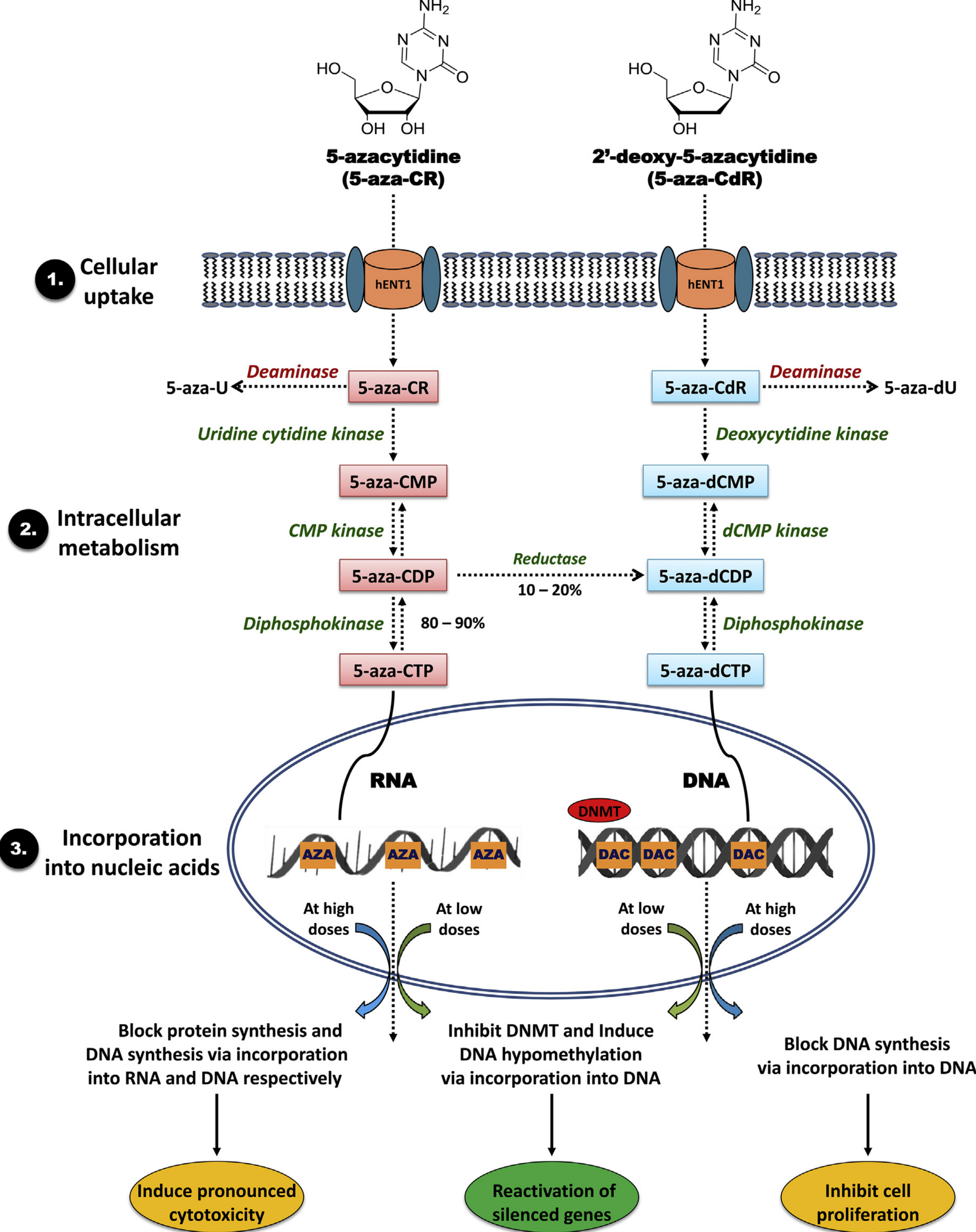

Fig. 2. Mechanism of molecular action of azanucleosides Azacytidine and its congener decitabine are transported into the cell by human eqilibrative nucleoside transporter 1 (hENT1). After their cellular uptake, these AZN are metabolically converted into their active triphosphate forms, 5-aza-2′-cytidine-triphosphate (5-aza-CTP) and 2′-deoxy-5-azacytidine-triphosphate (5-aza-dCTP) respectively, through phosphorylation by the different kinase. During replication, decitabine-derived 5-aza-dCTP is incorporated into newly synthesized DNA, whereas, 80–90% of azacytidine is incorporated into RNA as 5-aza-CTP, and only 10–20% is incorporated into DNA after multistep conversion by the enzyme ribonucleotide reductase to 5-aza-dCTP.

siRNA-mediated knockdown of DNMT1 in H1299 lung cancer cells resulted in induction of various TSGs such as *p16, RASSF1A,* and *E-cadherin* implicated in pathogenesis of lung cancer (Suzuki et al., 2004). siRNA-mediated simultaneous knockdown of DNMT1 and DNMT3B in hepatocellular carcinoma cell lines reduced cell proliferation and sensitized hepatoma cells to TRAIL-mediated apoptosis by upregulating TRAIL-R2/DR5 and hypermethylation-silenced pro-apoptotic protein, caspase-8 (Kurita et al., 2010).

However, apart from mechanism-based inhibition of DNA methylation, the AZN-induced effects may also be via DNMT-independent mechanisms. Recently, AZN have been shown to induce specific immune responses in cancer cells (Li et al., 2014; Wrangle et al., 2013)

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which highlight their significance in cancer immunotherapy. Further, AZN have been reported to impair de novo synthesis of pyrimidine through inhibition of uridine monophosphate synthase (Cihak, 1974).

4.1. 5-Azacytidine

5-Azacytidine (Azacytidine, Vidaza®, Celgene, Summit, NJ, USA) is the first hypomethylating agent to receive regulatory approval by the USA FDA in 2004 for the treatment of myelodysplastic syndrome (MDS), following the first successful clinical trial (Silverman et al., 2002) which demonstrated superiority of azacytidine over best supportive care (BSC) in MDS patients, at recommended dose of 75 mg/m^2 administered over a prolonged period of 7 days in a 4-week cycle. Presently, azacytidine has received regulatory approval for the treatment of MDS and acute myeloid leukemia (AML) with 20–30% bone marrow (BM) blasts in USA, Canada, and European Union (EU), and for the treatment of AML with >30% BM blasts in EU and several other countries. The complete list of clinical trials (296 studies until May 2017) registered with ClinicalTrials.gov for azacytidine, as single-agent therapy, and in combination with various chemotherapeutic, epigenetic or immunomodulatory agents can be found at https://clinicaltrials.gov/ct2/results?cond=&term=5azacytidine&cntry1=&state1=&SearchAll=Search+all+studies&recrs. The data presented in Tables 1 and 5 summarizes 15 years of experience and outcomes in clinical trials with azacytidine as single agent (Table 1) or in combinatorial therapies (Table 5). The data collectively indicate the effectiveness of azacytidine at increasing overall survival (OS) to similar or greater extent in comparison to currently approved AML treatment but with less toxicity, and recommends the use of azacytidine in the treatment of AML, especially for elderly patients who are unfit and ineligible for intensive chemotherapy regimens. Furthermore, the ongoing and future investigations of azacytidine in combinatorial therapies may lead to better treatment outcomes in hematologic malignancies as well as in various solid tumors.

4.2. 2′-Deoxy-5-azacytidine

2′-deoxy-5-azacytidine (Decitabine, Dacogen®, MGI Pharma, Bloomington, MN, USA) is the second hypomethylating agent, to be approved by FDA in 2006 for the treatment of higher-risk MDS, after showing its clinical effectiveness over BSC in treating elderly patients with intermediate- or high-risk MDS, ineligible for intensive chemotherapy (Lubbert et al., 2011), at low-dose schedule of 15 mg/m^2 every 8 h for 3 days in a 6-week cycle. Later, the lower-dose regimen with the higher-dose intensity of 20 mg/m^2 over 5 days in a 4-week cycle was suggested as a superior regimen (Kantarjian et al., 2007). Presently, decitabine has received regulatory approval for the treatment of MDS in the USA and for the treatment of elderly AML in EU so far. The complete list of clinical trials (225 studies until May 2017) registered with ClinicalTrials.gov for decitabine, as single-agent therapy, and in combination with various chemotherapeutic, epigenetic or immunomodulatory agents can be found at https://clinicaltrials.gov/ct2/results?cond=&term=2%27-deoxy-5-azacytidine&cntry1=&state1=&Search=Search. The data presented in Tables 2 and 6 summarizes 17 years of experience and outcomes in clinical trials with decitabine as single agent (Table 2) or in combinatorial therapies (Table 6). The data collectively indicate the effectiveness of decitabine at prolonging median time to progression (TTP) to AML or death, but no improvement in OS in comparison with BSC. The inferior outcome in terms of OS might be due to higher cytotoxicity observed. Nevertheless, the ongoing and future investigations of decitabine in combinatorial therapies may lead to better treatment outcomes in hematologic malignancies as well as in various solid tumors.

Apart from beta-*D*-anomer of 2′-deoxy-5-azacytidine, the alpha-*D*-anomer of this agent was also characterized for putative anti-leukemic effects and toxicity in mouse and human leukemic cells. The results of the studies indicated lower anti-leukemic activity as well as the toxicity of alpha-anomer (Fojtova et al., 2007; Vesely & Piskala, 1984). But the efficient ability of alpha-anomer to hypomethylate genomic DNA (Fojtova et al., 2007; Matousova et al., 2011) or induce demethylation of specific tested gene (Agrawal et al., 2017) at concentrations comparable to beta form, combined with low cytotoxicity (Agrawal et al., 2017; Fojtova et al., 2007; Matousova et al., 2011; Vesely & Piskala, 1984) indicates towards the potential use of alpha-anomer in epigenetic therapy.

5. First generation nucleosidic DNMTIs in pre-clinical or clinical development

The first nucleosidic modulators of DNMTs, azacytidine, and decitabine are undeniably the most effective hypomethylating drugs with exceptional epigenetic modulatory effects and substantial anti-proliferative activity. On the other hand, apart from these prototypal drugs, various other nucleoside analogs that work by a similar mechanism, targeting DNMTs have shown promising DNA hypomethylation activity during pre-clinical studies or have entered into clinical trials. These include cytosine analogs with modification at 5C position of the pyrimidine ring: 5-fluoro-2′-deoxycytidine, pseudoisocytidine, 5,6-dihydro-5-azacytidine, fazarabine, 2′-deoxy-5,6-dihydro-5-azacytidine, and 5-aza-4′-thio-2′-deoxycytidine, as well as other molecular variations which do not incorporate 5C modification of the pyrimidine ring: 6-thioguanine, zebularine, and 4′-thio-2′-deoxycytidine (Fig. 3, Table 3).

5.1. 6-Thioguanine

6-thioguanine also known as Thioguanine; Tioguanine; Thioguanine Tabloid ® or 2-amino-1,7-dihydro-6H-purine-6-thione (6-tG), is a synthetic guanosine analog antimetabolite with remarkable anti-neoplastic and immuno-suppressive activity, used in maintenance therapy of childhood acute lymphoblastic leukemia (ALL) and lymphoblastic non-Hodgkin's lymphoma (Munshi, Lubin, & Bertino, 2014). Chemically, 6-tG is synthesized by substitution of oxygen with sulfur at carbon 6 of guanine, Fig. 3 (Hitchings & Elion, 1954). The mechanism of action of 6-tG involves incorporation into DNA and RNA as a 6-tG nucleotide. At the nucleotide level, 6-tG competes with hypoxanthine and guanine for the enzyme hypoxanthine-guanine phosphoribosyltransferase and is converted to 6-thioguanilyic acid (TGMP). At therapeutic doses, TGMP reaches high intracellular concentrations and interferes with the synthesis of guanine nucleotide by inhibiting several enzymes involved in purine biosynthesis, and consequently resulting in blockade of DNA and RNA synthesis and cell death (Nelson, Carpenter, Rose, & Adamson, 1975). Moreover, a study using human embryonic kidney cell line suggested that cytotoxic effects induced by thiopurine drugs may also be contributed in part by inhibition of DNA methylation, as evidenced by dose-dependent decrease in global DNA methylation and DNMT activity following exposure of cells to 6-tG, which was comparable to decitabine (Hogarth et al., 2008). Eventually, another study conducted in human embryonic kidney cell line and leukemia-derived cell lines demonstrated an appreciable decrease in the level of global cytosine methylation following treatment with 6-tG (Wang & Wang, 2009; Yuan et al., 2011). The study also reported promoter demethylation and 4-fold increases in mRNA levels of epigenetically silenced genes *DCC*, *KCNK2*, *LRP1B*, *NKX6-1*, *NOPE*, *PCDHGA12*, and *RPIB9* in ALL cells following treatment with 6-tG (Yuan et al., 2011). The underlying mechanism behind the global cytosine demethylation was substantiated using ALL derived Jurkat-T cells. The study showed that epigenetic effect of 6-tG was mediated by down-regulation of histone lysine-specific demethylase 1 expression which stimulated lysine methylation of DNMT1, and triggered its degradation via the ubiquitin-proteasomal pathway (Yuan et al., 2011). Yet, another study conducted in canine malignant lymphoid cells further confirmed the demethylation activity of 6-tG, evidenced by a decrease in the level of DNMT1 protein and global DNA methylation (Flesner, Kumar, & Bryan, 2014).

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Table 1
Azacytidine in clinical trials
This table summarizes all registered clinical trials of azacytidine as single agent therapy in past 15 years for which study results have been posted or are available as publications.

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
MDS	Phase 3	2003, Completed	Randomized study of azacytidine in high-risk MDS, for determining the effectiveness of azacytidine + BSC as compared to CCR (physician choice of low-dose cytarabine + BSC, standard chemotherapy + BSC or BSC only) at (i) improving survival (ii) response (iii) effect on DOR, and (iv) TTP to AML; • Azacytidine: 75 mg/m^2/day, *s.c.*, daily, 1–7 d, every 4 w • Result [(Azacytidine vs. CCR): ORR: 51/179 (28%) vs. 21/179 (12%), DOR: 13.6 months vs. 5.2 months, TTP to AML: 20.7 months vs. 15.4 months, OS: 24.5 months vs. 15.0 months], [SAE: Azacytidine: 114/175 (65%), BSC only: 71/102 (70%), Low-dose Cytarabine: 27/44 (61%), Standard Chemotherapy: 14/19 (74%)]	NCT00071799 (Fenaux et al., 2009)
MDS	Phase 2	2005, Completed	Randomized study of azacytidine in MDS, for determining the safety and effectiveness of three alternative dosing regimens of azacytidine in combination with BSC; • Regimen A: 75 mg/m^2, *s.c.*, 1–5 d, every 4 w; Result: ORR: 4/50 (8%), SAE: 18/50 (36%) • Regimen B: 75 mg/m^2, *s.c.*, 1–5 d and 8–9 d, every 4 w; Result: ORR: 3/50 (6%), SAE: 27/50 (54%) • Regimen C: 50 mg/m^2, *s.c.*, 1–5 d and 8–12 d, every 4 w; Result: ORR: 4/51 (8%), SAE: 22/48 (46%)	NCT00102687
Myelofibrosis	Phase 2	2005, Completed	Non-randomized study of the safety and effectiveness of azacytidine in myelofibrosis; • Azacytidine: 75 mg/m^2, *s.c.*, daily, 1–7 d, every 4 w • Result: ORR: 8/34 (24%), SAE: 17/34 (50%)	NCT00569660
AML	Phase 2	2006, Completed	Study of azacytidine as maintenance therapy for determining the effectiveness of azacytidine, at increasing survival and decreasing the rate of leukemia relapse in older patients >60 years with AML in CR after induction chemotherapy; • Azacytidine: 50 mg/m^2, *s.c.*, daily, 1–5 d, every 4 w • Result: DFS-1 year: 50%, OS: 20.4 months, SAE: 2/24 (8%)	NCT00387647
MDS	Phase 2	2006, Completed	Study of azacytidine for determining ORR in MDS; • Azacytidine: 75 mg/m^2, *i.v.*, daily, 1–5 d, every 4 w • Result: ORR: 6/22 (27%), DOR: 15.0 months, PFS: 11.3 months, OS: 14.8 months, SAE: 12/24 (50%)	NCT00384956
Myelofibrosis	Phase 2	2006, Terminated	Study of azacytidine in patients with myelofibrosis with myeloid metaplasia, for determining (i) safety and effectiveness of azacytidine (ii) pertinent biologic characteristics of myelofibrosis before and during azacytidine therapy (iii) effects of treatment on constitutional symptoms in these patients, and (iv) time to event distributions for OS and progression; • Azacytidine: 75 mg/m^2, *s.c.*, daily, 1–5 d, every 4 w • Result: ORR: 0/10 (0%), OS: 16.9 months, SAE: 4/10 (40%)	NCT00381693
Prolymphocytic leukemia	Phase 2	2006, Terminated	Study of the safety and effectiveness of azacytidine in fludarabine-resistant chronic lymphocytic leukemia, Richter's transformation, and T-cell-prolymphocytic leukemia; • Azacytidine: 75 mg/m^2, *s.c.*, daily, 1–7 d, every 3–8 w • Result: ORR: 0/9 (0%), SAE: 0/9 (0%)	NCT00413478 (Malik et al., 2013)
MDS	Phase 3	2007, Completed	Randomized study (an extension to study NCT00071799) allowing for continuation of azacytidine treatment in MDS for ethical and safety reasons until the commercial availability of the drug; • Azacytidine: 75 mg/m^2/day, *s.c.*, daily, 1–7 d, every 4 w • Result: ORR: 91/179 (51%), SAE: 20/40 (50%)	NCT01186939 (Silverman et al., 2011)
MDS, CMML, AML	Phase 1	2007, Completed	Non-randomized dose-escalation study of oral azacytidine in patients with MDS, CMML, and AML, for determining (i) long term safety and effectiveness (ii) PK and PD, and (iii) MTD and BED based on safety, PK, and PD data; • Azacytidine: 75 mg/m^2/day, *s.c.*, daily, 1–7 d, 4 w (cycle 1) followed by 120–600 mg/day, *p.o.*, daily, 1–7 d of each additional 4 w cycle • Result: ORR: 6/17 (35%) in previously treated and 11/15 (73%) in	NCT00528983 (Garcia-Manero et al., 2011)

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Table 1 (*continued*)

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
			untreated MDS and CMML patients and no response in AML patients, PK [Tmax: 0.5 h (*s.c.*) vs. 1.0 h (*p.o.*), mean elimination half-life: 1.6 h (*s.c.*) vs. 0.62 h (*p.o.*), mean relative oral bioavailability: 6.3% to 20%], PD: Azacytidine (*s.c.*, *p.o.*) decreased DNA methylation in blood with maximum effect at day 15 of each cycle, MTD: 480 mg, SAE: ≥ 20% of patients	
MDS	–	2008, Completed	Pilot study of pre-transplant azacytidine in patients with high-risk MDS who are candidates for allogeneic hematopoietic cell transplantation, for determining the effectiveness of azacytidine in preventing MDS relapse; • Azacytidine: 75 mg/m^2/day, *s.c.*, daily, 5–7 d, every 4 w • Result: ORR: 10/21 (48%), DFS-1 year: 52%, OS-1 year: 62%, SAE: 8/25 (32%)	NCT00660400 (Nishihori et al., 2014)
MDS	Phase 2	2008, Completed	Study of the feasibility and effectiveness of azacytidine as pre-transplant cytoreduction prior to allogeneic hematopoietic cell transplantation in patients with high-risk MDS; • Azacytidine: 75 mg/m^2, *s.c.* or *i.v.*, daily, 1–7 d • Result: EFS-1 year: 47%, EFS-2 year: 37%, OS-1 year: 47%, OS-2 year: 37%, SAE: 13/16 (81%)	NCT00721214
AML	Phase 2	2008, Completed	Study of the safety and effectiveness of azacytidine in elderly patients with newly diagnosed previously untreated or secondary AML who are unsuitable for intensive chemotherapy; • Azacytidine**:** 100 mg/m^2/day, *s.c.*, daily, 1–5 d, every 4 w • • Result**:** CR: 8/45 (18%), DOR: 8.0 months, OS: 6.0 months, SAE: 8/45 (18%)	NCT00739388 (Passweg et al., 2014)
MDS, CMML, AML, Lymphoma, Multiple myeloma	Phase 1	2008, Completed	Randomized study of azacytidine in patients with MDS, CMML, AML, lymphoma, and multiple myeloma, for determining PK and safety of different *p.o.* formulations versus *s.c.* formulations; • Study 1: 75 mg/m^2, *s.c.*, d 1, d 15; 180 mg, *p.o.* (IRT-A, IRT—B, ECT, or 200 mg CAP), d 3; 360 mg, *p.o.* (IRT-A, IRT—B, ECT, or 400 mg CAP), d 5; individualized doses in formulation IRT-A, IRT—B, ECT or CAP, calculated to deliver 80% on d 17 and 120% on d 19 of the mean *s.c.* azacytidine exposure (AUC d 1–15), not to exceed 1200 mg • Study 2 (Part 1**):** 3 way crossover: 3 × 100 mg IRT-B tablets (under fasted conditions), d 1; 2 × 150 mg IRT-C tablets (under fasted conditions), d 2; 2 × 150 mg IRT-C tablets (under fed conditions), d 3 • Study 2 (Part 2): 2 × 150 mg IRT-C tablets (under fasted conditions), d 1; 40 mg omeprazole, d 2–4; 2 × 150 mg IRT-C tablets after 1 h of 40 mg omeprazole, d 5 • Result**:** Oral azacytidine is rapidly absorbed with little or no effect of food on PK parameters, and does not require dose adjustments when taking a proton-pump inhibitor such as omeprazole	NCT00761722 NCT01519011 (Laille et al., 2014)
MDS, AML, Solid tumors, Multiple myeloma, Non-hodgkin's lymphoma, Hodgkin's disease	Phase 1	2008, Completed	Randomized study of azacytidine in adult cancer patients with and without impaired renal function, for determining (i) if azacytidine is absorbed in the body at the same rate or proportion for different concentrations (ii) the effect of renal impairment on azacytidine PK, and (iii) safety and tolerability of azacytidine in patients with renal function impairment; • Regimen A**:** 25 mg/m^2, *s.c.*, d 1–75 mg/m^2, *s.c.*, daily, 1–7 d, every 4 w • Regimen B**:** 50 mg/m^2, *s.c.*, d 1–75 mg/m^2, *s.c.*, daily, 1–7 d, every 4 w • Regimen C: 75 mg/m^2, *s.c.*, d 1–5 - 75 mg/m^2, *s.c.*, daily, 1–7 d, every 4 w • Regimen D: 100 mg/m^2, *s.c.*, d 1–75 mg/m^2, *s.c.*, daily, 1–7 d, every 4 w • Regimen E**:** 75 mg/m^2, *s.c.*, d 1–5 - 75 mg/m^2, *s.c.*, daily, 1–7 d, every 4 w; severe renal impairment • Result [Regimen A, B, C, D: Cmax (ng/mL): 34%, 61%, 58%, 39%, Tmax: 0.25 h, 0.25 h, 0.25 h, 0.27 h, T1/2: 1.38 h, 0.63 h, 1.19 h, 1.03 h], [Normal renal function vs. severe renal impairment: Cmax (ng/mL): 58% vs. 93% on d 1 and 46% vs. 92% on d 5, Tmax: 0.25 h vs. 0.50 h on d 1 and 0.38 h vs. 0.64 h on d 5, T1/2: 1.19 h vs. 0.97 h on d 1 and 1.03 h vs. 1.15 h on d 5], [SAE: Regimen A, B, C, D, E: 0/5 (0%), 0/5 (0%), 4/6 (67%), 1/5 (20%), 1/6 (17%)]	NCT00652626
AML	Phase 3	2010, Completed	Randomized study of the effectiveness of azacytidine versus CCR (physician choice of low-dose cytarabine + BSC, intensive chemotherapy + BSC or BSC only), for determining OS in older patients with newly diagnosed AML;	NCT01074047 (Dombret et al., 2015)

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Table 1 (*continued*)

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
			• Azacytidine**:** 75 mg/m^2, *s.c.*, daily, 1–7 d, every 4 w • Result [(Azacytidine vs. CCR): ORR: 67/241 (28%) vs. 62/247 (25%), DOR: 10.4 months vs. 12.3 months, EFS: 6.7 months vs. 4.8 months, RFS: 9.3 months vs. 10.5 months, OS: 10.4 months vs. 6.5 months, OS-1 year: 47% vs. 34%], [SAE: Azacytidine: 188/236 (80%), BSC only: 30/40 (75%), Low-dose Cytarabine: 118/153 (77%), Standard Chemotherapy: 27/42 (64%)]	
MDS, AML	–	2010, Recruiting	Study of azacytidine in patients with high-risk MDS and AML with multilineage dysplasia, for characterizing (i) molecular mechanism of action and resistance to azacytidine: role of apoptosis versus autophagy, and (ii) reversion of azacytidine resistance using different drugs targeting apoptosis and/or autophagy; • Result: *BCL2L10* was discovered as a predictive factor for resistance to azacytidine in MDS and AML patients	NCT01210274 (Cluzeau et al., 2012)
MDS	Phase 4	2010, Completed	Study of the safety, effectiveness, and PK of azacytidine in adult Taiwanese patients with high-risk MDS; • Azacytidine: 75 mg/m^2/day, *s.c.*, daily, 1–7 d, every 4 w • Result: ORR: 0/44 (0%), Cmax (ng/mL): 44%, Tmax: 0.29 h, T1/2: 1.0 h, SAE: 28/44 (64%)	NCT01201811
Non-small cell lung cancer	Phase 2	2011, Active	Pilot study of azacytidine in patients with previously treated advanced non-small cell lung cancer, for determining (i) the ability of azacytidine to cause DNA hypomethylation and re-expression of silenced TSGs when stratified for high or low expression of mir29a, b, c (ii) ORR, PFS, and OS, and (iii) correlation of miRNA profiles with response to azacytidine; • Azacytidine: 75 mg/m^2, *s.c.*, daily, 1–7 d, every 4 w • Result: SAE: 1/1 (100%)	NCT01281124
CMML	Phase 2	2011, Completed	Study of the safety and effectiveness of azacytidine in CMML, for determining (i) ORR, PFS, and OS (ii) to develop biomarkers for response and gain insights into mechanisms determining response, and (iii) the gene expression and promoter methylation profiling pre- and post-azacytidine therapy; Azacytidine: *s.c.* or *i.v.* 10–40 min, daily, 1–7 d Result: CR: 3/11 (27%), SAE: 2/11 (18%)	NCT01350947
MDS	Phase 2	2012, Active	Study of the safety, effectiveness, and PK of azacytidine in adult Chinese patients with high-risk MDS; • Azacytidine: 75 mg/m^2/day, *s.c.*, daily, 1–7 d, every 4 w • Result: ORR: 9/72 (13%), OS: 22.0 months, Cmax (ng/mL): 31%, Tmax: 0.25 h, T1/2: 0.8 h, SAE: 38/72 (53%)	NCT01599325

Altogether, inhibition of DNA methylation by thiopurine drugs may contribute in part to their cytotoxic activity.

5.2. 5-Fluoro-2′-deoxycytidine

5-Fluoro-2′-deoxycytidine (FdCyd) is a chemically stable fluoropyrimidine analog, currently undergoing phase 1/2 clinical trial in combination with the cytidine deaminase (CDA) inhibitor, tetrahydrouridine (THU). Structurally, FdCyd bears a chemical modification at position 5 of the pyrimidine ring, where fluorine replaces hydrogen, Fig. 3 (Wempen, Duschinsky, Kaplan, & Fox, 1961). The mechanism of action of FdCyd involves deamination by CDA to 5-fluoro-2′-deoxyuridine (FdUrd), phosphorylation by thymidine kinase to 5-fluoro-2′-deoxyuridine monophosphate, and subsequent inhibition of deoxythymidine monophosphate (dTMP) synthetase. The inhibition of dTMP synthetase results in the decreased production of dTMP which in turn leads to depletion of thymidine triphosphate, and inhibition of DNA synthesis and cell division (Newman & Santi, 1982). The tumor inhibitory activity of FdCyd was first reported by an in vitro study which evidenced complete growth inhibition of human cervical cancer cells in culture (Eidinoff, Rich, & Perez, 1959). In addition to its function as a prodrug for FdUrd, the specific mechanism of action of FdCyd involves inhibition of DNMT after incorporation into DNA as FdCyd triphosphate. The hypomethylation potential of FdCyd was confirmed by its ability to inhibit DNA methylation and induce muscle formation in cultured mouse embryo cells (Jones & Taylor, 1980). However, DNMT inhibitory properties of FdCyd are limited due to CDA mediated rapid conversion of FdCyd in vivo to pharmacologically active, yet unwanted metabolites, FdUrd, 5-fluorouracil (FU), and 5-fluorouridine (FUrd) which do not inhibit DNMT. In this context, a pre-clinical study characterizing the pharmacokinetics (PK) and metabolism of FdCyd in mice demonstrated that co-administration of FdCyd + THU significantly reduced the first pass effect of CDA on FdCyd, evidenced by increased exposure to FdCyd and decreased exposure to its metabolites (Beumer et al., 2006). Similar PK study conducted in cynomolgus monkeys and humans proved that co-administration of FdCyd + THU resulted in increased exposure to FdCyd and improved oral bioavailability (Holleran et al., 2015). Consequently, it was proposed that the degradation of FdCyd to inactive metabolites can be inhibited by combining with CDA inhibitor, THU, without inhibiting its activation by deoxycytidine kinase (dCK). Sequentially conducted clinical studies in patients, simultaneously treated with FdCyd and THU further showed that following co-administration of FdCyd + THU, the plasma concentrations of FdCyd required for in vitro inhibition of DNA

Table 2
Decitabine in clinical trials
This table summarizes all registered clinical trials of decitabine as single agent therapy in past 17 years for which study results have been posted or are available as publications.

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
MDS	Phase 3	2001, Completed	Randomized study for comparing the safety and efficacy profiles of decitabine versus supportive care in adults with advanced-stage MDS; • Decitabine: 15 mg/m^2, *i.v.*, 3 h, every 8 h × 3 d, every 6 w • Result: ORR: 44/157 (28%), DOR: 9.9 months, OS: 16.6 months, SAE: >4%	NCT00043381 (Jabbour et al., 2013)
MDS, CMML	Phase 2	2003, Completed	Randomized study of the safety and effectiveness of three different schedules of low-dose decitabine in MDS; • Schedule A: 10 mg/m^2, *i.v.*, 1 h daily, 1–10 d, every 4–8 w; Result: ORR: 10/17 (59%), SAE: 5/17 (29%) • Schedule B: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4–8 w; Result: ORR: 68/93 (73%), SAE: 32/93 (34%) • Schedule C: 20 mg/m^2, *s.c.*, twice daily, 1–5 d, every 4–8 w; Result: ORR: 8/14 (57%), SAE: 7/14 (50%)	NCT00067808 (Oki et al., 2008)
Thyroid cancer	Phase 2	2004, Completed	Study of decitabine in patients with metastatic papillary or follicular thyroid cancer unresponsive to iodine I 131 (131I), for determining (i) if decitabine can restore 131I uptake (ii) the efficacy of 131I therapy administered after restoration of 131I uptake (iii) the effect of decitabine on clinical and molecular markers of thyroid cancer cell differentiation, and (iv) the safety and tolerability of decitabine in patients undergoing thyroid hormone withdrawal-induced hypothyroidism and 131I therapy; • Decitabine: 6 mg/m^2, *i.v.*, 1 h, 1–5 d and 8–12 d with possible second course • 131I: thyrotropin-alfa stimulated radioactive iodine scan on w 3 • Result: Restoration of 131I uptake in metastatic lesions: 0/12 (0%), SAE: 9/12 (75%)	NCT00085293
Myelofibrosis	Phase 2	2004, Active	Study of the safety and ORR of decitabine in primary and secondary myelofibrosis, and determination of (i) the epigenetic effects including methylation status and re-expression of specific target genes, and (ii) the potential utility of CD34+ as surrogate biomarker for biological activity of decitabine in myeloid metaplasia with myelofibrosis; • Decitabine: 0.3 mg/kg/day, *s.c.*, 1–5 d and 8–12 d, every 6 w • Result: ORR: 7/19 (37%), SAE: 15/21 (71%)	NCT00095784
MDS, CMML	Phase 2	2005, Terminated	Non-randomized study of the ORR of low-dose decitabine in MDS following the failure of the standard azacytidine therapy; • Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4–8 w • Result: CR: 3/16 (19%), SAE: 6/16 (38%)	NCT00113321
AML	Phase 2	2005, Completed	Non-randomized study of decitabine for determining the rate of CR and OS in older patients with AML; • Decitabine: 20 mg/m^2, *i.v.*, daily, 1–5 d, every 4 w • Result: CR: 13/55 (24%), SAE: 40/55 (73%)	NCT00358644
AML, MDS	Phase 1	2005, Completed	Non-randomized PK study of decitabine in AML or MDS; • Decitabine: 15 mg/m^2, *i.v.*, 3 h, every 8 h × 3 d • Result: Cmax (ng/mL): 73.8 (d 1), 64.8 (d 2), 77.0 (d 3), Tmax: 2.49 h (d 1), 2.53 h (d 2), 2.29 (d 3), SAE: 9/16 (56%)	NCT01378416
MDS	Phase 2	2005, Completed	Non-randomized study of the ORR of decitabine in adults with advanced-stage MDS; • Decitabine: 20 mg/m^2, *i.v.*, daily, 1–5 d, every 4 w • Result: ORR: 33/99 (33%), SAE: 65/99 (66%)	NCT00260065 (Jabbour et al., 2013)
AML, MDS	Phase 2	2005, Completed	Randomized study of decitabine in AML or MDS (i) to generate additional information about the overall safety profile (ii) safety information of hepatically or renally impaired patients, and patients taking concomitant medications and/or therapies without trial restrictions; • Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4 w • Result: Patients with adverse events: 10/10 (100%), SAE: 6/10 (60%)	NCT00760084
AML	Phase 3	2005, Completed	Randomized study of decitabine versus supportive care or low-dose cytarabine for comparing the treatment results in older patients with newly diagnosed de novo or secondary AML; • Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4 w • Result (decitabine vs. cytarabine or supportive care): OS: 7.7 months vs. 5 months, SAE: 190/238 (80%) vs. 162/237 (68%)	NCT00260832 (Mayer et al., 2014)
AML	Phase 2/3	2006, Completed	Randomized study of decitabine as maintenance therapy for adults with unfavorable risk AML in first CR or with relapsed AML in second CR; • Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4–8 w • Result: DFS-1 year: 50%, SAE: 1/20 (5%)	NCT00398983
AML	Phase 2	2006, Active	Study of decitabine as maintenance therapy after standard therapy (chemotherapy: busulfan, cytarabine, daunorubicin hydrochloride, etoposide; bone marrow transplantation; allogeneic hematopoietic cell transplantation) in treating younger patients <60 years with previously untreated AML, for determining (i) efficacy, feasibility, and toxicities	NCT00416598 (Blum et al., 2017)

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Table 2 (*continued*)

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
			(ii) 1-year DFS rate (iii) biologic response to decitabine (iv) DNA demethylation, down-regulation of DNMT1, and gene re-expression; • Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 6 w • Result: DFS-1 year: 80%, SAE: 0/132 (0%)	
AML	Phase 2	2007, Completed	Study determining (i) the rate of CR (ii) rate of OS at 1-year (iii) ORR, and (iv) pharmacological and biological correlative studies of decitabine with clinical endpoints and/or response in patients with previously untreated AML; • Decitabine: 20 mg/m^2/day, *i.v.*, 1 h daily, 1–10 d, every 4 w • Result: CR: 25/55 (45%), SAE: 0/53 (0%)	NCT00492401 (Blum et al., 2010)
AML	Phase 1	2007, Completed	Non-randomized study of the feasibility, safety, and biologic activity of epigenetic priming with decitabine prior to standard cytarabine, daunorubicin (7 + 3) induction chemotherapy in younger patients with less-than-favorable risk AML, for determining (i) the appropriate dose level (ii) safety and expected toxicities (iii) optimal dose schedule of decitabine, and (iv) molecular and cellular consequences of decitabine-induced hypomethylation; • Decitabine: 20 mg/m^2/day, *i.v.*, 1 h (Arm A) or 24 h (Arm B), 1–3/5/7 d • Result: ORR: 25/30 (83%), toxicity similar to standard induction chemotherapy	NCT00538876 (Scandura et al., 2011)
Myelofibrosis	Phase 2	2008, Terminated	Study of the safety and effectiveness of low-dose decitabine in patients with symptomatic primary myelofibrosis (PMF) or post essential thrombocythemic (ET) or polycythemic vera (PV) MF, and analysis of the ability of decitabine at decreasing pathologic angiogenesis and other stromal reactive features intrinsic to PMF or post ET/PV MF; • Decitabine: 20 mg/m^2/day, *i.v.*, 1 h daily, 1–5 d, every 4 w • Result: ORR: 1/4 (25%), SAE: 1/4 (25%)	NCT00630994
MDS	Phase 2	2008, Completed	Randomized study of the safety and effectiveness of two different schedules of low-dose decitabine in adults with low or intermediate-1 risk MDS; • Schedule A: 20 mg/m^2/day, *s.c.*, daily, 1–3 d, every 4 w; Result: ORR: 10/43 (23%), SAE: 18/43 (42%) • Schedule B: 20 mg/m^2/day, *s.c.*, d 1, d 8, d 15, every 4 w; Result: ORR: 5/22 (23%), SAE: 10/22 (45%)	NCT00619099 (Garcia-Manero et al., 2013)
MDS	Phase 1	2008, Completed	Non-randomized study of decitabine for determining the recommended dose level, safety and effectiveness in MDS; • Dose A: 15 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4 w; Result: ORR: 2/3 (67%), Cmax (ng/mL): 151.7 (d 1), 142 (d 5), SAE: 1/3 (33%) • Dose B: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4 w; Result: ORR: 3/6 (50%), Cmax (ng/mL): 166.4 (d 1), 190.6 (d 5), SAE: 1/6 (17%)	NCT00796003 (Oki et al., 2012)
MDS	Phase 2	2008, Completed	Study of the safety and ORR of decitabine in previously treated and untreated Taiwanese patients with MDS; • Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4 w • Result: ORR: 8/34 (24%), OS: 22.8 months, SAE: 28/37 (76%)	NCT00744757
CMML	Phase 2	2008, Completed	Study of the therapeutic efficacy of decitabine in patients with previously treated or untreated CMML; • Decitabine: 20 mg/m^2/day, *i.v.*, 1 h daily, 1–5 d, every 4–7 w • Result: ORR: 15/39 (38%), OS-2 year: 48%	NCT01098084 (Braun et al., 2011)
MDS	Phase 4	2008, Completed	Study of the safety and effectiveness of decitabine in MDS; • Decitabine: 20 mg/m^2, *i.v.*, daily, 1–5 d, every 4 w • Result: ORR: 56/101 (55%), DOR: 13.2 months, OS: 17.7 months	NCT01041846 (Lee et al., 2011)
MDS	Phase 4	2009, Terminated	Randomized study for demonstrating the effectiveness and safety of decitabine over azacytidine in patients with intermediate or high-risk MDS; • Decitabine: 20 mg/m^2/day, *i.v.*, daily, 1–5 d, every 4 w • Azacytidine: 75 mg/m^2/day, *s.c.*, daily, 1–7 d, every 4 w • Result (Decitabine vs. Azacytidine): ORR: 1/11 (9%) vs. 1/12 (8%), SAE: 7/13 (54%) vs. 7/13 (54%)	NCT01011283
MDS	Phase 3	2009, Completed	Randomized study of the safety and effectiveness of two different schedules of low-dose decitabine in MDS; • Schedule A: 15 mg/m^2, *i.v.*, 3 h, every 8 h × 3 d, every 6 w • Result: ORR: 10/34 (29%), OS-6 and 12 months: 91% and 76%, SAE: 8/34 (24%) • Schedule B: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4 w • Result: ORR: 25/98 (26%), OS-6 and 12 months: 85% and 66%, SAE: 26/98 (27%)	NCT01751867 (Wu et al., 2015)
MDS	Phase 1/2	2010, Completed	Study of decitabine as differentiation therapy in MDS, for demonstrating (i) the effectiveness of DNMT1 depleting but non-DNA damaging doses of decitabine	NCT01165996 (Saunthararajah et al., 2015)

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Table 2 (*continued*)

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
			(ii) the safety of the regimen (iii) response by aberrant methylation signature (iv) correlation of DNMT1 depletion, cytogenetic and methylome profile, and CDA genotype and expression with clinical response criteria; • Induction phase: 0.2 mg/kg/day, *s.c.*, twice weekly for 4 w or thrice weekly until achieving bone marrow blasts <5% • Maintenance phase: 0.2 mg/kg/day, *s.c.*, twice weekly for up to 52 w in the absence of disease progression or unacceptable toxicity • Result: CR: 4/25 (16%), SAE: 12/25 (48%)	
MDS, AML	Phase 1	2011, Active	Dose-escalation study of decitabine as maintenance therapy in patients with higher-risk MDS and MDS/AML receiving allogeneic stem cell transplantation; • Decitabine: 5–15 mg/kg/day, *i.v.*, 1 h daily, 1–5 d, every 4 w • Result: Median maintenance dose: 7 mg/m^2/day	NCT01277484 (Han et al., 2015)
MDS	–	2012, Completed	Study determining the prognostic impact of mutations in spliceosome machinery genes (*SRSF2*, *U2AF1*, and *ZRSR2*) on the outcomes of 1st line decitabine treatment in MDS; • Result (Spliceosome wild-type group vs. mutated group): ORR: 43% vs. 47%, OS: 22.0 months vs.15.9 months	NCT02060409 (Hong et al., 2015)
AML, MDS	Phase 2	2013, Recruiting	Study determining the potential genetic markers of decitabine response in patients with AML or MDS; • Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–10 d, every 4 w • Result: ORR (unfavorable-risk vs. favorable-risk cytogenetic profile): 29/43 (67%) vs. 24/71 (34%), ORR (TP53 mutations vs. wild-type TP53): 21/21 (100%) vs. 32/78 (41%)	NCT01687400 (Welch et al., 2016)

methylation was achieved, and accompanying plasma concentration of unwanted metabolites, FdUrd and FU was diminished. This resulted in less cytotoxic side effects and increased hypomethylation efficacy (Beumer et al., 2008). Apparently, studies were conducted in various cancer cell lines, to investigate the ability of FdCyd (i) to induce demethylation and cause re-expression of hypermethylation-silenced genes (ii) the association between hypomethylation activity and cellular biological activities, and (iii) the underlining molecular mechanism behind cytotoxicity. The study conducted in *MAGE-1* negative melanoma cell line demonstrated that FdCyd treatment resulted in decreased methylation of CpG sites in the *MAGE-1* promoter region, induced the expression of *MAGE-1* mRNA, and increased *MAGE-1* protein in a dose- and time-dependent manner (Hou & Newman, 2005). The demethylation effect of FdCyd was also proven by another study conducted in breast cancer cells, where, FdCyd treatment resulted in decreased methylation and increased mRNA expression of various originally silenced TSGs, specifically *TWIST1*, in a dose- and time-dependent manner (Li, Villacorte, & Newman, 2006). However, no correlation was found between cytotoxic activity and hypomethylation activity of FdCyd, studied in several human cancer cell lines, although incorporation of FdCyd into DNA was evidenced (Liu et al., 2009). Instead, a study conducted in FdCyd sensitive colon cancer cells showed that inhibition of cell proliferation by FdCyd which arrested cells in G2/M phase was mediated by activation of DNA damage response pathway (Zhao, Fan, Hong, Li, & Wu, 2012). Recently, an extensive study was conducted in vitro and in vivo to investigate the combination of FdCyd + THU as a demethylation regimen in tumor cells. The results of the study showed that continuous exposure to the combination of FdCyd + THU modified tumor cell growth by inhibiting DNMT1, and decreased long interspersed nuclear elements 1 (LINE1) promoter methylation in bladder cancer cells. DNMT1 and LINE1 methylation changes in tumor cells isolated from patients with FdCyd + THU treatment protocol, enrolled in a Phase 1 clinical trial further confirmed the mechanism of this combination regimen (Kinders et al., 2011). Besides, the study also showed the upregulation of *p16* expression in bladder cancer, following treatment with FdCyd + THU. Importantly, an immunofluorescence assay for *p16* expression in circulating tumor cells (CTCs) was developed and implemented in phase 1 trial. Determination of DNMT1 and LINE1 methylation in tumor biopsies, and *p16* expression in CTCs will also be included in phase 2 trial of this regimen (Kinders et al., 2011). The first-in-human phase 1 trial of FdCyd was conducted in patients with advanced solid tumors, to establish the best dose of FdCyd which can be combined with THU, and to determine the side effects of the combination. The maximum tolerated dose (MTD) of the combination was established at 134 mg/m^2 FdCyd +350 mg/m^2 THU, 1–5 and 8–12 days, every 4 weeks, with the recommended phase 2 dose of 100 mg/m^2/day FdCyd +350 mg/m^2/day THU (Newman et al., 2015). Recently, in an attempt to develop preclinical drug development pipeline to reduce the attrition of drugs in clinical trials, the combination of FdCyd + THU was tested in pediatric brain tumor models. The results of the study revealed that despite potent in vitro activity and in vivo PK properties, FdCyd showed no significant in vivo therapeutic response, and therefore was deprioritized for the treatment of pediatric brain tumors in clinic (Morfouace et al., 2016). The lack of in vivo therapeutic response following systemic administration of FdCyd may be due to chemoresistance of glioma tumors stem-like cells, arising from tumor cell-intrinsic changes such as drug efflux by ABCG2 transporter, contributing to blood-brain barrier (Bleau et al., 2009; Morfouace et al., 2015, 2016) or tumor cell-extrinsic factors such as the impact of cytokines and growth factors in the tumor-microenvironment (Gilbert & Hemann, 2010; Hao et al., 2012; Morfouace et al., 2016).

5.3. Pseudoisocytidine

Pseudoisocytidine or 2-amino-5-β-*D*-ribofuranosylpyrimidin-4 (1H)-one (ψ ICyd), an isostere of cytidine and 5-aza-CR is a synthetic pyrimidine C-nucleoside with hydrolytically stable ring structure (Fig. 3). The exceptional stability of ψ ICyd (stable at pH 7.4 for 6 days at 22 °C and for at least 3 days at 37 °C) may be due to the substitution of C—N glycosyl linkage with the C—C bond between C-1 of the β-*D*-ribofuranose moiety and C-5 of the aglycon (Chu, Watanabe, Kyoichi, & Fox, 1975). The mechanism of action is similar to 5-aza-CR, however, ψ ICyd has been reported to be comparatively less cytotoxic (Burchenal et al., 1976). Also, ψ ICyd is resistant to enzymatic deamination by cytidine deaminase (CDA), in comparison with 5-aza-CR and 1-beta-*D*-arabinofuranosylcytosine (Woodcock et al., 1980) and was recently discovered as an inhibitor of CDA (Costanzi et al., 2011). The low

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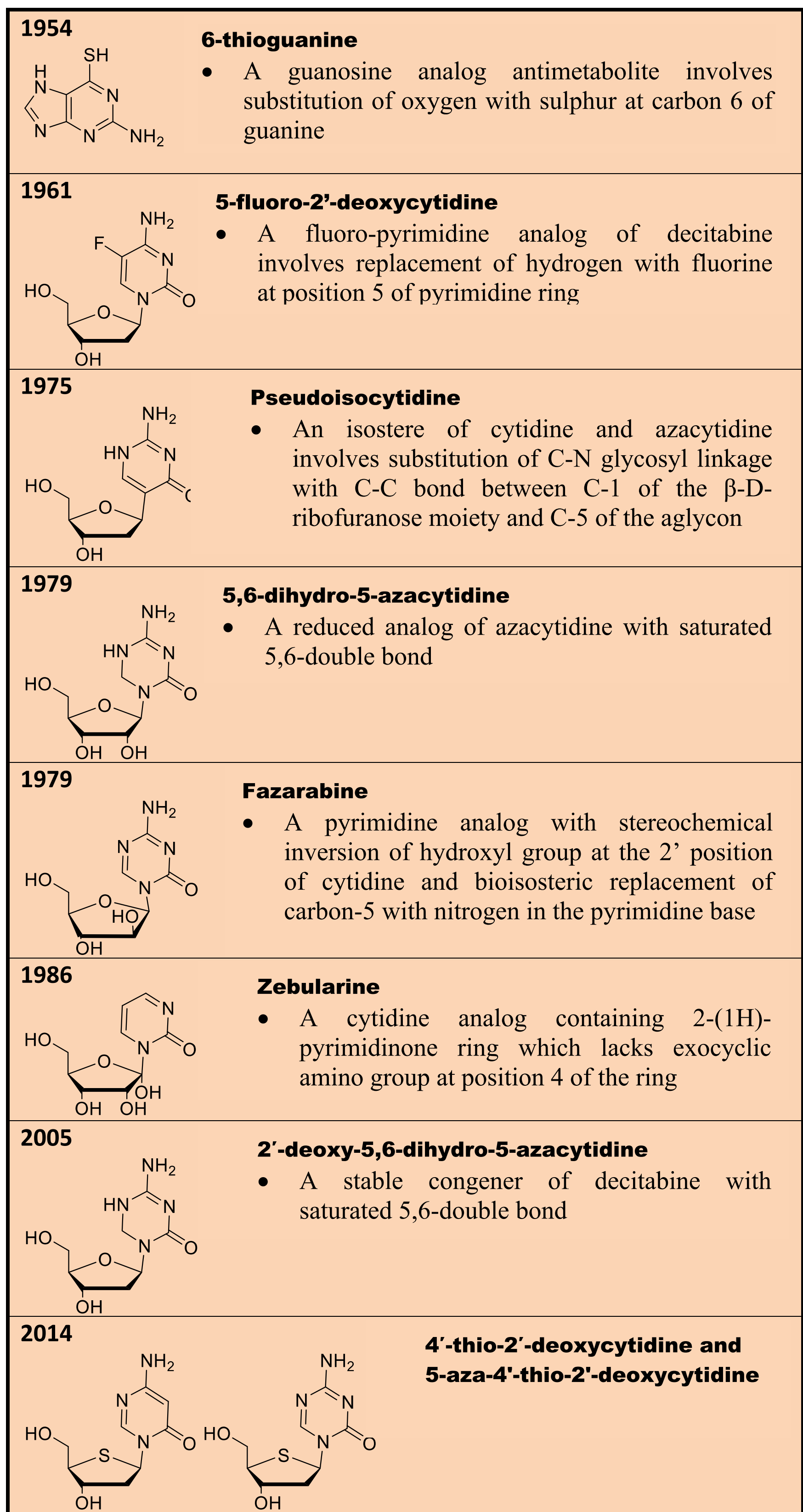

Fig. 3. First generation nucleosidic DNMTIs in developmental stage.

cytotoxicity combined with stability against both enzymatic and chemical catabolism supported biological evaluation of ψ ICyd, in vitro and in vivo. The study conducted in human and mouse leukemic cell lines demonstrated equal or significantly higher anti-leukemic effects of ψ ICyd, compared to 5-aza-CR. Remarkably, ψ ICyd showed no cross-resistance to cytosine arabinoside or cytarabine (Ara—C), but exhibited strong inhibitory effects in Ara-C resistant mouse leukemias, in contrast to 5-aza-CR. Further, the in vivo anti-leukemic activity of *i.p.* or *p.o.*

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Table 3
Nucleoside analogs as DNA methylation inhibitors in pre-clinical or early clinical development stage
This table presents the beneficial characteristics of first-generation nucleosidic DNA methylation inhibitors, their in vitro cellular potency in various cancer types, in vivo anti-tumor activity, and current phase of clinical development.

Drug	Specific characteristics	Types of cancer (*in vitro*)	*In vitro* cellular potency	Pre-clinical activity	Clinical phase	References
6-tG	• Incorporates into DNA and RNA and inhibits DNA and RNA synthesis • Inhibits DNA methylation via proteasomal degradation of DNMT1	Leukemia, kidney	1–4 μM	–	–	Hogarth et al. (2008), Nelson et al. (1975), Wang and Wang (2009), Yuan et al. (2011)
FdCyd	• Stable in aqueous solution • Orally bioavailable when co-administered with CDA inhibitor, THU	Cervix, melanoma, breast, colon, bladder, brain	25 nM-10 μM	• The combination of 6 mg/kg FdCyd +100 mg/kg THU, *i.v.* dose showed no significant in vivo activity	Phase 1/2	Beumer et al. (2006), Beumer et al. (2008), Eidinoff et al. (1959), Holleran et al. (2015), Hou and Newman (2005), Kinders et al. (2011), Li et al. (2006), Morfouace et al. (2016), Newman et al. (2015), Wempen et al. (1961), Zhao et al. (2012)
ψICyd	• Exceptional hydrolytic stability • Resistant to enzymatic deamination by CDA • Reduced cytotoxicity • Orally bioavailable	Leukemia	0.04–3.8 μg/mL	• Anti-leukemic activity at 60–150 mg/kg, *i.p.* or 100–150 mg/kg, *p.o.* doses in leukemia mouse models	–	Burchenal et al. (1976), Chu et al. (1975), Costanzi et al. (2011), Jones and Taylor (1980), Woodcock et al. (1980)
DHAC	• Stable in aqueous solution • Modest hematologic toxicity profile	Lymphoma, leukemia, breast, prostate	10–200 μM	• 25.1% and 46.3% decrease in DNA methylation at 1500 mg/kg, *i.p.* dose in dCK(0) and dCK(−) mouse leukemic cancer xenografts	Phase 1/2	Antonsson et al. (1987), Avramis et al. (1989), Beisler et al. (1977), Creagan et al. (1993), Curt et al. (1985), Dhingra et al. (1991), Holoye et al. (1987), Izbicka, Davidson, et al. (1999), Izbicka, MacDonald, et al. (1999), Kees and Avramis (1995), Powell and Avramis (1988), Traganos et al. (1981), Yogelzang et al. (1997)
Ara-AC	• Protected from deamination by CDA • Orally bioavailable	Colon, leukemia	0.75–10 μM	• Ara-AC, administered *i.p* or *i.v.* demonstrated wide therapeutic activity against several murine leukemias, and human xenografts of the NCI tumor panel	Phase 1/2	Ahluwalia et al. (1986), Amato et al. (1992), Bailey et al. (1991), Ben-Baruch et al. (1993), Bernstein et al. (1993), Casper et al. (1992), Dalai et al. (1986), Glazer and Knode (1984), Goldberg et al. (1997), Heideman et al. (1989), Hubbard et al. (1992), Kuebler et al. (1991), Manetta, Blessing, and Look (1995), Manetta, Blessing, Mann, and Smith (1995), Selby et al. (1994), Surbone et al. (1990), Vesely and Piskala (1986), Wallace et al. (1989), Walters et al. (1992), Wilhelm et al. (1999), Williamson et al. (1995)
Zeb	• Stable in aqueous solution • Potent inhibitor of CDA • Selective specificity for cancer cells ensures minimal general toxicity • Continuous long-term treatment possibility prevents gene re-methylation • Orally bioavailable	Bladder, leukemia, stomach, cervix, lung, pancreas, breast, liver, colon, cholangiocarcinoma, brain, osteosarcoma, oral squamous cell carcinoma	10 μM-1 mM	• Significant reduction in tumor volume at high dose of 1000 mg/kg, *i.p.* or *p.o.* in human bladder cancer xenografts • Improved OS in radiation-induced T-cell lymphoma mouse model at 400 mg/kg, *i.p.* dose • Significant inhibition of tumor volume at 10, 50, and 100 mg/kg, *p.o.* doses in human gastric cancer xenografts • 68.2% tumor growth inhibition at high dose of 1000 mg/kg, *i.p.* in human pancreatic cancer xenografts • A significant delay in tumor growth at 750 mg/kg, *p.o.* dose in human mammary tumors • Significant inhibition of tumor growth and tumor volume at 750 mg/kg, *p.o.* dose in human colorectal cancer xenografts	–	Andrade et al. (2017), Billam et al. (2010), Calvisi et al. (2006), Chen et al. (2012), Cheng et al. (2003), Cheng, Weisenberger, et al. (2004), Cheng, Yoo, et al. (2004), Herranz et al. (2006), Holleran et al. (2005), Kim et al. (1986), Meador et al. (2010), Nakamura et al. (2013), Nakamura et al. (2015), Neureiter et al. (2007), Ruiz-Magana et al. (2012), Savickiene et al. (2012), Scott et al. (2007), Suzuki et al. (2008), Tan et al. (2013), Yang et al. (2013), Ye et al. (2016), Yoo et al. (2008), You and Park (2012, 2013, 2014)
DHDAC	• High aqueous stability • Minimal cytotoxicity	Leukemia	50–100 μM	–	–	Agrawal et al. (2017), Matousova et al. (2011)
TdCyd **5-aza-TdCyd**	• Longer half-life • Minimal off-target toxicity • Orally bioavailable	Leukemia, lung, ovary, colon	TdCyd: 0.6–100 μM 5-aza-TdCyd: 0.06–58 μM	• TdCyd at 1.3 and 0.9 mg/kg and 5-aza-TdCyd at 6.7 and 10 mg/kg, *i.p.* doses caused significant tumor growth inhibition in human lung cancer xenografts	Phase 1	Thottassery et al. (2014)

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administered ψ ICyd was also proven to be equal or better than comparatively toxic doses of 5-aza-CR (Burchenal et al., 1976). Apart from interesting anti-leukemic activity, ψ ICyd displayed effective DNA demethylation activity and perturbed the cellular differentiated state (Jones & Taylor, 1980). The encouraging pre-clinical results indicated towards the clinical evaluation of ψ ICyd, especially in AML patients resistant to Ara—C. Unfortunately, the phase 1 clinical evaluation of ψ ICyd was precluded due to dose-limiting accumulative hepatotoxicity (Woodcock et al., 1980).

5.4. 5,6-Dihydro-5-azacytidine

5,6-Dihydro-5-azacytidine (DHAC) is a reduced analog of 5-aza-CR that surpasses the disadvantage of hydrolytic instability due to saturated 5,6-double bond, Fig. 3 (Beisler, Abbasi, & Driscoll, 1979), and facilitates prolonged *i.v.* infusion, potentially avoiding acute toxicities associated with bolus administration of 5-aza-CR (Curt et al., 1985). The mechanism of action is similar to the parent drug that involves phosphorylation by uridine cytidine kinase (uCK) and incorporation into nucleic acids, resulting in inhibition of RNA synthesis and DNA methylation (Avramis, Powell, & Mecum, 1989). The therapeutic potential of DHAC has been mainly characterized in lymphoid and leukemic cell lines. The studies demonstrated the defined effects of DHAC on cell survival and cell cycle kinetics (Traganos, Staiano-Coico, Darzynkiewicz, & Melamed, 1981), and inhibition of DNA methylation (Antonsson, Avramis, Nyce, & Holcenberg, 1987; Avramis et al., 1989; Kees & Avramis, 1995) resulting in induced dCK re-expression (Antonsson et al., 1987). The hypomethylation activity of DHAC was further confirmed in vivo, where *i.p.* administered DHAC significantly reduced DNA methylation levels in a xenografted mouse model of leukemic cells. In addition, the hypomethylation level correlated with dCK expression in these cells (Powell & Avramis, 1988). But, the comparative studies of the parent drug, 5-aza-CR and DHAC established lower hypomethylation activity (Jones & Taylor, 1980; Matousova et al., 2011), as well as less potency of DHAC as cytotoxic agent, and requirement of 10-fold higher drug concentration to achieve similar growth inhibitory activity as the parent drug (Voytek, Beisler, Abbasi, & Wolpert-DeFilippes, 1977). The lower potency of the reduced analog may be due to its greater affinity towards CDA causing rapid deamination at lower therapeutic concentrations, and consequently inefficient conversion to the active metabolite, 5-aza-dCTP resulting in poor DNA incorporation (Futterman, Derr, Beisler, Abbasi, & Voytek, 1978). However, the advantage of increased stability in aqueous solution over a wide range of pH necessitated clinical investigation of DHAC. During the phase 1 study, MTD was attained at 7 g/m^2 of DHAC, administered as a 24 h constant *i.v.* infusion, every 4 weeks, demonstrating pleuritic chest pain as the dose-limiting toxicity. Other toxicities included nausea and vomiting with no evidence for myelosuppression, nephrotoxicity or hepatotoxicity. Transient disease responses were observed in two patients with aggressive lymphoma, and one patient with progressive Hodgkin's lymphoma showed disease stabilization for 7 treatment cycles (Curt et al., 1985). Subsequently, phase 2 trials were conducted in extensive, untreated non-small cell lung cancer, pleural malignant mesothelioma, and disseminated malignant melanoma. However, low response rate during initial clinical trials, accompanied by cardiac toxicity ceased further development of DHAC (Creagan, Schaid, Hartmann, & Loprinzi, 1993; Dhingra, Murphy, Winn, Raber, & Hong, 1991; Holoye et al., 1987; Yogelzang et al., 1997). Nevertheless, definite antitumor activity in chemo-refractory malignant mesothelioma (Yogelzang et al., 1997), and meaningful regressions in disseminated malignant melanoma (Creagan et al., 1993), combined with modest hematologic toxicity profile favors the use of DHAC with other agents and warrants further trials testing synergistic combination regimens. But caution regarding cardiac arrhythmias and pericardial effusion is essential. Recently, the studies conducted in estrogen- and androgen-refractory, breast and prostate cancers respectively evidenced the effectiveness of DHAC to restore estrogen and androgen sensitivity. This suggests the clinical application of DHAC in treatment of hormone-refractory breast and prostate cancer patients by re-sensitizing them to conventional therapies with estrogen and androgen antagonists (Izbicka, Davidson, Lawrence, MacDonald, & Von Hoff, 1999; Izbicka, MacDonald, et al., 1999).

5.5. Fazarabine

Fazarabine, also known as Kymarabine or 1-β-*D*-arabinofuranosyl-5-azacytosine (Ara-AC), is a pyrimidine analog, synthesized by combining the structural features of cytotoxic nucleosides, Ara-C and 5-aza-CR. Structurally, Ara-AC bears stereochemical inversion of the hydroxyl group at the 2′ position of cytidine, analogous to Ara-C, and bioisosteric replacement of carbon-5 with nitrogen in the pyrimidine base, analogous to 5-aza-CR, Fig. 3 (Beisler et al., 1979). Similar in the mechanism of action to Ara-C, Ara-AC is phosphorylated by dCK to triphosphate form which incorporates into DNA in place of thymidine and exerts its anti-neoplastic effect by causing DNA hypomethylation, and direct cytotoxicity by inhibiting DNA synthesis (Barchi et al., 1996). A study investigating the mechanisms of native and acquired resistance to Ara-AC further showed dCK as the important determinant of tumor sensitivity to this drug. It was shown that leukemic and solid tumor cell lines exhibiting resistance towards Ara-C due to a marked decrease in dCK level, also showed cross-resistance towards Ara-AC. But, Ara-AC is protected from deamination by CDA, unlike Ara-C (Ahluwalia et al., 1986). During pre-clinical evaluations, Ara-AC showed cytocidal activity against human colon cancer cells in vitro by inhibition of DNA synthesis (Glazer & Knode, 1984), and in vivo Ara-AC demonstrated marked antitumor activity in wide spectrum of murine leukemias and solid tumors, and human tumor xenografts of National Cancer Institute (NCI) tumor panel. The studies also demonstrated the equal effectivity of Ara-AC by the *p.o.* route, compared with *i.p.* administration (Dalai et al., 1986; Vesely & Piskala, 1986; Wallace, Lindh, & Durr, 1989). Further, an in vitro study conducted in Ara-C sensitive and resistant human leukemia cell lines confirmed the DNA hypomethylation potency of Ara-AC (Kees & Avramis, 1995). Consequently, several Phase 1 studies were conducted in past decades, in cases of both, children and adult with refractory or solid tumor malignancies. The studies aimed to determine the toxicity, MTD, and therapeutic efficacy for low-dose 72 h continuous *i.v.* infusions, as well as for high dose short infusions, using a daily bolus administration for 5 days. In both schedules, predominant dose limiting toxicities (DLT) observed was myelosuppression including reversible granulocytopenia and thrombocytopenia. Other toxicities observed were moderate nausea and vomiting which did not appear to be dose-dependent. A rare case of one patient with stable disease for 65 days was noted (Amato, Ho, Schmidt, Krakoff, & Raber, 1992; Bailey et al., 1991; Bernstein et al., 1993; Goldberg et al., 1997; Heideman et al., 1989; Surbone et al., 1990; Wilhelm et al., 1999). After promising pre-clinical activity and reasonable toxicity in phase 1 clinical trials, several Phase 2 studies of Ara-AC were also published in solid tumors. Using the continuous *i.v.* infusions for 3 days, no major clinical responses were observed in advanced colorectal and pancreatic adenocarcinoma, metastatic breast and colon cancer, and advanced non-small cell lung carcinoma (Ben-Baruch, Denicoff, Goldspiel, O'Shaughnessy, & Cowan, 1993; Casper, Schwartz, & Kelsen, 1992; Hubbard et al., 1992; Walters, Theriault, Holmes, Hortobagyi, & Esparza, 1992; Williamson, Crowley, Livingston, Panella, & Goodwin, 1995). The studies employing the bolus regimens for five days in advanced head and neck cancer, high grade gliomas, advanced squamous cell carcinoma of the cervix, and ovarian cancer also reported unsatisfactory results (Kuebler, Metch, Schuller, Keppen, & Hynes, 1991; Manetta, Blessing, & Look, 1995; Manetta, Blessing, Mann, & Smith, 1995; Selby, Upchurch, Townsend, & Eyre, 1994). No significant activity of this drug in various phase 2 clinical trials blocked further investigation.

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5.6. Zebularine

Zebularine or 1-(β-*D*-ribofuranosyl)-1,2 dihydropyrimidin-2-one (Zeb) is a mechanism-based inhibitor of DNA methylation, without apparent modification at position 5 of the pyrimidine ring. Structurally, Zeb is a cytidine analog containing 2-(1H)-pyrimidinone ring which lacks exocyclic amino group at position 4 of the ring, originally designed as the potent inhibitor of CDA, Fig. 3 (Kim, Marquez, Mao, Haines, & McCormack, 1986). In addition to concomitant CDA inhibitory activity, Zeb has also been reported to induce selective inhibition of DNMTs. Its mechanism of action is similar to AZN analogs and involves incorporation into DNA, and subsequent formation of a covalent adduct with DNMTs at position 6 of the pyrimidinone ring, resulting in proteasomal-mediated enzyme degradation (Zhou et al., 2002). Further, Zeb features a remarkable property of being preferentially selective towards the tumor cells, in terms of incorporation into DNA, cell growth inhibition, demethylation, and depletion of DNMTs, suggesting minimal toxicity (Cheng, Yoo, et al., 2004; Tan, Zhou, Yu, Luo, & Shen, 2013). Furthermore, unlike AZN drugs Zeb is chemically stable in aqueous solutions which enables its oral administration (Holleran et al., 2005). Also, due to low toxicity Zeb can be used for long-term treatment with minimal side effects (Yoo et al., 2008). Thus, favorable pharmacological properties of Zeb, stability combined with minimal toxicity allows for continuous treatment. This sustains demethylation effects for prolonged periods and prevents gene re-silencing, demonstrated by induction and maintenance of *p16* expression, global demethylation, and complete depletion of DNMT1 following Zeb treatment in bladder cancer cells (Cheng, Weisenberger, et al., 2004). During pre-clinical studies, the hypomethylation and anti-tumor activity of Zeb were evaluated in a wide range of cancer cell lines, including myeloid malignancies and selected solid tumors. The studies demonstrated the potential role of Zeb as a demethylating agent in epigenetic therapy, as well as via cell cycle arrest and induction of apoptosis by various other pathways independent of DNA methylation. The first study characterizing Zeb as a demethylating agent reported slight cytotoxicity and demethylation-mediated reactivation of silenced *p16* gene in bladder cancer cells, in vitro. Also, Zeb administered *i.p.* or *p.o.* induced *p16* re-expression and significantly reduced tumor volume in in vivo established human bladder cancer xenografts(Cheng et al., 2003). The study designed in radiation-induced T-cell lymphoma mouse model showed the positive effects of *i.p.* administered Zeb with minimal toxicity against the development of thymic lymphoma, evidenced by longer OS, and accompanied therapeutic changes including global genomic hypomethylation, DNMT1 depletion, and demethylation-induced re-expression of *p16INK4a*, *MGMT*, *MLT-1*, and *E-cadherin* genes (Herranz et al., 2006). The study in AML cell lines and primary patient samples demonstrated demethylation and dose-dependent increase in *p15INK4B* expression, along with inhibition of cell proliferation, the blockade in G2/M phase, and induction of apoptosis (Scott et al., 2007). Another study using human promyelocytic leukemia cell lines again reported decreased DNMT1 expression, time-dependent expression of *pan-cadherin* and partial demethylation of *E-cadherin*, together with dose- and time-dependent cell growth inhibition, dose-dependent apoptosis manifested by procaspase-3 and PAR-1 cleavage, and the onset of early apoptosis (Savickiene, Treigyte, Borutinskaite, & Navakauskiene, 2012). Yet, another study in p53 mutant leukemic T cells reported caspase-mediated apoptosis induction and activation of the intrinsic apoptotic pathway by inducing mitochondrial alterations such as *BAK* activation, loss of transmembrane potential, and generation of reactive oxygen species, paralleled by induction of DNA damage, following Zeb treatment (Ruiz-Magana et al., 2012). In gastric cancer cell lines, Zeb treatment caused DNMT inhibition and re-expression of hypermethylation silenced *p16* in a dose- and time-dependent manner. This most likely activated mitochondrial apoptosis pathway by upregulating pro-apoptotic *BAX* and inhibiting anti-apoptotic *Bcl-2* expression associated with an increase of caspases-3 activity. The study also reported the anti-tumor effect of *p.o.* administered Zeb in human gastric cancer xenografted mouse model (Tan et al., 2013). In cervical cancer cells, Zeb inhibited cell growth in a dose-dependent manner by causing S-phase arrest of the cell cycle, accompanied by increased levels of S-phase marker, *Cyclin A*/*CDK2* proteins, and induction of apoptosis, accompanied by loss of mitochondrial membrane potential (MMP), PARP-1 cleavage, and activation of caspase-3, −8 and −9 (You & Park, 2012). In lung cancer cell lines, Zeb induced cell death in a dose-dependent manner, accompanied by loss of MMP, *Bcl-2* decrease, *BAX* and *p53* increase (You & Park, 2014), and/or caspase-3 and -8 activations, and S-phase arrest of the cell cycle (You & Park, 2013, 2014). Besides, Zeb treatment resulted in depletion of glutathione (GSH) levels in both cervical and lung cancer cell lines, and GSH content was inversely correlated with apoptotic effect induced by Zeb (You & Park, 2012, 2013, 2014). The study in pancreatic cancer models reported dose- and time-dependent decrease in cell proliferation and increase in apoptosis, associated with up-regulation of *BAX* and increased expression of *CK7,* in vitro. Also, *i.p.* administered Zeb caused the delayed growth of in vivo established human pancreatic xenografts, accompanied with up-regulation of *CK7* and down-regulation of dedifferentiation markers (Neureiter et al., 2007). In human mammary tumors, Zeb-induced inhibition of cell growth was associated with increased *p21* expression, decreased expression of *cyclin D*, and induction of S-phase arrest in a dose- and time-dependent manner. At high doses, Zeb mediated alterations in apoptotic proteins, *caspase-3*, *BAX*, *Bcl-2*, and PARP cleavage. However, at low doses, Zeb inhibited DNMTs and induced re-expression of epigenetically silenced estrogen and progesterone receptor mRNA (Billam, Sobolewski, & Davidson, 2010). The anti-tumor study conducted in genetically engineered mouse model of breast cancer further evidenced high apoptotic index and significantly delayed growth of mammary tumors following *p.o.* administration of Zeb. The study also reported the depletion of DNMTs and up-regulation of various methylation regulated as well as cancer related cell cycle regulatory genes (Chen et al., 2012). In hepatocellular carcinoma cells, Zeb induced cell cycle arrest independent of DNA methylation via MAPK pathway, and induced apoptosis by decreasing the activity of *PKR* resulting in *Bcl-2* down-regulation and apoptotic cell death (Nakamura et al., 2013), and via DNA methylation pathway by reactivating *Ras* and *Jak*/*Stat* inhibitors resulting in cell growth suppression and extensive cell death (Calvisi et al., 2006). The study conducted in colorectal cancer further described the anti-cancer activity of Zeb via induction of *p53* dependent apoptosis, by down-regulation of the increased expression of pro-survival marker of endoplasmic reticulum stress, *GRP78* and autophagy, *p62,* and by up-regulating the pro-apoptotic CHOP in colorectal cancer patients and tumor-derived stem cells. Also, *p.o.* administered Zeb significantly inhibited both tumor weight and tumor volume in human colorectal cancer xenografts (Yang et al., 2013). The anti-cancer activity of Zeb was also explored in cholangiocarcinoma. The study demonstrated that Zeb treatment resulted in DNMT depletion, and to an extent, the alteration in DNA methylation status was associated with suppression of the Wnt signaling pathway leading to apoptotic cell death. In addition, decrease in β-catenin protein levels was also reported in Zeb treated cells (Nakamura et al., 2015). The anti-cancer effects of Zeb were further characterized in brain cancers. In glioblastoma cells, Zeb induced cytotoxic effects in a dose-dependent manner and caused rapid global and gene-specific demethylation. The major determinant for cellular response to Zeb was found to be combination of DNA repair and cell cycle checkpoint defects (Meador, Su, Ravanat, & Balajee, 2010). In pediatric medulloblastoma cell lines, Zeb treatment inhibited cell proliferation and clonogenicity by increasing expression of TSGs, *p53* and *p21*, induced S-phase cell cycle arrest and decreased expression of *cyclin A*, and induced apoptosis by increasing *BAX* and decreasing *Bcl-2* and *survivin* proteins. In addition, Zeb treatment also modulated the activation of SHH pathway, and altered global gene expression profile, significantly upregulating *BATF2* expression in medulloblastoma cells (Andrade et al., 2017). In human osteosarcoma cells, Zeb treatment inhibited viability and promoted apoptosis in a dose- and time-dependent manner by disturbing the interaction between DNMT1 and histone methyltransferase, G9a, thereby causing

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demethylation-induced expression of hypermethylation silenced TSG, *ARHI* (Ye et al., 2016). In oral squamous cell carcinoma, treatment with Zeb inhibited *VEGF* expression via proteasome-based ubiquitination of the *HIF-1α* pathway, which suggests the potential of Zeb in modulation of angiogenic properties in these cells (Suzuki, Shinohara, & Rikiishi, 2008). Altogether, the spectrum of Zeb effects in myeloid malignancies and wide range of solid tumors demonstrate the anti-cancer mechanisms of Zeb, as demethylating agent and as a promising adjuvant chemotherapy agent via DNMT independent pathways, and provide strong rationale to continue the research with Zeb. However, the poor bioavailability of Zeb, resulting from its complex metabolism into endogenous inactive compounds and its limited DNA incorporation (Ben-Kasus, Ben-Zvi, Marquez, Kelley, & Agbaria, 2005), and secondly, requirement of higher dose to induce similar levels of demethylation as 5-aza-CR and 5-aza-CdR, due to lack of permanent covalent complex with DNMTs (Champion et al., 2010) has prevented Zeb from entering into clinical trials, yet. Nevertheless, the combinatorial therapy of Zeb with other demethylating agents may lower its required dose for clinical approaches, and provide effective anti-cancer treatment. Moreover, the depletion of cancer-related antigen genes suggests anti-tumor potential of Zeb in combination with immunotherapy (Cheng, Yoo, et al., 2004).

5.7. 2′-Deoxy-5,6-dihydro-5-azacytidine

2′-Deoxy-5,6-dihydro-5-azacytidine (DHDAC, KP-1212) is another recently developed, hydrolytically stable congener of DAC with advantages of high aqueous stability and minimal cytotoxicity (Fig. 3). DHDAC has already been known for its anti-HIV activity mediated by lethal mutagenesis of the viral genome (Harris, Brabant, Styrchak, Gall, & Daifuku, 2005) and has also been tested in phase 2 clinical trial against HIV (Mullins et al., 2011). However, the demethylation potential of DHDAC in cellular models was discovered very recently. The study reported the efficient ability of DHDAC to decrease the methylation level of two epigenetically silenced genes, *CDKN2B* and *THBS-1*, and increase mRNA expression of *THBS-1* in human leukemic cell lines, similar to DAC. The study also demonstrated that hypomethylation activity of DHDAC was comparable to DAC (Matousova et al., 2011). Furthermore, the studies proved DHDAC as less toxic alternative of DAC, evidenced by time-dependent increase in DAC toxicity against negligible or no effect of DHDAC on cell cycle progression at 100-fold higher concentration or at dose that induced DNA hypomethylation and gene reactivation comparable to DAC (Agrawal et al., 2017; Matousova et al., 2011). Overall, efficient hypomethylation activity combined with low toxicity and aqueous stability might represent DHDAC as a superior hypomethylating agent over DAC. But, further pre-clinical studies and clinical trials validating DHDAC as feasible alternative of DAC is clearly required.

α-Anomer of DHDAC (α-DHDAC) was reported with no significant hypomethylation potency. This may be due to absence of the 5,6-double bond, required for spontaneous conversion of α-DHDAC to corresponding β-anomer. Also, incorporation of α-anomer into DNA is unlikely (Matousova et al., 2011).

5.8. 4′-Thio-2′-deoxycytidine and 5-aza-4′-thio-2′-deoxycytidine

4′-Thio-2′-deoxycytidine (TdCyd) was synthesized as a 5′-protected phosphoramidite (Fig. 3), and was initially discovered as inhibitor of methylation by bacterial *Hha*I methyltransferase (Kumar et al., 1997). Recently, TdCyd and its 5-aza analog (Fig. 3), 5-aza-4′-thio-2′-deoxycytidine (5-aza-TdCyd) were reported for their potential activity in depleting human DNMT1 and concomitant inhibition of tumor growth, in both in vitro and in vivo cancer models (Thottassery et al., 2014). The study demonstrated that both TdCyd and 5-aza-TdCyd decreased cell viability and caused marked depletion of DNMT1 in leukemia and solid tumor cells, and effectively induced CpG demethylation and re-expression of TSG, *p15* in leukemia cells. Both TdCyd and 5-aza-TdCyd administered *i.p.* also showed DNMT1 depleting activity in human leukemia and lung cancer xenograft models, and caused efficient reduction of tumor growth in lung cancer xenografts (Thottassery et al., 2014). Furthermore, the study also indicated better tolerance of 5-aza-TdCyd as compared to decitabine, evident by at least 10-fold greater selectivity index (ratio of MTD to that of minimal DNMT1 depleting dose) than decitabine. The data suggest minimal off-target toxicity of 5-aza-TdCyd, however, the reason of less toxicity remains ununderstood (Thottassery et al., 2014). It was also distinguished that 5-aza-TdCyd was indefinitely stable in aqueous solution with three times longer half-life over decitabine, thereby supporting adequate bioavailability of oral formulations (Thottassery et al., 2014). Collectively, the data emphasize towards further development of 4′-thio modified deoxycytidine analogs as novel clinically effective DNA methylation inhibitors with less toxicity and increased stability, and approval of their use in treatment of solid tumors. Now, TdCyd has entered into phase 1 clinical trial, to establish safety, tolerability, and MTD of oral TdCyd in patients with refractory solid tumors (NCT02423057).

6. Second generation pro-drugs

Despite, immense clinical development of azacytidine and decitabine in epigenetic cancer therapy, the efficacy of these nucleoside drugs is limited due to significant challenges arising from low bioavailability, metabolic instability, and reduced cellular uptake of both compounds. Based on the current mechanistic understanding about metabolization and cellular drug uptake, efforts are underway to identify novel AZN derivatives with better PK and pharmacodynamics (PD) profile, exemplified by SGI-110 and CP-4200. SGI-110, dinucleotide of decitabine with increased metabolic stability, and CP-4200, azacytidine variant affording improved cellular delivery, are recently developed second generation nucleoside analogs with enhanced therapeutic efficacy over FDA approved DNA methylation inhibitors. Apart from SGI-110 and CP-4200, other potential pro-drugs of decitabine and azacytidine, NPEOC-DAC and 2′3′5′triacetyl-5-azacytidine respectively, and a novel cytidine analog, RX-3117 have also been investigated in pre-clinical trials and/or have entered into clinical trials (Fig. 4, Table 4).

6.1. RX-3117

RX-3117 or TV-1360; Fluorocyclopentenylcytosine (Rexahn Pharmaceuticals Inc., Rockville, MD, USA) is a next generation novel cytidine analog, currently being investigated in phase 1/2 clinical trial in solid tumors. The synthesis of RX-3117 involves replacement of sugar moiety with a cyclopentenyl group, Fig. 4 (Jeong et al., 2007; Zhao et al., 2005). The metabolism and mechanism of action of RX-3117 are distinct from other (deoxy) cytidine analogs. Unlike existing nucleoside analogs which are phosphorylated by dCK, RX-3117 has a different activation pathway that involves phosphorylation by uCK to its monophosphate and subsequently to its diphosphate (RX-DP) and triphosphate (RX-TP) forms. RX-TP is incorporated into RNA and inhibits RNA synthesis, whereas, RX-DP is further reduced by ribonucleotide reductase to dRX-DP, converted to its triphosphate form dRX-TP, and incorporated into DNA where it inhibits DNA synthesis. Apart from exerting its cytotoxic effects by inhibition of DNA and RNA synthesis, RX-3117 also mediates down-regulation of DNMT1 (Peters et al., 2013). Remarkably, due to the unique specificity of uCK for cancer cells in contrast to dCK which is highly expressed in both cancer and normal cells, RX-3117 has improved efficacy and safety profile in cancer patients (Peters et al., 2013). Moreover, a recent study evidenced significant correlation of RX-3117 phosphorylation in intact cells specifically with uCK2 expression, but not with uCK1. This may be implicated in the clinic to potentially select the patients sensitive to RX-3117 (Sarkisjan et al., 2016). Also, RX-3117 is protected from extensive deamination by CDA and therefore has long half-life and high oral bioavailability (Peters et al., 2013). The potent anti-tumor activity of RX-3117 was reported in vitro in broad range of tumor cell lines (Choi et al., 2012; Jeong

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et al., 2007; Zhao et al., 2005), and was further correlated with in vivo anti-tumor effects in tumor xenograft model of human lung cancer cells, where, *i.p.* administered RX-3117 significantly inhibited the tumor growth, tumor volume, and tumor weight in a dose-dependent manner(Choi et al., 2012). The mechanism of action involved in potent anti-tumor activity was discovered to be dose-dependent inhibition of DNMT1, demonstrated in breast cancer cells (Choi et al., 2012). The *p. o.* administered RX-3117 further demonstrated high bioavailability and superior toxicity profile across a wide variety of human tumor xenografts in contrast to gemcitabine, an orally unavailable chemotherapeutic drug of the same class (Yang et al., 2014). RX-3117 also showed potent efficacy against human pancreatic xenograft, relatively resistant to gemcitabine. This indicated the therapeutic potential of RX-3117 for treatment of gemcitabine in-sensitive tumors (Yang et al., 2014). The study also demonstrated the positive correlation between the efficacy of RX-3117 and uCK levels in xenograft models (Yang et al., 2014). After the promising results of the pre-clinical studies, RX-3117 is currently being evaluated in phase 1/2 dose-escalation open-label clinical trial. The study aims to evaluate the MTD of RX-3117 in patients with advanced or metastatic solid tumors in phase 1, and anti-tumor activity in patients with relapsed or refractory pancreatic or advanced bladder cancer in phase 2 (NCT02030067).

6.2. SGI-110

SGI-110 or S-110; Decitabine deoxyguanosine; Decitabine deoxyguanosine dinucleotide; Guadecitabine (Astex Pharmaceuticals, Dublin, CA, USA) is a second generation hypomethylating pro-drug of decitabine in advanced clinical development. SGI-110 is a dinucleotide of decitabine formulated by binding active decitabine with its chemically modified form, deoxyguanosine, by a natural phosphodiester linkage, Fig. 4 (Yoo et al., 2007). Unlike decitabine which is susceptible to in vivo deamination by CDA which compromises the plasma level of the drug resulting in low bioavailability, a SGI-110 dinucleotide is highly protected from inactivation by CDA (Yoo et al., 2007). The mechanism of action of SGI-110 to inhibit DNA methylation via depletion of DNMT1, its aqueous stability and cytotoxicity remain similar to the parent compound (Stresemann & Lyko, 2008; Yoo et al., 2007). However, the differentiated PK and PD profile of SGI-110 offers improved clinical efficacy over existing hypomethylating agents, not only in patients with hematologic malignancies but also marks the drug useful for the treatment of solid tumors for which first generation drugs are not approved. The first biological study of SGI-110 was conducted in bladder and colon cancer cells, where, SGI-110 caused dose-dependent demethylation and increase in *p16* expression at both mRNA and protein level (Yoo et al., 2007). The

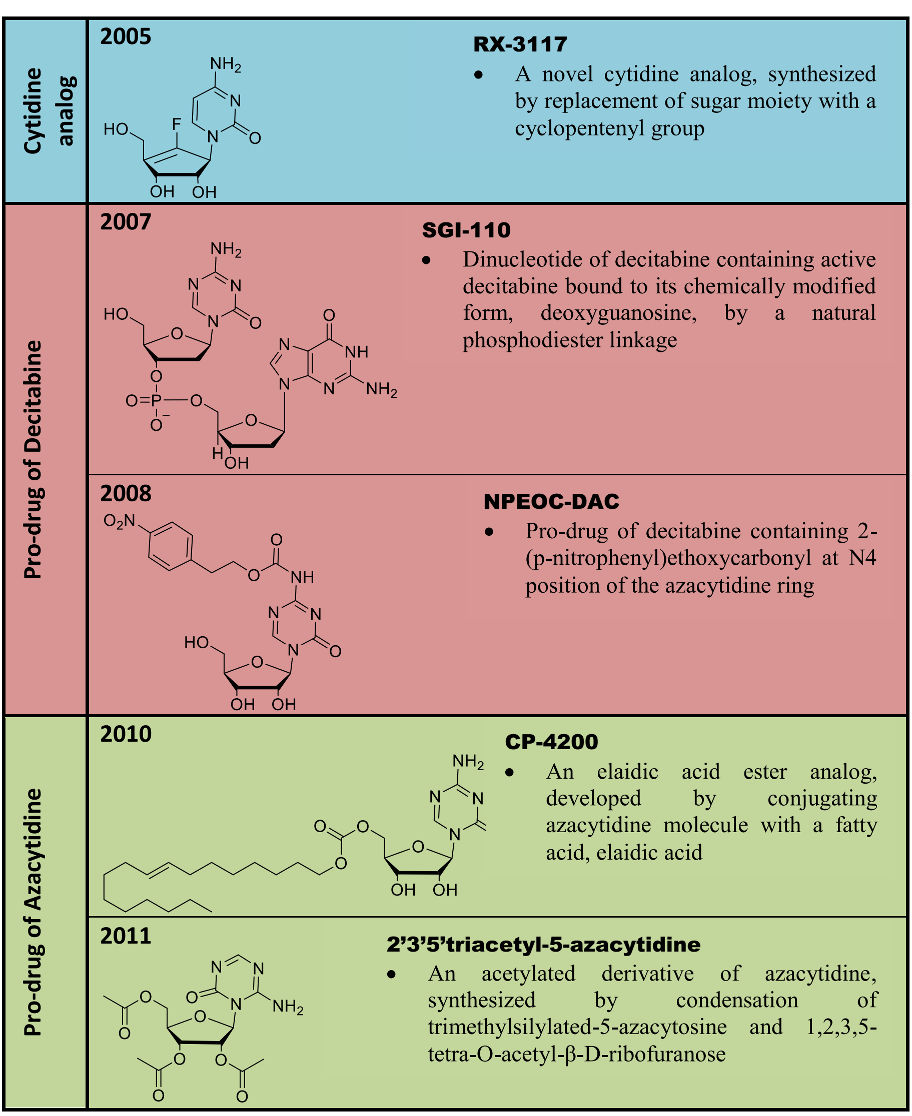

Fig. 4. Second generation pro-drugs.

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Table 4
Second generation DNA demethylating pro-drugs in pre-clinical or clinical development
This table presents the beneficial characteristics of second generation DNA methylation inhibitors, their in vitro cellular potency in various cancer types, in vivo anti-tumor activity, and current phase of clinical development.

Drug	Specific characteristics	Types of cancer (*in vitro*)	*In vitro* cellular potency	Pre-clinical activity	Clinical phase	References
RX-3117	• Long half-life • Improved efficacy and safety profile in cancer patients • Orally bioavailable	Breast, Colon, Lung, Stomach, Pancreas, Prostate, Liver, Ovary, Leukemia, Kidney, Brain, Cervix, Melanoma	0.18–2.67 μM	• 31.8% and 58.1% tumor growth inhibition at 3 and 10 mg/kg, *i.p.* doses in human lung cancer xenograft model • 100%, 78%, 62%, and 66% tumor growth inhibition in human colon, non-small cell lung, small cell lung, and cervical cancer xenograft models, and 76% tumor growth inhibition in gemcitabine-resistant human pancreatic xenografts by *p.o.* administration	Phase 1/2	Choi et al. (2012), Peters et al. (2013), Sarkisjan et al. (2016), Yang et al. (2014)
SGI-110	• Longer in vivo half-life • Prolonged in vivo exposure to decitabine by small volume *s.c.* administrations • Improved PK and PD profile, and clinical efficacy over existing hypomethylating agents	Bladder, Colon, Melanoma, Renal cell carcinoma, Mesothelioma, Sarcoma, Leukemia, Ovary	1 μM	• Retardation in tumor growth at 10 mg/kg, *i.p.* and 12.2 mg/kg, *s.c.* doses in human bladder cancer xenografts • Reduction in tumor mass at 2 mg/kg, *s.c.* dose in human hepatocellular carcinoma xenografts • Enhanced antigen-specific CD8+ T cell anti-tumor response at 3 mg/kg, *s.c.* dose in human epithelial ovarian cancer xenografts	Phase 1/2 Phase 3	Cardenas et al. (2014), Chuang et al. (2010), Coral et al. (2013), Issa et al. (2015), Jueliger et al. (2016), Yoo et al. (2007)
NPEOC-DAC	• Increased plasma half-life • Decreased cytotoxicity • Orally bioavailable	Liver	≥10 μM	–	–	Byun et al. (2008)
CP-4200	• Low dependence on nucleoside transporters involved in drug uptake mechanisms • Increased epigenetic potential	Leukemia, Colon, Breast	2–15 μM	• Significant decrease in spleen weight at 15 and 20 mg/kg, *i.v.* or *i.p.* doses in orthotopic ALL mouse tumor model	–	Brueckner et al. (2010), Hummel-Eisenbeiss et al. (2013)
TAC	• Higher solubility and stability across wide range of pH • Longer half-life • Minimal general toxicity • Orally bioavailable	Leukemia	No cellular toxicity	• 50% increased lifespan at 38 mg/kg, *p.o.* dose in human lymphocytic leukemia animal model	–	Ziemba et al. (2011)

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Table 5
Azacytidine in combinatorial therapies
This table represents all registered clinical trials of azacytidine in combination with various chemotherapeutic, epigenetic or immunomodulatory agents for which study results have been posted or are available as publications.

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
➢ **Standard chemotherapy drugs: Cytarabine, cisplatin, docetaxel, daunorubicin (+prednisone)**				
AML, MDS	Phase 1/2	2005, Completed	Randomized study of azacytidine in combination with cytarabine in patients with relapsed or refractory AML or high-risk MDS, for determining (i) MTD of azacytidine in combination, and (ii) safety and effectiveness of the combination treatment; • Group 1: Azacytidine: 37.5 mg/m^2, *i.v.*, 20–30 min, daily, 1–7 d + Cytarabine: 100 mg/m^2, c.*i.v.*, daily, 1–7 d, every 4–8 w • Group 2: Azacytidine: 75 mg/m^2, *i.v.*, 20–30 min, daily, 1–7 d + Cytarabine: 100 mg/m^2, c.*i.v.*, daily, 1–7 d, every 4–8 w • Group 3: Azacytidine: 37.5 mg/m^2, *i.v.*, 20–30 min, daily, 1–7 d + Cytarabine: 1 g/m^2, c.*i.v.*, daily, 1–4 d (age < 65 y) or 1–3 d (age ≥ 65 y), every 4–8 w • Group 4: Azacytidine: 75 mg/m^2, *i.v.*, 20–30 min, daily, 1–7 d + Cytarabine: 1 g/m^2, c.*i.v.*, daily, 1–4 d (age < 65 y) or 1–3 d (age ≥ 65 y), every 4–8 w • Result (Group 1 vs. Group 2 vs. Group 3 vs. Group 4): CR: 0/6 (0%) vs. 0/6 (0%), vs. 0/11 (0%) vs. 2/11 (18%), SAE: 5/6 (83%) vs. 5/6 (83%) vs. 8/11 (73%) vs. 3/11 (27%)	NCT00569010
Squamous cell carcinoma	Phase 1	2007, Terminated	Non-randomized dose-escalation study of azacytidine in combination with cisplatin in patients with recurrent or metastatic squamous cell carcinoma of the head and neck, for determining the safety and toxicity of the combination; • Azacytidine: 37–110 mg/m^2/day, *s.c.*, daily, 1–5 d + Cisplatin: 75 mg/m^2, *i.v.*, d 8, every 4 w • Result: SAE: 1/1 (100%)	NCT00443261
Prostate cancer	Phase 1/2	2007, Terminated	Non-randomized study of azacytidine in combination with docetaxel and prednisone in patients with previously treated hormone refractory metastatic prostate cancer, for determining (i) a safe and potentially efficacious phase 2 dose of azacytidine in combination with docetaxel and prednisone (ii) the therapeutic efficacy of the combination (iii) toxicity profile (iv) DOR, and (v) PFS and OS; • Phase 1: Azacytidine (*i.v.*, 30 min, daily, 1–5 d, every 3 w) + Docetaxel (*i.v.*, 1 h, d 6, every 3 w): 75 mg/m^2 + 60 mg/m^2 (level 1) – 75 mg/m^2 + 75 mg/m^2 (level 2) – 100 mg/m^2 + 75 mg/m^2 (level 3) – 150 mg/m^2 + 75 mg/m^2 (level 4) + Prednisone: 5 mg, *p.o.*, twice daily, 1–21 d • Phase 2: Azacytidine + Docetaxel with 5 mg of prednisone at initial recommended phase 2 dose level (RPTD) • Phase 2: Reduced dose of Azacytidine + Docetaxel with 5 mg of prednisone at RPTD • Result: [Initial and Reduced RPTD: Azacytidine: 150 and 75 mg/m^2, Docetaxel: 75 and 75 mg/m^2, Prednisone: 5 and 5 mg], [ORR: Phase 1 (Level 1): 0/2 (0%), Phase 1 (Level 2): 0/0 (0%), Phase 1 (Level 3): 1/2 (50%), Phase 1 (Level 4): 1/3 (33%), Phase 2 (initial RPTD): 1/3 (33%)], PFS: 4.9 months, OS: 19.5 months, [SAE: Level 1: 1/3 (33%), Level 2: 0/4 (0%), Level 3: 1/3 (33%), Level 4: 4/12 (33%)]	NCT00503984 (Singal et al., 2015)
AML	Phase 2	2009, Completed	Randomized study of the effectiveness of azacytidine added to standard primary therapy in older patients with newly diagnosed AML; • Induction therapy: Azacytidine: 75 or 37.5 mg/m^2/day, *i.v.*, 30 min, daily, −5 to −1 d + Cytarabine: 100 mg/m^2/day, c.*i.v.*, daily, 1–7 d + Daunorubicin: 45 mg/m^2/day, *i.v.*, daily, 3–5 d • Consolidation therapy: Azacytidine: 75 or 37.5 mg/m^2/day, *s.c.*, daily, −5 to −1 d + Cytarabine: 1 g/m^2, *i.v.*, twice a day, d 1, d 3, d 5 • Maintenance therapy: Azacytidine: 75 or 37.5 mg/m^2/day, *s.c.*, daily, 1–5 d, every 4 w • Result: CR: 7/12 (58%), OS: 8.9 months, EFS: 7.2 months	NCT00915252 (Krug et al., 2012)
➢ **Histone deacetylase inhibitors: Phenylbutyrate, entinostat, valproic acid, vorinostat (+All-Trans retinoic acid, carboplatin, gemtuzumab ozogamicin, lenalidomide)**				
Solid tumors	Phase 1/2	2000, Completed	Study of azacytidine in combination with phenylbutyrate in patients with advanced or metastatic solid tumors, for determining (i) safety and toxicity of the combination (ii) MTD of this treatment regimen where maximal gene re-expression occurs in these patients (iii) PK, and (iv) minimal effective dose of azacytidine in combination with phenylbutyrate that elicits a biological or clinical response in these patients; • Regimen A: Azacytidine: 25–18.75-15-10 mg/m^2/day, *s.c.*, daily, 1–14 d + Phenylbutyrate: 400 mg/m^2/day, c.*i.v.*, d 6, d 13, every 5 w • Regimen B: Azacytidine: 75 mg/m^2/day, *s.c.*, daily, 1–7 d + Phenylbutyrate: 200–400 mg/m^2/day, c.*i.v.*, d 8, d 14, every 5 w • Regimen C: Azacytidine: 10–12.5 mg/m^2/day, *s.c.*, daily, 1–21 d + Phenylbutyrate: 400 mg/m^2/day, c.*i.v.*, d 6, d 13, d 20, every 6 w • Result: The combination of azacytidine and phenylbutyrate across three dose schedules was generally well tolerated and safe, but lacked any real evidence for clinical benefit	NCT00005639 (Lin et al., 2009)
MDS, CMML, AML	Phase 1	2004, Active	Study of azacytidine in combination with entinostat in patients with MDS, CMML, and AML, for determining (i) safety and toxicity of the combination (ii) MTD and optimal phase 2 dose of entinostat when combined with azacytidine (iii) therapeutic efficacy of the regimen, and (iv) correlate PK of entinostat with clinical response and laboratory correlative endpoints; • Arm 1: Azacytidine: 50 mg/m^2/day, *s.c.*, daily, 1–10 d, every 4 w • Arm 2: Azacytidine: (Arm 1) + Entinostat: 4 mg/m^2/day, *p.o.*, d 3, d 10, every 4 w • Result: (Arm 1 vs. Arm 2): ORR: 12/24 (50%) vs. 4/23 (17%), DOR: 8 months vs. 5 months, OS: 13 months vs. 6 months	NCT00101179 (Prebet et al., 2016)
MDS, CMML, AML	Phase 2	2006, Completed	Randomized study of azacytidine with or without entinostat in patients with de novo MDS, CMML (dysplastic type) or AML with multilineage dysplasia, for determining (i) ORR and the major response rate of azacytidine	NCT00313586 (Prebet et al., 2016)

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Table 5 (*continued*)

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
			monotherapy versus combination (ii) toxicity of the combination (iii) to identify the changes in gene promoter methylation and expression, and (iv) the molecular mechanisms associated with response to azacytidine and entinostat such as DNA damage; • Arm 1: Azacytidine: 50 mg/m^2/day, *s.c.*, daily, 1–10 d, every 4 w • Arm 2: Azacytidine: (Arm 1) + Entinostat: 4 mg/m^2/day, *p.o.*, d 3, d 10, every 4 w • Result (Proportion of patients with clinical response; Arm A vs. Arm B): [Non-treatment induced cohort: 74 (0.32) vs. 75 (0.27)], [Treatment induced cohort: 24 (0.46) vs. 23 (0.17)], [SAE: 92/99 (93%) vs. 93/98 (95%)]	
Colorectal cancer	Phase 2	2010, completed	Study of azacytidine in combination with entinostat in patients with metastatic colorectal cancer, for determining (i) ORR (ii) TTP, and (iii) toxicity of the combination; • Azacytidine: 40 mg/m^2, *s.c.*, 1–5 d and 8–10 d + Entinostat: 7 mg, *p.o.*, d 3, d 10, every 4 w • Result: ORR: 0/22 (0%), TTP: 1.9 months, SAE: 5/22 (23%)	NCT01105377
Breast cancer	Phase 2	2011, Active	Study of azacytidine in combination with entinostat in patients with advanced breast cancer, for determining (i) ORR (ii) safety and tolerability, and (iii) PFS, OS, and clinical benefit rate of the combination; • Azacytidine: 40 mg/m^2, *s.c.*, 1–5 d and 8–10 d + Entinostat: 7 mg, *p.o.*, d 3, d 10, every 4 w • Result: ORR: 4%, OS: 6.6 months, PFS: 1.4 months, SAE: 2/40 (5%)	NCT01349959
MDS, AML	Phase 2	2005, Completed	Study of azacytidine in combination with valproic acid and all-trans retinoic acid in patients with high-risk MDS and AML, for determining (i) MTD of valproic acid in combination (ii) the safety and effectiveness of the combination therapy, and (iii) the in vivo molecular and biological effects of the combination such as analysis of changes in DNA methylation, histone modifications, and gene expression; • Azacytidine: 75 mg/m^2, *s.c.*, daily, 1–7 d + Valproic Acid: 50–62.5-75 mg/kg, *p.o.*, daily, 1–7 d + All-Trans Retinoic Acid: 45 mg/m^2, *p.o.*, daily (in two divided doses), 3–7 d, every 3 w • Result: ORR: 22/34 (65%), SAE: 31/34 (91%)	NCT00326170 (Soriano et al., 2007)
AML, MDS	Phase 2	2005, Completed	Randomized study of azacytidine in combination with valproic acid versus low-dose cytarabine in older patients ≥60 years with untreated AML or high-risk MDS not eligible for other therapies, for determining (i) EFS of either therapies, and (ii) to determine if the ability of azacytidine + valproic acid combination to induce demethylation or acetylation correlates with response; • Arm 1: Azacytidine: 75 mg/m^2, *s.c.*, daily, 1–7 d + Valproic Acid: 50 mg/m^2, *p.o.*, daily, 1–7 d, every 4–6 w • Arm 2: Cytarabine: 20 mg, *s.c.*, twice daily, 1–10 d, every 4–6 w • Result (Arm 1 vs. Arm 2): ORR: 0/4 (0%) vs. 0/5 (0%), SAE: 4/4 (100%) vs. 6/6 (100%)	NCT00382590
Solid tumors	Phase 1	2007, Completed	Study of the safety and effectiveness of azacytidine in combination with carboplatin and valproic acid in patients with advanced solid tumors; • Azacytidine: 75 mg/m^2, *s.c.* or *i.v.*, daily, 1–5 d + Valproic Acid: 20–50 mg/kg, *p.o.*, daily, 5–11 d + Carboplatin: AUC 2–3, *i.v.*, d 3, d10 (not given in cycle 1), every 4 w • Result: MTD: 75 mg/m^2 (Azacytidine) + 20 mg/kg (Valproic Acid) + AUC 3.0 (Carboplatin), DLT: 6/32 (19%), Minor response or stable disease lasting ≥4 months: 6/32 (19%)	NCT00529022 (Falchook et al., 2013)
AML, MDS	Phase 2	2009, Active	Randomized study of the safety and effectiveness of azacytidine in combination with vorinostat as compared to azacytidine alone in patients with newly-diagnosed AML or MDS who are ineligible for other leukemia protocols; • Azacytidine: 75 mg/m^2/day, *i.v.*, 15–30 min, daily, 1–5 d, w/ or w/o Vorinostat: 200 mg, *p.o.*, thrice daily, 1–5 d, every 3–8 w • Result (Azacytidine vs. Azacytidine + Vorinostat): CR: 8/26 (31%) vs. 11/51 (22%), Survival-60 days: 18/27 (67%) vs. 43/51 (84%), SAE: 18/27 (67%) vs. 36/52 (69%)	NCT00948064
AML	Phase 1/2	2009, Completed	Non-randomized dose-escalation study of vorinostat in combination with azacytidine and gemtuzumab ozogamicin in older patients ≥50 years with relapsed or refractory AML, for determining (i) MTD and DLT of vorinostat in combination therapy (ii) CR and DFS, and (iii) in vitro correlative and mechanistic studies; • Phase 1: Azacytidine: 75 mg/m^2/day, *s.c.* or *i.v.*, 10–40 min, daily, 1–7 d + Vorinostat: 200–300-400 mg/day, *p.o.*, daily, 1–9 d + Gemtuzumab Ozogamicin: 3 mg/m^2/day, *i.v.*, 2 h, d 8 or d 4 and d 8, every 3 w • Phase 2 (MTD defined in phase 1): Azacytidine: 75 mg/m^2/day, 1–7 d + Vorinostat: 400 mg/day, 1–9 d + Gemtuzumab Ozogamicin: 3 mg/m^2/day, d 4, d 8 • Result (Phase 1 and Phase 2): CR: 4/9 (44%) and 18/43 (42%), SAE: 6/10 (60%) and 13/43 (30%)	NCT00895934 (Walter et al., 2014)
MDS, CMML	Phase 2	2012, Active	Randomized study of azacytidine alone or in combination with lenalidomide or vorinostat in patients with higher-risk MDS and CMML, for determining (i) ORR for azacytidine alone versus combinations (ii) OS, RFS, cytogenetic response rate, and toxicity profile of each regimen, and (iii) association of cytogenetic abnormalities with clinical outcomes; • Arm 1: Azacytidine: *s.c.* or *i.v.*, 1–7 d or 1–5 d and 8–9 d, every 4 w • Arm 2: Azacytidine (Arm 1) + Lenalidomide: *p.o.*, daily, 1–21 d, every 4 w • Arm 3: Azacytidine (Arm 1) + Vorinostat: *p.o.*, twice daily, 3–9 d, every 4 w • Result (Arm 1 vs. Arm 2 vs. Arm 3): ORR: 35/92 (38%) vs. 46/93 (49%) vs. 25/92 (27%), OS: 15.0 months vs. 19.6 months vs. 17.6 months, RFS: 10.4 months vs. 14.5 months vs. 15.2 months, SAE: 8/91 (9%) vs. 37/89 (42%) vs. 47/91 (52%)	NCT01522976
➢ Immunomodulatory agents: Lenalidomide, gemtuzumab ozogamicin, lintuzumab, filgrastim (+darbepoetin alfa)				
MDS	Phase 1/2	2006, Completed	Study of azacytidine in combination with lenalidomide in patients with advanced MDS, for determining (i) MTD and DLT of the combination (ii) ORR (iii) TTP to AML or death (iv) DOR, and (v) to determine the effect of this regimen on hematologic status;	NCT00352001 (Sekeres et al., 2012)

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Table 5 (*continued*)

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
			• Azacytidine: 75 mg/m^2/day, *s.c.*, daily, 1–5 d or 1–5 and 8–12 d + Lenalidomide: 10 mg/day, *p.o.*, daily, 1–14 or 1–21 d, every 4 w • Result: ORR: 26/36 (72%), DOR: 17.0 months, OS: 13.6 months	
AML	Phase 1/2	2009, Completed	Study of azacytidine in combination with lenalidomide in older patients with previously untreated AML, for determining MTD of lenalidomide administered after azacytidine in phase 1, and the effectiveness of the combination treatment in phase 2; • Azacytidine: 75 mg/m^2, *s.c.* or *i.v.*, daily, 1–7 d + Lenalidomide: 5–10–25-50 mg, *p.o.*, daily, 8–28 d, every 4 w • Result: MTD of lenalidomide: 50 mg, CR: 12/43 (28%), ORR: 18/43 (42%), DOR: 1.4 months, Survival-4 weeks: 84%, OS: 4.7 months, SAE: 36/43 (84%)	NCT00890929 (Pollyea et al., 2012; Pollyea et al., 2013)
MDS, AML	Phase 1/2	2009, Completed	Study of azacytidine in combination with lenalidomide in patients with high-risk MDS and AML, for determining (i) MTD of lenalidomide in combination with azacytidine, and (ii) safety and effectiveness of the combination; • Azacytidine: 75 mg/m^2/day, *i.v.*, 15–30 min, daily, 1–5 d + Lenalidomide: 10–15–20-25-50-75 mg, *p.o.*, daily, 6–10 or 6–15 d, every 3–8 w • Result: MTD of lenalidomide: 25 mg for 5 days, CR: 31/88 (35%), ORR: 27/60 (45%), SAE: 21/40 (53%)	NCT01038635 (DiNardo et al., 2015)
Multiple myeloma	–	2010, Completed	Pilot study of autologous lymphocyte (ALI) mobilization following immuno-modulatory therapy comprising azacytidine and lenalidomide in multiple myeloma, for determining (i) the feasibility of mobilizing and infusing ALI following immuno-modulatory therapy (ii) the ability to proceed with autologous stem cell transplantation in these patients (iii) CR, OS, PFS, TTP, and toxicity profile following transplant in patients treated with this regimen (iv) pre- and post-ALI immune response to cancer testis antigens (CTA), and (v) the expression of CTA in multiple myeloma before and after azacytidine therapy; • Azacytidine: 75 mg/m^2, *s.c.*, daily, 1–5 d + Lenalidomide: 15 mg, *p.o.*, daily, 6–21 d, every 4 w • Result: CR-6 months: 50%, OS-1 year: 93.3%, OS-2 year: 86.1%, PFS-1 year: 87.5%, PFS-2 year: 67.3%, TTP: 14.9 months, CTA-specific T cell response: 3/17 (18%), CTA up-regulation: 6/17 (35%), SAE: 9/17 (53%)	NCT01050790
AML	Phase 1/2	2010, Completed	Non-randomized study of azacytidine in combination with lenalidomide in AML, for determining (i) toxicity and feasibility of the combination in patients with relapsed or refractory AML ≥ 18 years or untreated AML ≥ 60 years in phase 1 (ii) CR and DOR (iii) ORR, OS, EFS, RFS, and TTP in untreated AML ≥ 60 years, and (iv) toxicity profile of the combination in phase 2; • Induction regimen: Azacytidine: 25–50-75 mg/m^2, *i.v.*, daily, 1–5 d + Lenalidomide: 50 mg, *p.o.*, daily, 1–28 d • Maintenance regimen: Azacytidine: 75 mg/m^2, *i.v.*, daily, 1–5 d + Lenalidomide: 10 mg, *p.o.*, daily, 1–28 d • Result: MTD of azacytidine: 75 mg/m^2, CR: 2/9 (22%), DOR: 12.2 months, ORR: 7/9 (78%), OS: 4.3 months, EFS: 2.9 months, RFS: 12.2 months, TTP: 3.7 months, SAE: 12/12 (100%)	NCT01016600
Lymphoma	Phase 2	2010, Terminated	Non-randomized study of the safety and effectiveness of azacytidine in combination with lenalidomide in patients with relapsed or refractory follicular or marginal zone lymphoma; • Azacytidine: 75 mg/m^2, *s.c.* or *i.v.*, daily, 1–5 d + Lenalidomide: 15 mg, *p.o.*, daily, 1–21 d, every 4 w; Arm 1: Azacytidine followed by lenalidomide, Arm 2: lenalidomide followed by azacytidine • Result (Arm 1 vs. Arm 2): ORR: 2/4 (50%) vs. 0/1 (0%), SAE: 2/6 (33%) vs. 2/3 (67%)	NCT01121757
AML	Phase 2	2012, Active	Randomized study for comparing the safety and effectiveness of three different regimens (i) high-dose lenalidomide (ii) lenalidomide + azacytidine, and (iii) azacytidine in older patients ≥65 years with newly-diagnosed AML; • Regimen A: Lenalidomide: 50 mg (cycle 1, 2) – 25 mg (cycle 3, 4) – 10 mg (remaining cycles), *p.o.*, daily for 4 w + BSC • Regimen B: Azacytidine: 75 mg/m^2/day, *s.c.*, daily, 1–7 d + Lenalidomide: 50 mg, *p.o.*, daily, 8–28 d followed by a 14-days break + BSC • Regimen C: Azacytidine: 75 mg/m^2/day, *s.c.*, daily, 1–7 d followed by a 21-days break + BSC • Result (Regimen A vs. Regimen B vs. Regimen C): Survival-1 year: 3 months vs. 9.6 months vs. 13.7 months, SAE: 13/14 (93%) vs. 29/38 (76%) vs. 25/32 (78%)	NCT01358734
AML	Phase 2	2008, Active	Study of azacytidine in combination with gemtuzumab ozogamicin as induction and post-remission therapy in older patients ≥60 years with previously untreated non-M3 AML, for determining (i) Phase 3 trial justification based on outcomes (ii) toxicity profile in good- and poor-risk patients (iii) DFS and cytogenetic response rate, and (iv) the effects of cytogenetic abnormalities, promoter and global methylation changes, and multidrug resistance on OS and response to the combination therapy; • Remission induction therapy: Azacytidine: 75 mg/m^2, *s.c.* or *i.v.*, 10–40 min, daily, 1–7 d + Gemtuzumab Ozogamicin: 3 mg/m^2, *i.v.*, 2 h, d 8 • Consolidation therapy: Azacytidine: 75 mg/m^2, *s.c.*, daily, 1–7 d + Gemtuzumab Ozogamicin: 3 mg/m^2, *i.v.*, 2 h, d 8 • Maintenance therapy: 75 mg/m^2, *s.c.*, daily, 1–7 d, every 4 w • Result [Good-risk vs. Poor-risk patients: CR: 35/79 (44%) vs. 19/54 (35%), Survival-30 days: 92% vs. 87%, RFS: 8 months vs. 7 months], [SAE: Remission induction therapy: 55/133 (41%), Consolidation therapy: 1/32 (3%), Maintenance therapy: 3/27 (11%)]	NCT00658814 (Nand et al., 2013)
MDS	Phase 2	2009, Terminated	Study of azacytidine in combination with lintuzumab in patients with previously untreated MDS, for determining (i) CR, ORR, and toxicity profile of the combination regimen (ii) the correlation between pre-treatment and drug-induced changes in expression of *Syk* and clinical response (iii) biological activity of	NCT00997243

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Table 5 (*continued*)

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
			azacytidine as demethylating agent (iv) exploratory studies of azacytidine-triphosphate with global DNA methylation, and (vi) the biologic role of miRNA in determining clinical response and other PD endpoints; • Azacytidine: 75 mg/m^2, *s.c.* or *i.v.*, daily, 1–7 d + Lintuzumab: 600 mg, *i.v.*, d 2, d 7, d 15, d 22, every 4 w (cycle 1) – d 7, d 22, every 4 w (subsequent cycles) • Result: CR: 1/7 (14%), ORR: 1/7 (14%), SAE: 7/7 (100%)	
MDS	Phase 2	2006, Terminated	Non-randomized study of azacytidine in combination with hematopoietic growth factors, darbepoetin alfa and filgrastim, for determining (i) the hematological response rate (ii) time to progression to AML or death (iii) OS and PFS, and (iv) changes in apoptotic index of bone marrow in patients treated with this regimen; • Azacytidine: 100 or 125 mg/m^2, *s.c.*, daily, 1–5 d, every 4 w + Filgrastim: 300 μg (weight < 100 kg) or 450 μg (weight ≥ 100 kg), *s.c.*, thrice weekly, w 2–4 + Darbepoetin Alfa: 500 μg, *s.c.*, d 8, every 4 w • Result: CR: 0/3 (0%), SAE: 1/3 (33%)	NCT00398047
MDS	Phase 2	2012, Terminated	Study of the safety and effectiveness of azacytidine in combination with filgrastim in patients with low- or intermediate-1- risk MDS; • Azacytidine: 40 mg/m^2, *s.c.* or *i.v.*, 15–30 min, daily, 1–4 d + Filgrastim: 250 μg/m^2, *s.c.* or *i.v.*, 15 min, daily, 5–7 d, every 4–6 w • Result: ORR: 0/8 (0%), SAE: 3/8 (38%)	NCT01542684
➢ Targeted therapies: Ilorasertib, sorafenib, midostaurin (+deferasirox)				
AML, MDS, CMML	Phase 1	2010, Completed	Non-randomized study for determining the safety, PK, and MTD of ilorasertib as monotherapy and in combination with azacytidine in patients with advanced hematologic malignancies; • Arm 1: Ilorasertib: 10–690 mg, *p.o.*, once weekly, d 1, d 8, d 15, every 4 w • Arm 2: Ilorasertib: 320 or 480 mg, *p.o.*, twice weekly, d 1, d2, d 8, d 9, d 15, d 16, every 4 w • Arm 3: Ilorasertib: 440 mg, *p.o.*, once weekly, d 1, d 8, d 15, every 4 w + Azacytidine: *s.c.* or *i.v.*, daily, 1–7 d, every 4 w • Arm 4: Ilorasertib: 32 mg (starting dose), *i.v.*, once weekly, d 1, d 8, d 15, every 4 w • Result: MTD: not determined, Recommended phase 2 oral monotherapy dose: 540 mg once weekly or 480 mg twice weekly, Half-life of oral Ilorasertib: 15 h, ORR (Arm 1, 2): 3/52 (6%), TTP: 1.8 months	NCT01110473 (Garcia-Manero et al., 2015)
AML, MDS	Phase 1/2	2011, Completed	Study of the safety and effectiveness of azacytidine in combination with sorafenib in patients with relapsed or refractory AML and MDS; • Azacytidine: 75 mg/m^2, *s.c.* or *i.v.*, 10–40 min, daily, 1–7 d, every 4 w + Sorafenib: 200–400 mg, *p.o.*, twice daily, continuously, 12 h apart • Result: MTD of sorafenib: 400 mg, ORR: 25/48 (52%), SAE: 0/57 (0%)	NCT01254890 (Ravandi et al., 2013)
AML, MDS	Phase 1/2	2011, Completed	Study of the safety and effectiveness of azacytidine in combination with midostaurin in patients with relapsed or refractory AML and MDS; • Azacytidine: 75 mg/m^2/day, *s.c.* or *i.v.*, 30 min, daily, 1–7 d, every 4 w + Midostaurin: 25 or 50 mg, *p.o.*, twice daily, 8–21 d, every 4 w (cycle 1) – daily continuously, as of cycle 2 • Result: MTD of midostaurin: 50 mg, ORR: 14/54 (26%), SAE: Phase 1 (Azacytidine +25 mg Midostaurin): 5/6 (83%), Phase 1 (Azacytidine + 50 mg Midostaurin): 5/8 (63%), Phase 2: 29/40 (73%)	NCT01202877
MDS	Phase 2	2014, Terminated	Randomized study for determining the ORR in patients with higher-risk MDS treated with azacytidine alone or in combination with deferasirox; • Azacytidine: 75 mg/m^2, *s.c.*, daily, 1–7 d, every 4 w, w/ or w/o Deferasirox: 10 mg/kg/day • Result: SAE: 1/1 (100%)	NCT02159040

demethylation activity of SGI-110 was also confirmed in vivo by its ability to reduce DNA methylation and induce *p16* expression in human bladder cancer xenografts. Also, SGI-110 retarded tumor growth of xenografts by both *i.p.* and *s.c.* deliveries, and offered less toxicity in tumor-free nude mice as compared to decitabine (Chuang et al., 2010). The *s.c.* administered SGI-110 further showed anti-tumor efficacy in human hepatocellular carcinoma xenografts by impeding tumor growth and inhibiting angiogenesis. The study also demonstrated the pronounced demethylation effects of SGI-110 in a subset of TSGs, *CDKN2A*, *DLEC1*, and *RUNX3*. (Jueliger et al., 2016). In another study conducted in ovarian cancer, SGI-110 proved its demethylation potential by inducing expression of significant epithelial-mesenchymal transition genes silenced by hypermethylation (Cardenas et al., 2014). The demethylation activity of SGI-110 was also evidenced in primates (Lavelle et al., 2010). Furthermore, studies investigating the immunomodulatory potential of SGI-110 revealed that SGI-110 improved the immunogenic potential and immune recognition of treated cancer cells. It was demonstrated in wide variety of human cancer cell lines that SGI-110 treated neoplastic cells showed induced expression of various methylated cancer-testis antigen genes by promoter demethylation, up-regulated expression of HLA class I antigens, MHC class I, and co-stimulatory molecule expression in a dose-dependent manner, both in vitro and in vivo (Coral et al., 2013; Srivastava et al., 2014, 2015). Moreover, the immunomodulatory potential of SGI-110 was found to be significantly higher than azacytidine or decitabine (Srivastava et al., 2014). These key findings emphasize towards the clinical application of SGI-110 in cancer immunotherapies and provide a strong rationale for the development of novel anti-cancer chemo-immunotherapies, utilizing SGI-110 in combination with immunotherapeutic drugs. After encouraging demethylation and anti-tumor effects in pre-clinical models, SGI-110 entered into clinical trial phases. At first, a multicenter randomized dose escalation Phase 1study of SGI-110 formulated as low volume and pharmaceutically stable *s.c.* injections was conducted in previously treated relapsed or refractory, intermediate or high-risk MDS or AML patients. The results of the study demonstrated the drastic increase in therapeutic exposure window (beyond 8 h) compared to decitabine *i.v.* infusions (3–4 h) and prolonged half-life (~2.4 h) which is 4 -fold higher than achieved by *i.v.* administered decitabine (Issa et al., 2015). The clinical response was observed in 31% of MDS

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patients and 8% of AML patients, and DNA demethylation was confirmed as PD marker for clinical response. The most common grade ≥ 3 adverse events were febrile neutropenia, pneumonia, thrombocytopenia, anemia, and sepsis and of which febrile neutropenia, pneumonia, and sepsis were recorded as serious adverse events (Issa et al., 2015). Two dose-limiting toxicities were observed for MDS at 125 mg/m^2 daily × 5, and MTD for MDS was established as 90 mg/m^2 daily × 5, however, MTD was not attained for AML patients. Notably, optimal biologically effective dose (BED) of SGI-110, 60 mg/m^2 daily × 5 was lower than MTD of the drug in either case (Issa et al., 2015). The outcomes of the study warranted further phase 2 trials with the recommended dose of 60 mg/m^2 daily × 5. Presently, SGI-110 is being evaluated in phase 1/2 and/or phase 3 clinical trials in MDS/AML and in phase 1/2 clinical trials for various solid tumors. The complete list of ongoing clinical studies can be found at https://clinicaltrials.gov/ct2/results?term=SGI-110&Search=Search. Apart from its clinical progress as a single agent in leukemia, SGI-110 has also gained significant interest in combinatorial therapies and as a priming agent in solid tumors (Table 8).

6.3. NPEOC-DAC

NPEOC-DAC or 2′-deoxy-N4-[2-(4-nitrophenyl)ethoxycarbonyl]-5-azacytidine is another analog of DAC that was developed to circumvent the metabolic instability of the drug. NPEOC-DAC was synthesized by binding 2-(*p*-nitrophenyl)ethoxycarbonyl at the N4 position of the azacytidine ring (Fig. 4). This modification at N4 position protects the exocyclic amine of DAC from deamination by plasma CDA, rendering increased plasma half-life to the drug (Byun et al., 2008). In addition, unlike DAC, NPEOC-DAC is highly hydrophobic with very low aqueous solubility which further improves the PK profile of the drug, including oral bioavailability. The orally available mechanistic inhibitor of DNMT thus allows for continuous drug administration, adding to its clinical effectiveness (Byun et al., 2008). The demethylation activity of the pro-drug was demonstrated by the ability of NPEOC-DAC to significantly decrease global DNA methylation, reverse hypermethylation and reactivate expression of TSG, *ID4*. The DNA demethylation ability was found to be specific for the liver cancer cell lines and dependent on the activity of the carboxylesterase 1 (CES1) enzyme (Byun et al., 2008). While NPEOC-DAC at doses ≥10 μM was comparatively more effective at inhibiting DNA methylation, the potency of the pro-drug to inhibit DNA methylation at low doses (<10 μM) was found to be significantly lower than DAC. Besides, a 3-day delay in the effect of NPEOC-DAC was also reported, along with less toxicity than observed with DAC. It is assumed that the low potency and the delayed effect could result from the inefficient or slow conversion of NPEOC-DAC to active drug, DAC. The fact that NPEOC-DAC is dependent on the activity of CES1 enzyme for its metabolization to DAC has limited the development of the prodrug because expression of CES1 is variable in different tissues and also may not be 100% efficient in converting NPEOC-DAC to DAC (Byun et al., 2008). Nevertheless, it is speculated that substitution of N4-NPEOC group of NPEOC-DAC with a smaller carbon chain may lead to a molecule which can inhibit DNA methylation much more efficiently. Furthermore, the prodrug NPEOC-DAC facilitates the attachment of another epigenetic agent such as histone deacetylase inhibitors at the N4 position, and release two active agents on cleavage of the carboxylester bond, thereby extending the possibility of combined epigenetic therapy (Byun et al., 2008).

6.4. CP-4200

CP-4200 or 5-azacytidine-5′-elaidate (Clavis Pharma, ASA, Oslo, Norway) is a 5-azacytidine variant with modified chemical properties, currently in the pre-clinical research phase for MDS. The pro-drug is essentially an elaidic acid ester analog developed by conjugating azacytidine molecule with a fatty acid, elaidic acid (Fig. 4). CP-4200 was designed to decrease the drug dependency on conventional nucleoside transporters involved in azacytidine uptake and to overcome transport-related drug resistance (Brueckner et al., 2010). An extensive study characterizing the mode of action and therapeutic efficacy of CP-4200 was conducted in a panel of human cancer cell lines. The results of the study proved that cellular uptake mechanism of CP-4200 was fundamentally different from that of azacytidine. Also, it was shown that despite extensive chemical modification CP-4200 retained its epigenetic potency. This was well evident by a significant depletion of DNMT protein, genome-wide DNA demethylation, and widespread DNA demethylation of hypermethylated markers causing robust reactivation of epigenetically silenced TSGs, *TIMP-3,* and *DAPK-1* in colon cancer and leukemia cells, respectively. Importantly, during in vivo study conducted in orthotopic ALL mouse tumor model, *i.v.* or *i.p.* administered CP-4200 demonstrated significantly higher anti-tumoral activity compared with equitoxic doses of azacytidine (Brueckner et al., 2010). Furthermore, it was shown during a study that inhibition of human equilibrative nucleoside transporter 1 (hENT1) resulted in strong abolishment of cytotoxic and demethylation drug effects of azacytidine, however, CP-4200 effectively retained its cellular activity, thereby explaining its effectiveness in overcoming hENT-related resistance (Hummel-Eisenbeiss et al., 2013). Thus, pre-clinical studies which evidenced the low dependence of CP-4200 on nucleoside transporters combined with increased epigenetic potential have marked the pro-drug as an intriguing candidate for epigenetic cancer therapy. Currently, further pre-clinical studies are ongoing but clinical trials have not been initiated yet.

6.5. 2′3′5′Triacetyl-5-azacytidine

2′3′5′Triacetyl-5-azacytidine (TAC) is another potential pro-drug of 5-azacytidine with improved PK profile over parent drug. Structurally, TAC is an acetylated derivative of AZA, synthesized by condensation of trimethylsilylated-5-azacytosine and 1,2,3,5-tetra-*O*-acetyl-β-*D*-ribofuranose, Fig. 4 (Ziemba, Hayes, Freeman, Ye, & Pizzorno, 2011). During in vitro and in vivo characterization, TAC demonstrated favorable physio-chemical characteristics in contrast to its parent compound. In vitro, TAC showed higher solubility and stability across a wide range of pH which confirmed efficient drug absorption in the gastrointestinal tract, and increased bioavailability over AZA which is rapidly degraded in an acidic environment (Ziemba et al., 2011). In vivo, the terminal phase half-life (9.2 h vs. 6.8 h) and the alpha phase half-life (0.73 h vs. 0.32 h) of *p.o.* administered TAC was longer than *i.v.* administered AZA, respectively (Ziemba et al., 2011). However, during in vitro assessment of anti-proliferative and demethylation effects, TAC showed no cellular toxicity in leukemia cells and less effect on methylation level of *P15INK4B* as compared to AZA. The reduced in vitro efficacy is predicted to be due to lack of necessary esterase activity in cultured cells, required for conversion and activation of pro-drug to AZA (Ziemba et al., 2011). The analysis of the anti-leukemic activity of *p.o.* administered TAC in human lymphocytic leukemia animal model demonstrated significantly increased survival time with minimal general toxicity, but it was less effective than AZA at improving lifespan. The less effectivity may be due to higher C_{max} achieved by *i.p.* administered AZA in comparison to *p.o.* administered TAC. While the ability of *p.o.* TAC to suppress global methylation was comparable with *i.v.* AZA, further studies are required to confirm the demethylation efficacy of TAC in cancer cells (Ziemba et al., 2011). Altogether, higher solubility, stability, and bioavailability combined with minimal toxicity encourage further pre-clinical investigation of its mechanism of action, epigenetic modulatory effect, and possible clinical evaluation to establish it as an effective pro-drug for AZA.

7. Mechanisms of drug resistance to azanucleosides

Drug resistance to AZN is an ongoing intractable problem which accounts for limited success and durability of AZN-based therapy. The failure of treatment with AZN drugs can be divided into two broad categories: primary resistance in which case patients do not show a response

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Table 6
Decitabine in combinatorial therapies
This table represents all registered clinical trials of decitabine in combination with various chemotherapeutic, epigenetic or immunomodulatory agents for which study results have been posted or are available as publications.

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
➢ **Standard chemotherapy drugs: Arsenic trioxide, carboplatin, clofarabine, cytarabine, vincristine sulfate, doxorubicin hydrochloride, PEG asparaginase, methotrexate (+ascorbic acid, filgrastim, aclacinomycin, omacetaxine mepesuccinate, vorinostat, prednisone, imatinib mesylate, cytokine-induced killer cells)**				
MDS	Phase 2	2007, Completed	Non-randomized pilot study of decitabine in combination with As_2O_3 and ascorbic acid in MDS for determining the safety of the combination; • Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4 w + As_2O_3: 0.25 mg/kg, *i.v.*, daily, 1–5 d for cycle 1(4w) followed by 0.25 mg/kg, twice weekly (Mon-Thu or Tue-Fri) for all remaining cycles + Ascorbic Acid: 1000 mg in 100 mL solution of 5% dextrose in water, *i.v.*, 15–30 min, administered within 30 min of As_2O_3 administration • Result: ORR: 0/6 (0%), SAE: 4/6 (67%)	NCT00621023
MDS, AML	Phase 1	2008, Completed	Non-randomized study of the safety and effectiveness of decitabine in combination with As_2O_3 and ascorbic acid in order to improve response rate in patients with de novo or secondary MDS and AML; • Decitabine: 20 mg/m^2/day, *i.v.*, daily, 1–5 d, every 4 w + As_2O_3: 0.1 mg/kg/day, *i.v.*, daily, 1–5 d followed by 0.1 mg/kg, *i.v.*, weekly or: 0.2 mg/kg/day, *i.v.*, daily, 1–5 d followed by 0.2 mg/kg, *i.v.*, weekly or: 0.3 mg/kg/day, *i.v.*, daily, 1–5 d followed by 0.3 mg/kg, *i.v.*, weekly + Ascorbic Acid: 1000 mg, *i.v.*, following each dose of As_2O_3 • Result: MTD of As_2O_3 in combination: 0.2 mg/kg, CR: 1/13 (8%)	NCT00671697 (Welch et al., 2011)
Ovarian cancer	Phase 1/2	2007, Completed	Study of decitabine as a sensitizer to carboplatin in patients with platinum refractory or platinum resistant recurrent ovarian cancer, for determining the safety and biologic activity of the combination; • Decitabine: 10 mg/m^2 (dose level 1) - 20 mg/m^2 (dose level 2), *i.v.*, 1 h daily, 1–5 d + Carboplatin: Dose ~ AUC 5, *i.v.*, 30 min, d 8, every 4 w • Result: MTD: 10 mg/m^2, ORR: 6/17 (35%), PFS: 10.2 months, SAE: Phase 1: 3/11 (27%), Phase 2: 4/17 (24%)	NCT00477386 (Fang et al., 2010; Matei et al., 2012)
AML, MDS	Phase 2	2008, Completed	Study of the safety and effectiveness of clofarabine in combination with low-dose cytarabine and decitabine in older patients ≥60 years with AML or high-risk MDS; • Clofarabine: 20 mg/m^2, *i.v.*, 1–2 h daily, 1–5 d + Cytarabine: 20 mg, *s.c.*, twice daily, 1–10 d, administered 3–6 h following the start of the clofarabine infusions + Decitabine: 20 mg/m^2, *i.v.*, 1–2 h daily, 1–5 d • Result: ORR: 73/118 (62%), OS: 11.1 months, DFS: 15.9 months, EFS: 7.7 months, SAE: 12/119 (10%)	NCT00778375
AML, MDS	Phase 2	2008, Terminated	Non-randomized study of the feasibility and toxicity of decitabine in combination with low-dose cytarabine and filgrastim in patients with high-risk MDS, refractory AML or AML patients with significant co-morbidities; • Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d + Cytarabine: 20 mg/m^2, *s.c.*, daily, 1–5 d + Filgrastim: 5 μg/kg, *s.c.*, daily, 1–5 d • Result: ORR: 1/9 (11%), SAE: 8/9 (89%)	NCT00740181
MDS, AML	Phase 1/2	2012, Completed	Study of the effectiveness of decitabine-based chemotherapy followed by haploidentical lymphocyte infusion (HLI) in elderly patients with intermediate-high risk MDS or AML; • Decitabine: 20 mg/m^2, *i.v.*, daily, 1–5 d + Aclacinomycin: 20 mg, *i.v.*, every second day for 5 days + Cytarabine: 10 mg/m^2, *s.c.*, every 12 h for 5 days + Filgrastim: 300 μg/day, *s.c.*, from day 0 to neutrophil recovery, every 4 w + HLI (36 h after the last dose of chemotherapy) • Result: CR: 21/29 (72%), OS-1 year: 72.2%, OS-2 year: 59.6%, DFS-1 year: 47.3%, DFS-2 year: 36.9%	NCT01690507 (Jing et al., 2016)
AML	Phase 2	2014, Terminated	Study of decitabine in combination with OAG (cytarabine, omacetaxine mepesuccinate) in older patients ≥65 years with newly diagnosed AML who are ineligible for intensive induction therapy, for determining (i) CR (ii) toxicity, and (iii) DFS and OS of these regimens; • Induction chemotherapy: OAG: *s.c.*, twice daily, 1–14 d, every 4 w • Consolidation therapy (alternative courses between decitabine and OAG): Decitabine: *i.v.*, daily, 1–5 d, every 4 w; OAG: *s.c.*, twice daily, 1–7 d, every 4 w • Result: SAE: 2/2 (100%)	NCT02029417
ALL, Lymphoblastic lymphoma	Phase 2	2009, Terminated	Study of the effectiveness of decitabine and vorinostat in combination with chemotherapy in patients with relapsed or refractory acute lymphoblastic leukemia or lymphoblastic lymphoma; • Decitabine: 15 mg/m^2, *i.v.*, 1 h daily, 1–4 d + vorinostat: 230 mg/m^2, *p.o.*, divided twice (max dose 400 mg daily), 1–4 d + prednisone: 40 mg/m^2/day, *p.o.*, divided twice, 5–33 d + vincristine sulfate: 1.5 mg/m^2 (max 2 mg), *i.v.*, d 5, d 12, d 19, d 26 + doxorubicin hydrochloride: 60 mg/m^2, *i.v.*, 15 min, d 5 + PEG asparaginase: 2500	NCT00882206 (Burke et al., 2014)

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Table 6 (*continued*)

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
			IU/m^2, *i.m.* or *i.v.*, d 6, d 12, d 19, d 26 + cytarabine: 30–70 mg (depending upon age), *i.t.*, d 5 + methotrexate: 8–15 mg (depending upon age), i.t., d 12, d 33 + imatinib mesylate (for patients with Philadelphia chromosome-positive disease): 340 mg/m^2 (age < 18 years) and 400 mg (age > 18 years), *p.o.*, daily, 5–33 d • Result: ORR: 6/8 (75%), SAE: 8/13 (62%)	
Solid tumors, Lymphoma	Phase 1/2	2012, Recruiting	Study of the safety and effectiveness of low-dose decitabine alone or in combination with chemotherapy and/or autologous cytokine-induced killer cells (CIK) in patients with relapsed or refractory solid tumors and B cell lymphomas; • Decitabine: 7 mg/m^2, *i.v.*, daily, 1–5 d, every 4 w or Decitabine + chemotherapy or decitabine + CIK: 1–5 × 10^9/L for two days in 4 w cycle • Result (decitabine vs. decitabine + chemotherapy vs. decitabine + CIK; 6 cycles): ORR: 1/2 (50%) vs. 7/11 (64%) vs. 4/5 (80%)	NCT01799083 (Fan et al., 2014)
➢ **Histone deacetylase inhibitors: Valproic acid, vorinostat, panobinostat (+Temozolomide)**				
Leukemia, MDS	Phase 1/2	2004, Completed	Study of decitabine in combination with valproic acid in patients with relapsed or refractory leukemia or MDS, for determining the MTD of the valproic acid in combination; • Decitabine: 15 mg/m^2, *i.v.*, 1 h daily, 1–10 d, w/ or w/o Valproic Acid: 20–35-50 mg/kg, *p.o.*, daily, 1–10 d • Result: MTD: 50 mg/kg, ORR: 12/53 (22%), DFS: 5.6 months, OS: 6 months	NCT00075010 (Garcia-Manero et al., 2006)
AML, Chronic lymphocytic leukemia, Small lymphocytic lymphoma	Phase 1	2004, Completed	Study of decitabine in combination with valproic acid in patients with relapsed or refractory AML or previously treated chronic lymphocytic leukemia or small lymphocytic lymphoma, for determining (i) BED of decitabine (ii) MTD and BED of valproic acid in combination with BED of decitabine (iii) toxic effects and therapeutic response of decitabine alone and in combination with valproic acid (iv) PK of the combined regimen (v) kinetics of DNMTs and re-expression of selected methylated genes, and histone deacetylase enzyme inhibition and changes in the acetylation status of histones, and (vi) correlate baseline and post-treatment changes in DNMTs expression and in histone code with disease response in these patients; • Decitabine: 15–20 mg/m^2/day, *i.v.*, 1 h daily, 1–5 or 1–10 d, every 4 w, w/ or w/o Valproic Acid: 15–20-25 mg/kg, *p.o.*, thrice daily, 5–21 d, every 4 w • Result: BED of decitabine: 20 mg/m^2/d (1–10 d), MTD: Decitabine; 20 mg/m^2/d (1–10 d) + Valproic Acid; 20 mg/kg/d (5–21 d), ORR: 11/21 (52%)	NCT00079378 (Blum et al., 2007)
MDS, AML	Phase 2	2006, Completed	Randomized study for determining the safety and effectiveness of low-dose decitabine with or without valproic acid in MDS or AML; • Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4–8 w, w/ or w/o valproic acid: 50 mg/kg, *p.o.*, daily, 1–7 d, every 4–8 w • Result (decitabine vs. decitabine + valproic acid): ORR: 28/70 (40%) vs. 39/79 (49%), SAE: 43/71 (61%) vs. 49/79 (62%)	NCT00414310
AML, MDS	Phase 1	2007, Completed	Non-randomized study of the safety and tolerability of vorinostat in combination with decitabine, and in vivo molecular and biological effects of vorinostat in patients with refractory or relapsed AML and intermediate or high-risk MDS; • Sequential: vorinostat: 400 mg, *p.o.*, once daily, 1–7 or 1–10 or 1–14 d + decitabine: 20 mg/m^2, *i.v.*, daily, 1–5 d, every 4 w • Concurrent: vorinostat: 400 mg, *p.o.*, once daily, 1–7 or 1–7 and 15–21 or 1–14 d + decitabine: 20 mg/m^2, *i.v.*, daily, 1–5 d, every 4 w • Result (sequential vs. concurrent): ORR (refractory or relapsed AML): 0/15 (0%) vs. 1/14 (7%), ORR (untreated or intermediate AML): 3/22 (14%) vs. 7/20 (35%), SAE: sequential (vorinostat; 1–7 d): 2/3 (67%), sequential (vorinostat; 1–10 d): 4/4 (100%), sequential (vorinostat; 1–14 d): 25/31 (81%), concurrent (vorinostat; 1–7 d): 3/3 (100%), concurrent (vorinostat; 1–7 and 15–21 d): 1/3 (33%), concurrent (vorinostat; 1–14 d): 21/28 (75%)	NCT00479232 (Kirschbaum et al., 2014)
MDS, AML	Phase 1/2	2008, Completed	Non-randomized study of the safety and effectiveness of decitabine in combination with panobinostat in older patients ≥60 years with high-risk MDS or AML; • Decitabine: 20 mg/m^2, *i.v.*, daily, 1–5 d + Panobinostat: 10 mg/day (Level 1) or 15 mg/day (Level 2) or 20 mg/day (Level 3) or 30 mg/day (Level 4) or 40 mg/day (Level 5), thrice weekly on nonconsecutive days or 40 mg/day (Level 5B), thrice weekly on nonconsecutive days for the first 2 w in a 4 w cycle • Result: MTD of panobinostat in combination: 40 mg/day (Level 5B), ORR: 6/51 (12%), DOR: 12.0 months, EFS: 3.5 months, OS: 6.4 months, SAE: Level 1: 0/4 (0%), Level 2: 0/3 (0%), Level 3: 0/6 (0%), Level 4: 0/8 (0%), Level 5: 0/10 (0%), Level 5B: 0/6 (0%), Phase 2: 0/14 (0%)	NCT00691938
Melanoma	Phase 1/2	2009, Terminated	Study of decitabine and temozolomide in combination with panobinostat for the treatment of resistant metastatic melanoma, for determining (i)safety and tolerability	NCT00925132 (Xia et al., 2014)

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Table 6 (*continued*)

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
			of the proposed schedule of decitabine, temozolomide, and panobinostat (ii) DLT and MTD of the combination (iii) OS, and (iv) TTP of patients treated with the combination in comparison to patients treated historically with the current standard of care; • Decitabine: 0.1–0.2 mg/kg, *s.c.*, thrice weekly for 2 w (starting on d 1) + Panobinostat: 10–20-30 mg, *p.o.*, every 96 h for 2 w (starting on d 8) + Temozolomide: 150 mg/m^2/day, *p.o.*, 9–13 d (cycle 1) -200 mg/m^2/day, *p.o.*, 9–13 d (after cycle 1, if neutropenia or thrombocytopenia had not occurred) • Result: DLT: 0/15, MTD: not reached, CR: 1/8 (13%), DOR: 8 months, SAE: 5/39 (13%)	
Breast cancer	Phase 1/2	2010, Terminated	Trial of tamoxifen following the epigenetic re-expression of estrogen receptor, using the combination of decitabine and panobinostat in patients with triple negative metastatic breast cancer; • Decitabine (*i.v.*, daily, 1–5 d, every 4 w) + Panobinostat (*i.v.*, d 1, d 8, every 4 w): 5 mg/m^2 + 10 mg/m^2 (dose level − 1) – 10 mg/m^2 + 10 mg/m^2 (dose level 0) – 10 mg/m^2 + 15 mg/m^2 (dose level + 1) – 10 mg/m^2 + 20 mg/m^2 (dose level + 2) – 15 mg/m^2 + 20 mg/m^2 (dose level + 3) – 20 mg/m^2 + 20 mg/m^2 (dose level + 4) • Result: SAE: 4/5 (80%)	NCT01194908
➢ **Immunomodulatory agents: Romiplostim, IFNα-2b, gemtuzumab ozogamicin, panitumumab, Rapamycin**				
MDS, Thrombocytopenia	Phase 2	2006, Completed	Randomized, double blind placebo controlled study for evaluation of the safety and effectiveness of romiplostim, at reducing the incidence of clinically significant thrombocytopenic events in low or intermediate risk MDS patients receiving hypomethylating agents, azacytidine or decitabine; • Regimen A: Azacytidine: 75 mg/m^2, *s.c.*, daily, 1–7 d + Romiplostim: 500 μg, *s.c.*, once weekly, every 4 w • Regimen B: Azacytidine: 75 mg/m^2, *s.c.*, daily, 1–7 d + Romiplostim: 750 μg, *s.c.*, once weekly, every 4 w • Regimen C: Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d + Romiplostim: 750 μg, *s.c.*, once weekly, every 4 w • Regimen D: Azacytidine: 75 mg/m^2, *s.c.*, daily, 1–7 d, every 4 w + Placebo: *s.c.*, once weekly • Regimen E: Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4 w + Placebo: *s.c.*, once weekly • Result (A, B, C, D, E): ORR: 1/13 (8%), 1/14 (7%), 5/15 (33%), 2/13 (15%), 3/14 (21%); Occurrence of a clinically significant thrombocytopenic event: 8/13 (62%), 10/14 (71%), 12/15 (80%), 11/13 (85%), 11/14 (79%); SAE: 4/13 (31%), 10/14 (71%), 8/15 (53%), 9/13 (69%), 8/14 (57%)	NCT00321711 (Greenberg et al., 2013; Kantarjian et al., 2010)
Renal cell carcinoma	Phase 2	2007, Terminated	Study of low-dose decitabine in combination with IFNα-2b in advanced renal cell carcinoma, for determining (i) ORR, OS, and PFS (ii) toxicity of the combination (iii) the effects on DNA methylation and gene expression, and (iv) modulation of cellular immunity in correlation with clinical outcomes; • Decitabine: 15 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4 w + IFNα-2b: 0.5 million U, *s.c.*, twice daily continuously, d 1, as of cycle 3 • Result: SAE: 0/1 (0%)	NCT00561912
AML, MDS	Phase 2	2008, Completed	Study of the safety and effectiveness of decitabine in combination with gemtuzumab ozogamicin in AML or high-risk MDS; • Decitabine: 20 mg/m^2, *i.v.*, 1–1/2 h daily, 1–5 d + Gemtuzumab Ozogamicin: 3 mg/m^2, *i.v.*, 1 h, d 5, every 4–6 w • Result: CR: 3/71 (4%), SAE: 15/71 (21%)	NCT00968071
AML, MDS, Myelofibrosis	Phase 2	2009, Completed	Study of the safety and effectiveness of decitabine in combination with gemtuzumab ozogamicin in AML, high-risk MDS or myelofibrosis; • Decitabine: 20 mg/m^2, *i.v.*, 1–1/2 h daily, 1–5 d + Gemtuzumab Ozogamicin: 3 mg/m^2, *i.v.*, 1 h, d 5, every 4–8 w • Result: CR: 10/40 (25%), SAE: 1/40 (3%)	NCT00882102
Colorectal cancer	Phase 1	2009, Completed	Study of the safety and feasibility of the sequential use of decitabine with panitumumab for KRAS wild-type advanced metastatic colorectal cancer, for determining (i) demethylation-induced re-expression of TSGs involved in colorectal cancer or EGFR signaling pathway, (ii) ORR, and (iii) PFS of patients with panitumumab and decitabine vs. patients treated with previous anti-EGFR therapy; • Decitabine: 45 mg/m^2, *i.v.*, 2 h, d 1, d 15 + Panitumumab: 6 mg/kg, *i.v.*, 1 h, d 8, d 22, every 4 w • Result: ORR: 2/20 (10%), PFS: 7 patients had a longer PFS with panitumumab and decitabine compared to their previous anti-EGFR treatment regimen	NCT00879385 (Garrido-Laguna et al., 2013)
AML	Phase 1	2010, Completed	Non-randomized study of the safety and feasibility of decitabine in combination with escalating doses of rapamycin in patients with relapsed or refractory AML;	NCT00861874 (Liesveld et al., 2013)

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Table 6 (*continued*)

Conditions	Phase	Study start, Status	Brief summary	NCT number (References)
			• Decitabine: 20 mg/m^2, *i.v.*, daily, 1–5 d + Rapamycin: 2–4-6 mg/daily, *p.o.*, daily, 6–25 d, every 4 w • Result: MTD: not reached, ORR: 1/13 (8%)	
➢ **Targeted therapy: Bortezomib**				
AML	Phase 1	2008, Completed	Study of decitabine in combination with bortezomib in AML, for determining (i) MTD of bortezomib in combination with decitabine (ii) specific toxicities and the DLT of the combination (iii) ORR and CR rate (iv) to correlate the biological activity of decitabine as demethylating agent with clinical endpoints and PK of decitabine, and intracellular concentration of decitabine triphosphate with biological endpoints and clinical response (v) to characterize the biological activity of bortezomib as a potential demethylating agent, and (vi) the biologic role of microRNAs in determining clinical response to the decitabine plus bortezomib combination and achievement of the other PD endpoints; • Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d or 1–10 d + Bortezomib: 0.7 mg/m^2, *i.v.*, d 5, d 8 (dose level 1); 0.7 mg/m^2, *i.v.*, d 5, d 8, d 12, d 15 (dose level 2); 1.0 mg/m^2, *i.v.*, d 5, d 8, d 12, d 15 (dose level 3); 1.3 mg/m^2, *i.v.*, d 5, d 8, d 12, d 15 (dose level 4), every 4 w • Result: MTD of bortezomib in combination: 1.3 mg/m^2 (dose level 4), ORR: 7/19 (37%)	NCT00703300 (Blum et al., 2012)
AML	Phase 2	2011, Active	Randomized study of decitabine with or without bortezomib in older patients ≥60 years with AML, for determining (i) the effectiveness of combination therapy at improving the OS times as compared to decitabine alone (ii) CR, OS, PFS, and DFS for both regimens (iii) if ongoing treatment with these regimens prolongs OS even in the absence of CR (iv) the frequency and severity of adverse events and tolerability of both regimens; • Arm 1 (Decitabine): [Remission induction therapy: 20 mg/m^2, *i.v.*, 1 h daily, 1–10 d, every 4 w], [Continuation/Maintenance therapy: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d, every 4 w] • Arm 2 (Decitabine + Bortezomib): [Remission induction therapy: Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 2–11 d + Bortezomib: 1.3 mg/m^2, *s.c.*, d 1, d 4, d 8, d 11, every 4 w], [Continuation/Maintenance therapy: Decitabine: 20 mg/m^2, *i.v.*, 1 h daily, 1–5 d + Bortezomib: 1.3 mg/m^2, *s.c.*, d 1, every 4 w] • Result (Arm 1 vs. Arm 2): CR: 33/82 (40%) vs. 31/81 (38%), OS: 9.3 months vs. 8.8 months, DFS: 8.5 months vs. 15.3 months, PFS: 7.3 months vs. 8 months, SAE: 43/80 (54%) vs. 45/79 (57%)	NCT01420926

to therapy for at least 4–6 cycles of treatment, and acquired resistance in which patients relapse during long-term treatment. To this end, the researches in past few years have identified some possible reasons for worst outcomes of these drugs in the clinic. Since the therapeutic efficacy of AZN is largely dependent on drug uptake by cells, metabolic activation, as well as degradation by a cascade of enzymes (Stresemann & Lyko, 2008), Fig. 2, each of these steps makes available a mechanism by which cells exhibit primary or secondary resistance to these agents. The first indication towards the involvement of metabolic pathways in AZN resistance came from the study of native and acquired resistance towards fazarabine in a panel of tumor cell lines which suggested dCK as an important determinant of sensitivity towards fazarabine, demonstrated by the markedly decreased level of dCK in resistant cells (Ahluwalia et al., 1986). Several years later, loss of dCK was established as a mechanism behind resistance to decitabine in a panel of cultured human cancer cell lines (Qin, Jelinek, Si, Shu, & Issa, 2009) which was further confirmed in vivo in a subset of MDS patients (Qin et al., 2011). The study suggested decreased levels of dCK (decreased phosphorylation of decitabine) and increased levels of CDA (increased deamination) as the marker of primary resistance to decitabine, demonstrated by higher CDA/dCK ratio in non-responders than responders (Qin et al., 2011). Similar results were obtained for azacytidine in leukemic cell lines (Sripayap et al., 2014) and in MDS patients treated with azacytidine in which low levels of uCK (which phosphorylates azacytidine) correlated with poor clinical outcomes (Valencia et al., 2014). The implication of altered expression of AZN metabolizing enzymes on modulation of response to azacytidine or decitabine therapy was further demonstrated by decreased cytidine analog half-life and worse outcomes, as a consequence of increased CDA expression in another trial with MDS patients (Mahfouz et al., 2013). Recently, the change in expression levels of CDA and/or dCK during acquisition of resistance to decitabine has been shown in in vitro developed decitabine-resistant variant of colorectal cancer cells (Hosokawa et al., 2015). Apart from enzymes involved in metabolic activation, membrane proteins involved in drug uptake are potential mediators of drug resistance. In this context, the recent studies identified hENT1 expression as a key determinant of azacytidine-triggered cytotoxicity (Hummel–Eisenbeiss et al., 2013) and hCNT1, hCNT3, and hENT2 as the key transporters involved in cellular uptake of Zeb (Arimany-Nardi et al., 2014) which suggests the significance of these transporters as useful biomarkers that may predict the therapeutic efficacy of these drugs. Besides metabolic pathways, primary resistance to AZN have also been linked to methylation status and gene expression (Meldi et al., 2015; Merlevede et al., 2016), as demonstrated by a recent study which correlated differentially methylated DNA regions with response to decitabine at diagnosis in chronic myelomonocytic leukemia patients (Meldi et al., 2015). The study further demonstrated overexpression of cytokines, *CXCL4* and *CXCL7* in non-responders (Meldi et al., 2015).

On the other hand, pharmacological mechanisms (Ahluwalia et al., 1986; Arimany-Nardi et al., 2014; Hosokawa et al., 2015; Hummel-Eisenbeiss et al., 2013; Mahfouz et al., 2013; Qin et al., 2009, 2011; Sripayap et al., 2014; Valencia et al., 2014), and DNA methylation status (Meldi et al., 2015; Merlevede et al., 2016) involved in primary resistance to these nucleoside analogs are not related with secondary resistance to these drugs, evident by no significant difference in decitabine metabolism gene expressions between diagnosis and relapse (Qin et al., 2011), and significant hypomethylation at relapse compared to diagnosis (Qin et al., 2011). Instead, secondary resistance to these AZN may result from genetic activation of oncogenic survival and progression pathways. In the past years, studies have identified several aberrantly expressed oncogenes as predictors of response to DNA hypomethylating agents. Included in the list are *DNMT3B* gene amplification (Simo-Riudalbas, Melo, & Esteller, 2011), up-regulated

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Table 7
Zebularine in combinatorial therapies
This table represents the combinations of zebularine with various chemotherapeutic, epigenetic or immunomodulatory agents tested so far.

Combination drug	Types of cancer	Effects of combination therapies	References
Decitabine	Leukemia	• Zeb, when combined with decitabine, resulted in greater inhibition of cell growth and colony formation in leukemic cell lines, as compared to either agent alone • The combination of Zeb and decitabine further produced synergistic effects at inducing demethylation and re-expression of TSG, *p57KIP2* as compared to either drug alone • In vivo, the combination of Zeb with decitabine resulted in increased survival of mice bearing leukemia cells as compared to either drug alone	Lemaire et al. (2005)
Decitabine	Leukemia	• Zeb in combination with decitabine significantly enhanced the anti-neoplastic action of decitabine in vitro in leukemic cells expressing high levels of CDA • In vivo, in mice bearing leukemia cells, co-infusion of Zeb with decitabine significantly increased the plasma level of decitabine and enhanced the survival time of mice	Lemaire et al. (2009)
Decitabine, Vorinostat	Breast	• Low-dose Zeb in combination with decitabine or vorinostat significantly inhibited cell proliferation and colony formation in breast cancer cells, as compared with either drug alone	Billam et al. (2010)
Vorinostat	Osteosarcoma	• Zeb in combination with vorinostat showed additive and significant cytotoxic effects in human and canine osteosarcoma cells with aggressive biological behavior	Thayanithy et al. (2012)
Entinostat	Leukemia	• Zeb in combination with entinostat increased the effect of histone deacetylase inhibition at inducing the re-expression of TSG, *AKAP12* in leukemic cells with dense *AKAP12* methylation	Flotho et al. (2007)
Depsipeptide	Lung	• Zeb alone or in combination with depsipeptide yielded additive or synergistic growth inhibitory effects via re-induction of silenced *CDKN2A* gene in lung cancer cell lines	Chen et al. (2010)
Retinoic acid, Sodium phenylbutyrate, BML-210	Leukemia	• Zeb alone inhibited cell proliferation in a dose- and time-dependent manner, elicited a dose-dependent increase in growth inhibition and cell death, and in combination with retinoic acid showed additive anti-proliferative and apoptotic effects in leukemia cells • Pre-treatment with Zeb or simultaneous combination of Zeb with sodium phenylbutyrate and BML-210 and retinoic acid accelerated cell differentiation caused by retinoic acid alone • Combination of Zeb with retinoic acid resulted in greater depletion of DNMT1 and greater re-expression of TSG, *E-cadherin* at both mRNA and protein levels, as compared to treatment with either single drug	Savickiene et al. (2012)
Retinoic acid	Pituitary	• Pre-treatment of pituitary cells with Zeb along with trichostatin rendered retinoic acid-augmented expression of silenced genes, *BMP-4* and *D2R*, potentially involved in mediating responsiveness to drugs commonly used in this tumor type	Yacqub-Usman et al. (2013)
Cisplatin	Ovary	• Zeb produced significant anti-proliferative effects against ovarian cancer cell lines, including cisplatin-resistant ovarian cancer cells in a dose-dependent manner, and induced demethylation and re-expression of various TSGs, *RASSF1A*, *hMLH1 ARHI*, and *BLU* • Zeb treatment significantly re-sensitized the cisplatin-resistant ovarian cancer cells to cisplatin, suggesting its potential in therapy of drug-resistant ovarian cancer	Balch et al. (2005)
Cisplatin, 5-fluorouracil	Oral squamous cell carcinoma	• Low-dose Zeb in combination with cisplatin significantly enhanced the cisplatin-induced apoptotic cell death in oral squamous cell carcinoma cells	Suzuki et al. (2007)
5-fluorouracil, Irinotecan, Oxaliplatin	Colorectal	• Zeb slightly potentiated the inhibitory effects of oxaliplatin and 5-fluorouracil, and the combination of Zeb with either chemotherapeutics, 5-fluorouracil, irinotecan, and oxaliplatin indicated synergistic or additive effects • Combination of Zeb with oxaliplatin and 5-fluorouracil increased the phosphorylation level of proteins of major signaling checkpoints in response to DNA damage and showed augmented effects on cell cycle arrest and induction of apoptosis	Flis et al. (2009) Flis et al. (2014) Ikehata et al. (2014)
Brostallicin	Prostate	• Pre-treatment of prostate cancer cells with Zeb enhanced the anti-proliferative activity of brostallicin, both in vitro and in vivo by inducing the re-expression of previously methylated *GST*	Sabatino et al. (2013)
Methotrexate	Leukemia	• Zeb alone significantly inhibited cell proliferation in a dose- and time-dependent manner and colony formation in a dose-dependent manner in pediatric leukemia cell lines and the combination of Zeb with methotrexate showed synergistic cytotoxic effects • Zeb treatment further induced and enhanced apoptotic cell death, decreased DNMT genes and protein levels, and induced *AhR* promoter demethylation and expression in pediatric leukemia cells	Andrade et al. (2014)
Vincristine	Medulloblastoma	• Zeb combined with vincristine showed synergistic cytotoxic effects against medulloblastoma cell lines	Andrade et al. (2017)
p53 retro-inverse peptide	Multiple myeloma	• Pre-treatment with Zeb followed by incubation with p53 retro-inverse peptide significantly reduced the cell viability and enhanced the apoptosis as compared to singular treatment with p53 activating peptide in myeloma cell line with methylated p53	Hurt et al. (2006)
Recombinant TRAIL	Leukemia, Breast, Prostate, Colon, Bladder	• Pre-treatment with Zeb sensitized leukemia, breast, prostate, colon, and bladder cancer cells to TRAIL-induced apoptosis by increasing the fucosylation level in a concentration-dependent manner, and the expression levels of several kinds of fucosylation-related genes in these cells	Moriwaki et al. (2010)

expression of anti-apoptotic *BCL2L10* (Cluzeau et al., 2012), constitutive activation of the ATM/BRCA1 pathway (Imanishi et al., 2014), simultaneous DNA re-methylation due to up-regulation of DNMT1, re-activation of tyrosine-protein kinase cascades (Yan et al., 2015), and very recently discovered, overexpression of a histone H2A variant macroH2A1.1, a marker of senescence-associated heterochromatic foci (Borghesan et al., 2016) as determinants of resistance to hypomethylating AZN. But so far none of these bonafide oncogenes/pathways have revealed clinical or molecular patterns that differentiate between responders and non-responders. Distinctly, another line of substantiation for secondary resistance to AZN comes from the inability of AZN to eliminate leukemia-stem cell containing population, the later growth of which leads to relapse, and secondary drug resistance over time (Craddock et al., 2013). Altogether, the investigation of response predicting biomarkers and mechanisms of primary and secondary resistance to hypomethylating agents is an unmet need towards the successful DNA-methylation based epigenetic therapy.

8. Mutations of epigenetic regulators: Predictive biomarkers of azanucleosides response

The identification of the patients who may derive the most clinical benefit from AZN therapy remains a challenge. If the response to AZN therapy depend upon genetic characteristics of underlying neoplasm

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Table 8
SGI-110 as priming agent or in combinatorial therapies for solid tumors
This table represents the synergistic effects of SGI-110 as priming agent or in combinatorial therapies with chemotherapeutic agents in the treatment of the solid tumor.

Combination drug	Types of cancer	Effects of combination therapies	References
Entinostat	Lung	• SGI-110 alone or in combination with entinostat significantly reduced the tumor burden against no effect of entinostat alone in orthotopically engrafted lung cancer model • Epigenetic therapy with SGI-110 alone or in combination with entinostat caused widespread re-programming of various genes involved in key cancer regulatory pathways such as TSG (*p21*), apoptotic gene (*BIK*), and EZH2 target genes, and various cancer-testis antigen genes which could sensitize tumor cells to immunotherapy	Tellez et al. (2014)
Cisplatin	Ovary	• Treatment with low-dose SGI-110 alone or in combination with cisplatin re-sensitized cisplatin-resistant ovarian cancer cells to cisplatin by decreasing subpopulation of ALDH(+) cells, responsible for cisplatin resistance, and induced re-expression of differentiation-associated genes • SGI-110 treatment alone or in combination with cisplatin markedly inhibited the spheroid forming ability of both parental and cisplatin-resistant ovarian cancer cell lines • In vivo, SGI-110 decreased the tumorigenesis of ovarian cancer stem cells by targeting ALDH (+) cells, and maintenance treatment with SGI-110 after carboplatin inhibited ovarian cancer stem cell growth, causing global tumor hypomethylation and decreased tumor progression	Wang et al. (2014)
Cisplatin	Ovary	• Priming with moderate- or low-doses of SGI-110 increased the sensitivity of a wide range of parental and platinum-resistant ovarian cancer cells to cisplatin, by inducing significant demethylation and re-expression of TSGs (*RASSF1A*), differentiation-associated genes (*HOXA10* and *HOXA11*), transcription factors (*STAT5B*), and putative drivers of ovarian cancer cisplatin resistance (*MLH1* and *ZIC1*) • Pre-treatment with SGI-110 significantly increased DNA damage, induced by cisplatin in parental as well as cisplatin-resistant ovarian cancer cells • SGI-110 alone or in combination with cisplatin was well tolerated in vivo and displayed increased antitumor effects in cisplatin-resistant ovarian cancer xenografts as compared to cisplatin alone	Fang et al. (2014)
Cisplatin	Testis	• Pre-treatment with low concentration of SGI-110 re-sensitized cisplatin resistant embryonal cancer cells (stem cells for testicular germ cell tumors) to cisplatin in a DNMT3B-dependent manner • Low concentration of SGI-110 caused transcriptional re-programming of embryonal cancer cells including induction of p53 targets genes (*GDF15*, *p21*, and *GADD45A*), hypermethylation silenced genes (*RASSF1* and *SOX15*), and repression of pluripotency genes which could be responsible for the anti-proliferation and anti-survival activity of SGI-110 • As a single agent, moderate-doses of SGI-110 induced complete abrogation and regression of embryonal cancer tumor growth in vivo, and the combination of low-dose SGI-110 with cisplatin sensitized refractory embryonal cancer cells to cisplatin, without any evident toxicity • The in-vivo antitumor activity of SGI-110 was found to be associated with genome-wide induction of p53 target and immune-related gene signatures	Albany et al. (2017)
Oxaliplatin	Liver	• Pre-treatment with low-dose SGI-110 or the combination of SGI-110 and oxaliplatin showed synergistic effects yielding enhanced cytotoxicity in wide range of hepatocellular carcinoma cells, by inhibiting the expression of genes involved in WNT/EGF/IGF signaling • SGI-110 as single agent or in combination with oxaliplatin significantly delayed tumor growth in hepatocellular carcinoma xenografts as compared to oxaliplatin alone, without causing any systemic toxicity	Kuang et al. (2015)

has been demonstrated through various studies that identified frequent inactivating mutations in epigenetically regulated genes, which directly impact DNA methylation and predict response to AZN. These include mutations in genes involved in DNA methylation (*TET2*, *DNMT3A*, *IDH1* and *IDH2*), chromatin modification (*ASXL1*, *EZH2*), transcriptional regulation (*RUNX1*, *CBL*), genes in spliceosome machinery (*SF3B1*, *SRSF2*), and *TP53* (Cedena et al., 2017; Traina et al., 2014). *TET2* is the first discovered active demethylating enzyme which converts 5-methylcytosine to 5-hydroxymethylcytosine. The *TET2* loss-of-function mutations impair the catalytic activity of the enzyme and diminish hydroxylation of 5-methylcytosine, leading to 5-methylcytosine accumulation at various genomic locations (Ko et al., 2010), and are frequently found in MDS (30%), myeloproliferative neoplasms (10%), secondary AML (25%), and CMML (40%) (Figueroa et al., 2010; Ko et al., 2010; Langemeijer et al., 2009). The association between *TET2* mutations and AZN response has been reported by several groups which found positive correlation between *TET2* mutations and sensitivity to AZN treatment in MDS, AML, and CMML (Bejar et al., 2014; Braun et al., 2011; Cedena et al., 2017; Itzykson et al., 2011; Traina et al., 2014; Voso et al., 2011). However, despite of being an independent predictor of better response and prolonged PFS, compared with wild-type, mutations in *TET2* did not relate with better OS (Bejar et al., 2014; Traina et al., 2014). Next in line, recurrent mutations in *DNMT3A*, a de novo methyltransferase have been identified in hematopoietic malignancies with an incidence of 20% in de novo AML (Ley et al., 2010; Yan et al., 2011) and 8–10% in de novo MDS patients (Walter et al., 2011). While the studies (Ley et al., 2010; Walter et al., 2011) associated *DNMT3A* mutations with worse outcomes and more rapid progression to AML, as compared with wild type patients, Metzeler et al. suggested that patients with *DNMT3A* inactivating mutations may benefit from treatment with hypomethylating AZN (Metzeler et al., 2012), however, further exploration of the relation between *DNMT3A* mutations and response to AZN is clearly warranted. Apart from loss-of-function mutations in *TET2* and *DNMT3A*, mutations targeting *IDH1* and *IDH2* are frequently found in gliomas, AML, and multiple cancer types, and have been exposed as promising biomarkers for disease prognosis and prediction of response to treatment (Megova et al., 2014; Yang, Ye, Guan, & Xiong, 2012). Mutations in *IDH1* and *IDH2* are known to obstruct normal histone and DNA methylation by mainly targeting α-ketoglutarate-dependent histone and DNA demethylases, and secondly *TET* family of DNA hydroxylases (Yang et al., 2012). In AML and MDS, mutations in *IDH1* and *IDH2* are known to induce hypermethylator phenotype and disrupt hematopoietic differentiation, driving leukemogenesis (Figueroa et al., 2010). A recent study (Cedena et al., 2017) which assessed the relation between mutations in aforementioned candidate genes and response to AZN predicted that patients with a total of ≤2 somatic mutations in candidate genes and ≥1 mutation in genes of the DNA methylation pathway (*TET2, DNMT3A, IDH1* and *IDH2*) showed better ORR (67%), as compared to typically expected response (40–50%), and further revealed *TET2* mutations as the strongest biomarker of clinical response (Cedena et al., 2017). Amongst other candidate genes, mutations in *ASXL1*, gene involved in histone modification are frequent in MDS (Gelsi-Boyer et al., 2009), and have been shown to confer partial resistance to AZN by lowering the likelihood of response (Bejar et al., 2014), and are associated with poorer OS (Traina et al., 2014). While mutations in *EZH2* or *RUNX1*, and *TP53* have been associated with shorter OS of MDS patients, the effect of mutational status of *EZH2* and *TP53* on OS was

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found to be independent of the response to AZN (Cedena et al., 2017; Müller-Thomas et al., 2014). Mutations in spliceosomal gene, *SF3B1* have been found to have favorable impact on PFS and OS of MDS patients (Traina et al., 2014). Altogether, these data suggest the role of molecular mutations as predictive biomarkers for response and survival in patients with hematological malignancies treated with AZN.

9. DNMTIs in rational combinations: An alternative strategy targeting drug resistance

DNA methylation is associated with silencing of critical drug-response genes and resistance of cancer cells to chemotherapeutic agents (Nyce, 1997). Significantly, the plasticity of the epigenome that provides the chance to correct gene expression, and the potential of DNA methylation inhibitors to restore expression of silenced genes is an added advantage that holds the promising possibility to re-sensitize resistant tumors to chemotherapeutics. Moreover, the success of single-agent clinical interventions of DNA methylation inhibitors has been limited, especially in solid tumors (Cowan et al., 2010) which indicate towards the unmet need for improvement of response rates and/or development of alternative therapies. To this end, the focus of researchers and clinicians have shifted towards combinatorial epigenetic therapies, and the rational combinations of epigenetic drugs with each other or with conventional agents have received considerable preclinical attention (Chiappinelli, Zahnow, Ahuja, & Baylin, 2016) The recent studies have suggested the synergistic activities of DNMTIs such as azacytidine and decitabine in overcoming intrinsic or acquired chemoresistance in several cancer types, especially in advanced solid tumors, when combined in low concentrations with chemo-drugs (Gravina et al., 2010). The relatively low doses of DNMTIs minimize their cytotoxic effects while producing synergistic effects on activation of silenced genes, resulting in impressive treatment outcomes (Fan et al., 2014; Juergens et al., 2011). Currently, several combination therapies of DNMTIs with other epigenetic drugs, immunomodulatory agents, and chemotherapeutics is being investigated in clinical trials. Tables 5-8 summarizes the registered clinical trials of azacytidine (Table 5) and decitabine (Table 6), and combinatorial effects of Zebularine (Table 7) and SGI-110, a recently discovered prodrug of decitabine (Table 8) in various rational combinations.

10. Future outlooks for DNA demethylating epigenetic drugs headway

DNMTIs which target the DNA methylation machinery and reverse the epigenetic alterations are promising anti-neoplastic strategies, that have been added to the growing list of anti-cancer drugs about 15 years ago with the regulatory approval of first hypomethylating agent in 2004. In this review, we performed an extensive search of published pre-clinical and clinical data concerning nucleosidic DNA hypomethylating drugs and assembled the available literature focusing on the efficacy of these agents in epigenetic cancer therapy. The first generation “Hypomethylating agents” azacytidine and decitabine are undeniably the most successful epigenetic modulatory drugs which are increasingly used in the clinic for myeloid malignancies and are gaining popularity as priming agents in the treatment of advanced solid tumors. But, the clinical response rate of these AZN drugs ranges between 30 and 50% only. The clinical effectiveness of these drugs is limited due to their low bioavailability and dependency on variably expressed nucleoside transporters for cellular uptake. Moreover, the application of these hypomethylating agents is confounded by high relative toxicity owing to their complex modes of action which not only involves DNA methylation but also additional non-specific effects, particularly at high doses, leading to genomic instability via activation of various genes with oncogenic roles. Besides, due to the poor oral absorption of these nucleoside analogs, these compounds have been administered by injection. The low response rates and adverse effects of these prototypal drugs continue to drive the discovery of newer DNMT depleting agents. In an effort to develop more efficacious DNA hypomethylating drugs, the active research in the past years led to the discovery of several other nucleoside analogs and second generation pro-drugs, reviewed here. The second generation pro-drugs with better PK and PD profile circumvent the potential drawbacks of prototypal hypomethylating agents, leading to higher response rates, and offer a wider window between hypomethylation and cytotoxicity, resulting in enhanced therapeutic indexes. But, a better understanding of the mechanisms responsible for treatment failure of FDA approved hypomethylating agents, azacytidine, and decitabine, and response predicting biomarkers is an unmet requirement to be fulfilled for improvement of clinical response, and/or development of alternative therapeutic regimens after the failure of treatment to these drugs. Nevertheless, the ongoing and future investigations of these hypomethylating drugs in combinatorial therapies may lead to better treatment outcomes in hematologic malignancies as well as in various solid tumors.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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