

# Development of DNA Methyltransferase Inhibitors for the Treatment of Neoplastic Diseases

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**Abstract:** Although chemotherapy is considered the mainstay of cancer therapy, unfortunate side effects of chemotherapy create a continuous demand for developing other novel and specific targets for cancer therapy. Re-expression of epigenetically silenced tumor suppressor genes is a rational strategy for the treatment of human neoplasms. Epigenetic modifiers like DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors induce the re-expression of epigenetically silenced genes *in vitro* and *in vivo*. Moreover, they demonstrate safety and efficacy against neoplastic diseases in clinical trials. DNMT inhibitors like 5-azacytidine and 5-aza-2'-deoxycytidine are currently FDA approved for the treatment of myelodysplastic syndrome. Nonetheless, the mechanism of action behind their clinical efficacy remains unclear. Ongoing clinical trials are attempting to identify tumor suppressor genes that upon re-expression can induce remission and cure in patients. On the other hand, the pleiotropic biological effects of DNMT inhibitors and recent reports demonstrating lack of association between clinical response and methylation reversal of candidate tumor suppressor genes, suggest a complex mechanism behind their clinical efficacy that may involve a cytotoxic effect.

**Keywords:** 5-azacytidine, decitabine, zebularine, RG108, DNA methylation, DNA methyltransferases.

## INTRODUCTION

The intense expansion in our knowledge about the molecular basis of cancer motivates and guides the development of molecularly targeted therapies. During the period between 1990 and 2005, 51 new agents have been approved by the FDA for the treatment of solid and hematologic tumors [1] (excluding hormonal therapy agents and newer indications of previously approved agents). The DNA methyltransferase (DNMT) inhibitors Vidaza® (azacitidine) and Dacogen® (decitabine) are among the new agents approved by the FDA for the treatment of myelodysplastic syndrome (MDS). In this review, the development of different DNMT inhibitors as epigenetic modifiers for the treatment of cancer and their mechanism of action are discussed.

The genetic basis of cancer as a disease is well established. However, the involvement of factors other than changes in nuclear DNA sequence in cancer development and progression gained much attention in the last three decades. Epigenetics refers to heritable reversible changes in gene expression that occur without any changes in the nuclear DNA sequence or DNA copy number. Epigenetic modifications affect the nuclear DNA and/or the nucleosomes-incorporated histones and consequently modify gene expression.

## HISTONE MODIFICATIONS

A diverse array of posttranslational modifications of the amino terminal tail of histones like methylation, acetylation, phosphorylation, ubiquitination and SUMOylation can directly affect the packing of nucleosomes and chromatin architecture [2-6]. Accordingly, the histone code hypothesis [7-9] proposes a combinatorial code of histone modifications that complement the information stored in the DNA sequence and mediate downstream events. Despite the diver-

sity of histones modifications, histone acetylation is the most recognized as a drug target for modulation of gene expression. Two enzymes control the process of histone acetylation; histone acetyltransferase (HAT) and histone deacetylase (HDAC). HAT is associated with a transcriptionally active state chromatin (euchromatin), while HDAC is associated with a transcriptionally repressed state chromatin (heterochromatin). Consequently, inhibition of HDAC enzymes to activate the transcription of silenced tumor suppressor genes (TSG) in cancer is a sound and a rational approach. However, global genomic approaches demonstrate that HDAC inhibitors induce and repress a small (~2%) set of genes [10]; emphasizing the coordinated role of other histone modifications and/or DNA cytosine methylation in remodeling the chromatin architecture. Additionally, HDAC inhibitors induce hyperacetylation of non-histone proteins like NF- $\kappa$ B [11], p53 [12] and Hsp90 [13]. Hyperacetylation of both histones and non-histone proteins by HDAC inhibitors indicate that these compounds are in fact "lysine deacetylase" inhibitors and not just HDAC inhibitors. Interestingly, hyperacetylation of non-histone proteins like Hsp90 by HDAC inhibitors induces DNMT1 protein downregulation by promoting the ubiquitin-dependent proteasomal degradation of DNMT1 [14]; highlighting an indirect effect of HDAC inhibitors on the DNA methylation machinery and adding another layer of complexity to the interactive effects of combined HDAC inhibitors and DNMT inhibitors therapy. The contribution of the acetylation of histones and non-histone proteins to the clinical efficacy of these compounds is not clear and beyond the scope of this review, interested readers are referred to them elsewhere [15-17].

Paradoxically, HAT inhibitors are also known as anticancer agents because aberrant lysine acetylation mediates oncogenesis [18]. The natural product anacardic acid has been used as a lead compound to develop HAT inhibitors [19]. HAT inhibitors demonstrate selective antitumor effect in cancer cell lines and prevent cardiac failure in rodent models [20]. In contrast to HDAC inhibitors, none of these compounds has advanced to clinical trials as anticancer agents.

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## MAMMALIAN DNA METHYLATION

DNA cytosine methylation and histone modifications comprise the cellular epigenetic changes that involve chromatin-remodeling [21]. Mammalian DNA methylation takes place at cytosine bases that are located 5' to a guanosine in a CpG dinucleotide (p indicates phosphodiester bond). CpG islands are short regions of approximately 0.5-4 kilobase in length and are rich in CpG dinucleotides. CpG island definition depends on several factors like the frequency of CpG dinucleotides and the length of the island. Several free software are available on the internet for predicting CpG islands, like the CpG island searcher ([www.uscnorris.com/cpgislands](http://www.uscnorris.com/cpgislands)) and the European Molecular Biology Open Software Suite (Emboss) CpG plot (<http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html>). Most CpG islands exist in the proximal promoter regions of almost half of the genes in the mammalian genome and are usually unmethylated in normal cells [22]. Promoter DNA methylation of different TSG in cancer cells plays a role in tumor initiation and progression and silences their expression. The biological effects of gene function loss caused by promoter hypermethylation are analogous to that caused by genetic mutations. For instance, according to the Knudson's two-hit hypothesis [23], the disruption of a TSG function requires a complete loss of function of both gene copies. In this context, genetic mutation or deletion can contribute to single allele loss followed by abnormal promoter hypermethylation and loss of function of the second allele [24]. Moreover, Hypermethylation also contributes to the loss of both alleles [25] and may predispose genetic point mutations by increasing the inci-

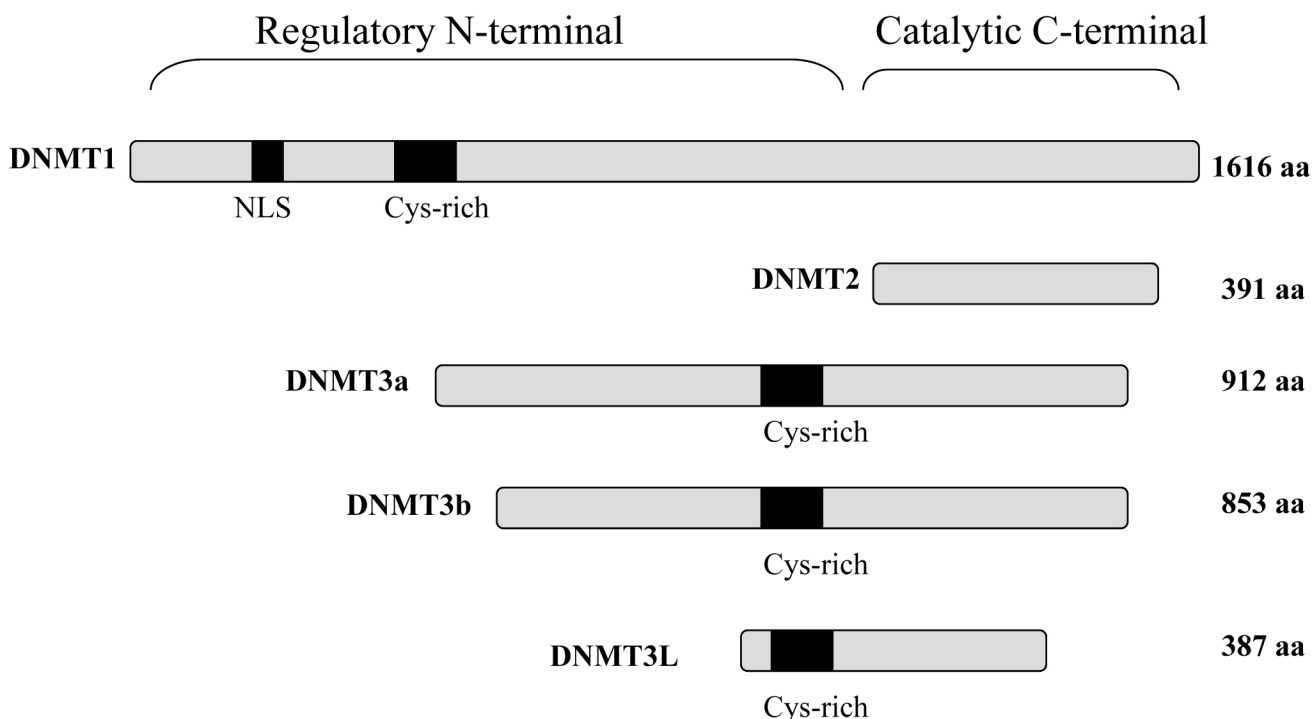
dence of cytosine deamination and consequent disruption of TSG function.

Gene silencing of hypermethylated CpG islands can be explained by 2 possible mechanisms. The first one proposes the involvement of methyl CpG-binding proteins such as MBD2 and MeCP2 and recruitment of transcriptional repressors [26]. The second mechanism postulates that methylated CpG islands inhibit the access and binding of transcription factors to their CpG-containing recognition sites [27].

## DNA METHYLTRANSFERASES (DNMTs)

The process of DNA cytosine methylation involves the addition of a methyl group to cytosine at the C5 position in CpG dinucleotides, using the universal methyl donor S-adenosyl-L-methionine (SAM). SAM is also a methyl donor for amino acid residues like arginine and lysine in proteins; however, protein methylation is not yet known as a target for drug intervention. The enzymes responsible for catalyzing the transfer of methyl group from SAM to cytosine DNA are known as DNMTs. Five different DNMTs are known in mammals; DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L. DNMT1 is considered maintenance DNMT by copying DNA methylation from parental strand to newly synthesized strand. DNMT1 also exhibits some de novo methylation activity *in vitro* [28]. DNMT3a and DNMT3b are also involved in both de novo and maintenance cytosine methylation [29]; however, their contribution to de novo methylation is much higher than DNMT1, which demonstrates 40-50 fold preference for hemimethylated over un-

# Mammalian DNA methyltransferases



**Fig. (1).** Sketch diagram of mammalian DNMT isotypes. NLS indicates nuclear localization sequence. Cys-rich indicates cysteine-rich region. Number of amino acid (aa) residues are listed on the right side.





The cytotoxicity and chemical instability associated with DAC and 5AC create incessant demand for the development of new DNMTs inhibitors, like zebularine [57]. Zebularine [1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one] (Fig. 2) is the first orally active drug that reactivates an epigenetically silenced gene with minimal toxicity [58]. Zebularine induces complete depletion of DNMT1 and partial depletion of DNMT3a and DNMT3b in human bladder cancer cells [59]. The preferential depletion of DNMT1 is also demonstrated by DAC, probably due to the differential nuclear localization of the DNMT enzymes rather than from inherent differences in reaction mechanisms. DNMT1 localizes with the replication fork during S phase, whereas DNMT3a and DNMT3b remain diffuse in the nucleus [60, 61]. Therefore, DNMT1 probably encounters DAC molecules incorporated into DNA more frequently than the diffusely localized DNMT3a and DNMT3b enzymes. Hence, 5AC is expected to exhibit such preferential depletion of DNMT1 but this remains to be proven. Zebularine inhibits DNMTs in a similar manner as DAC and 5AC through covalent DNMTs trapping with no direct effect on DNMTs transcription [59]. A major drawback of transient treatment with DNA demethylating agents is remethylation and resilencing of genes. The strategy of long term use of low doses of demethylating agents has been successful in overcoming this problem, especially with agents that exhibit minimal inherent cytotoxicity like zebularine [59]. Furthermore, chronic oral administration of zebularine to preweaned cancer prone *Apc<sup>Min/+</sup>* mice demonstrates safety and gender-specific efficacy against intestinal tumors [62].

Zebularine demonstrates selective incorporation into DNA of tumor cells rather than normal fibroblasts DNA [63]; the selective effect is not observed with the prototype analogues DAC and 5AC. The selectivity of zebularine is ascribed to the higher level of expression of the uridine/cytidine kinase (kinase activity is required for zebularine incorporation into DNA) in cancer cells than normal cells. DNA damage has been reported after zebularine treatment for 24 h [64]. *p21<sup>WAF1</sup>* upregulation by zebularine treatment in a demethylation-independent manner [63] may suggest the involvement of DNA damage in *p21<sup>WAF1</sup>* upregulation in a p53 dependent manner; similar to leukemia cells after DAC treatment [53]. Unfortunately, the pharmacokinetic profile of zebularine in mice reveals its rapid and extensive metabolism into endogenous compounds that are unlikely to induce demethylation at the concentrations observed [65].

Other nucleoside analogues like 5-fluorouracil and 2',3'-dideoxycytidine (zalcitabine) are FDA approved for the treatment of cancer and AIDS, respectively. However, their clinical activity is dependent on their cytotoxicity and the potential use of these drugs as demethylating agents is not well investigated yet.

#### NON-NUCLEOSIDE ANALOGUES DNA DEMETHYLATING AGENTS

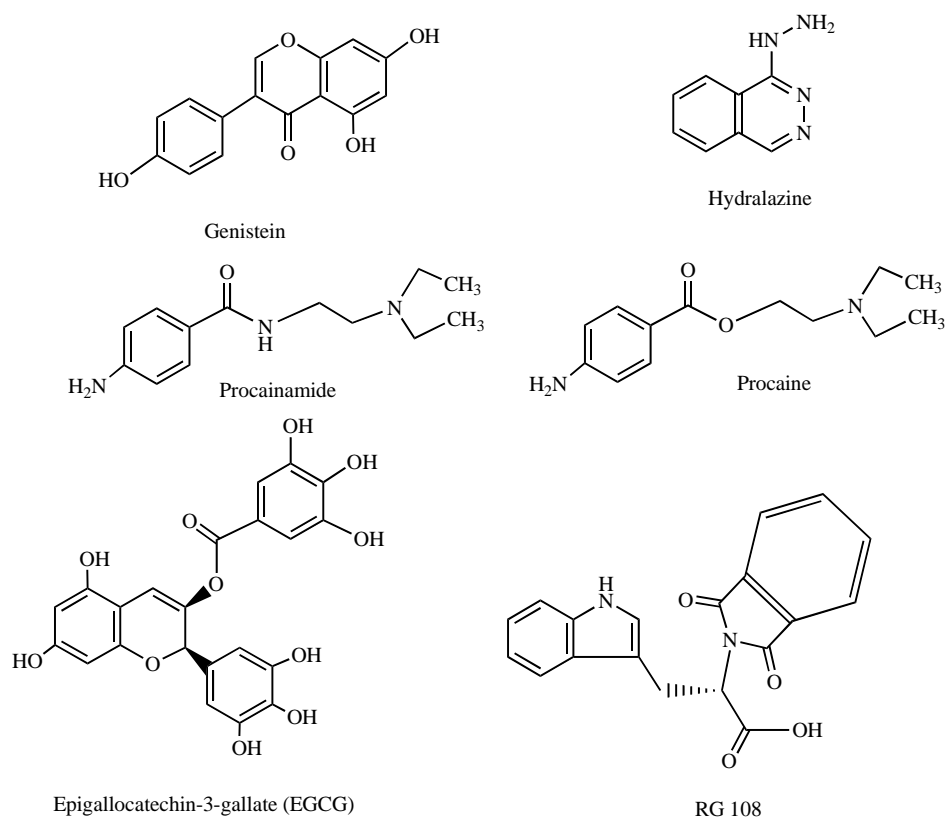
Direct inhibition of DNMTs is an optimum strategy for reversal of silenced hypermethylated genes. Specific and direct inhibition of DNMTs evades the drawbacks of the nucleoside analogues nonspecific cytotoxic effects.

Small interfering RNA (siRNA) or antisense mediated degradation of DNMTs can hypothetically achieve this mission. Antisense or siRNA oligonucleotides can be utilized to selectively deplete specific isoforms of DNMTs like DNMT1 in colorectal cells. Knock down of DNMT1 re-expresses the wild-type *CDKN2A (p16)* allele in colorectal cells and reactivates methylation-silenced genes like *RASSF1* in renal cancer cells [66]. These data indicate that DNMT1 depletion is sufficient to induce promoter demethylation in human cancer cells. On the contrary, the isogenic DNMT1<sup>-/-</sup> HCT116 colon cancer clones do not show significant alterations in DNA methylation or tumor-suppressor gene expression [67] from wild type HCT116 cells, most probably because DNMT3b maintains the CpG methylation pattern [68]. Furthermore, methylated alleles of *CDKN2A* and other genes like *SFRP1*, *GATA4* and *GATA5* are still detected after silencing DNMT1 in HCT116 and SW480 cells [69]; emphasizing the dispensable role of DNMT1 in maintaining the cell DNA methylation machinery.

Despite the confusing preclinical data regarding the validity of the approach of knocking down DNMT1 isotype only to reverse DNA methylation, clinical trials with the phosphorothioate antisense oligodeoxynucleotide (MG98) that targets the mRNA of DNMT1 has been initiated in both solid tumors and hematological malignancies. Phase I clinical trials with MG98 in solid tumors are not revealing; MG98 does not show antitumor activity or consistent dose related changes in DNMT1 mRNA levels in peripheral blood [70, 71]. Moreover, MG98 lacks antitumor activity in a phase II trial in patients with metastatic renal carcinoma [72]. Additionally, no antitumor activity in MDS and AML patients is observed after administering MG98 [73]. Taken together, the outcome of the clinical trials with MG98 is not encouraging and future studies should ensure the efficient intracellular uptake of MG98 and the consistent knockdown of DNMT1 mRNA.

Depletion of multiple DNMT isoforms provides an ideal alternative approach to completely block the DNA methylation machinery of the cell. Unfortunately, simultaneous depletion of multiple DNMT isoforms with oligonucleotides (antisense or siRNA) is not feasible because of the observed non-specific effects at the combined high doses of the different oligonucleotides [66].

Blocking the enzymatic activity of DNMTs by using small molecule inhibitors is another strategy to achieve gene demethylation. Hydralazine and procainamide (Fig. 4) are FDA approved for the treatment of hypertension and cardiac arrhythmia, respectively. Both drugs show demethylating activity [74] by binding to GC-rich DNA sequences; however the exact mechanism of DNMT inhibition and whether it involves direct blocking of DNMTs accessibility by these drugs is not clear. Procainamide reverses *GSTP1* hypermethylation and restores its expression in LNCaP human prostate cancer cells both *in vitro* and *in vivo* as xenograft tumors in athymic nude mice [75]. Procaine, a local anesthetic and structural analogue of procainamide, acts as a demethylating agent in breast cancer cells, induces global DNA hypomethylation, methylation reversal and reactivation of *RARB2* [76]. A major drawback of these drugs is the high concentrations required for their demethylating activity, which can elicit undesired toxic effects if administered clinically.



**Fig. (4). Chemical structures of non-nucleoside analogues DNMT inhibitors.** RG108 is the only rationally designed DNMT1 inhibitor; all other compounds demonstrate DNMT inhibition at clinically irrelevant high concentrations and can trigger a variety of other biological effects.

The use of natural products in cancer chemoprevention is currently receiving much attention. Tea polyphenols are strong antioxidants and tea preparations demonstrate inhibitory activity against carcinogenesis [77-79]. (-)-Epigallocatechin-3-gallate (EGCG), the major polyphenol from green tea (Fig. 4), is a potent inhibitor of catechol-*O*-methyltransferase activity (COMT) [80, 81]. The structural similarity between DNMTs and COMT suggests possible inhibition of DNMTs by EGCG. EGCG inhibits DNMTs activity in KYSE 510 esophageal cells in a dose-dependent manner and induces re-expression of hypermethylated genes like *CDKN2A*, *RAR $\beta$*  and *MGMT*. EGCG demethylating activity does not involve competitive binding to CG rich regions of DNA; instead, it is mediated by direct binding to DNMT1 through hydrogen bonding [82, 83]. The binding of EGCG to other DNMTs has not been tested but is likely to occur because DNMTs share a highly conserved catalytic pocket. Genistein, a polyphenolic compound (Fig. 4), demethylates DNA and increases histones acetylation at the transcription start sites of *CDKN2A* and *p21<sup>WAF1</sup>* with consequent induction of gene expression [84, 85]. Additionally, genistein enhances gene re-expression when combined with trichostatin A or DAC [86]. A major concern associated with the use of natural products is product standardization. Multiple sources can provide extracts with different activities and therefore create discrepancies in their reported demethylating activity.

Rational design of DNMT inhibitors that interact non-covalently with the active catalytic site of DNMTs, utilizing

a three-dimensional model of the human DNMT1 catalytic pocket, is a sound alternative approach to silence the DNA methylation machinery. RG108 [2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-3-(1*H*-indol-3-yl)propanoic acid] is the first rationally designed DNMT1 inhibitor that demonstrates demethylating activity both *in vivo* and *in vitro* (Fig. 4) [87]. Unlike DAC and 5AC, RG108 inhibits DNMT1 in cell-free *in vitro* assay and is not cytotoxic to HCT116 cells, even at high concentrations. Successful derivatization of RG108 by attaching a biotin group to the C5 position of its tryptophan moiety is feasible [88]. This position is chosen based on a docking study of RG108 and DNMT1, where the C5 position of tryptophan protruded out from the active site pocket, indicating that modifying this position will not hinder the drug-enzyme interaction. Biotinylated-RG108 demonstrates inhibitory activity against DNA methylation *in vitro* and provides a valuable future tool for identifying proteins that interact and associate with DNMT1. RG108 inhibition of other DNMTs isotypes is not determined; however, it is likely to interact with all of them as they share a highly conserved catalytic pocket. Although RG108 reactivates several epigenetically silenced TSG, the compound does not demethylate heterochromatic centromeric repeats, in contrast to 5AC and DAC [87]. The preference of RG108 for euchromatic regions may suggest that euchromatic and heterochromatic sequences are methylated by distinct pools of DNMTs or may be attributed to the different catalytic properties of DNMTs. The continuous association of DNMT1 to centromeric heterochromatin in a replication-independent manner during the G2 and

M phase of the cell cycle [89] can represent a novel and separate pathway for maintaining DNA methylation and may contribute to the preferential demethylating activity of RG108 for euchromatic regions.

### DEMETHYLATING ACTIVITY OF DIFFERENT DNA DEMETHYLATING AGENTS

Direct quantitative comparison of the demethylating activity of the nucleoside analogues versus non-nucleoside analogues reveals the higher activity of the former in demethylation and gene re-expression [90]. The non-nucleoside analogues induce minimal demethylation activity when used at relevant pharmacological concentrations. RG108 is the only non-nucleoside analogue with comparable demethylating activity to zebularine but less active than 5AC and DAC. Also, RG108 demonstrates concentration-dependent demethylation activity similar to the nucleoside analogues [50]. Interestingly, treatment of leukemia cells with different nucleoside analogues (DAC, 5AC and zebularine) shows unnoticeable gene transcription signature, indicating a distinct effect of each DNMT inhibitor on the cellular transcriptome [91]. Moreover, the changes in gene expression are observed in several genes with no CpG islands around its promoter region, further emphasizing the off-target effects of these compounds.

### DEMETHYLATION-INDEPENDENT REVERSAL OF DNA METHYLATION EPIGENETIC SILENCING

Aberrant DNA methylation may not have intrinsic silencing properties because transcriptional silencing requires the

recruitment of methyl CpG-binding transcription factors. Therefore, targeting methyl CpG-binding proteins is a rational approach for reversing epigenetic transcriptional silencing without inducing CpG demethylation. In support of this, MBD2 protein depletion in cancer cells reactivates methylated genes without CpG demethylation [92]. Additionally, treatment of breast cancer cells with an HDAC inhibitor re-expresses methylated estrogen receptor gene without inducing CpG demethylation [93]. Furthermore, depletion of the transcriptional repressor Kaiso restores *CDKN2A (p16)* expression without CpG demethylation of its methylated allele, restores cell cycle arrest and sensitizes colon cancer cells to chemotherapy [94]. Taken together, targeting methyl CpG-binding proteins is an effective strategy in reversing the silencing effect of DNA methylation without reversing promoter methylation or inducing global hypomethylation with consequent genomic instability.

### STRATEGIES FOR COMBINING EPIGENETIC MODIFIERS

Different strategies of epigenetic therapy can be formulated based on the pleiotropic effects of these agents (Fig. 5). The successful outcome of using epigenetic modifiers as single agents in the treatment of cancer encourages their use in sequential or simultaneous combinations to harness their additive or synergistic effect on gene re-expression and cytotoxicity. Tumor resistance to chemotherapy is a major obstacle in cancer therapy. Epigenetic silencing of TSG disrupts the apoptotic machinery in cancer cells with consequent resistance development to chemotherapy. Pretreatment with DNMT or HDAC inhibitors can restore the expression of

**Fig. (5). Schematic diagram showing the possible use of epigenetic modifiers as a single agent or in combination with other agents and their mechanism of action.** Sequential treatment with epigenetic modifiers and chemotherapeutic agent can be administered using DNMT or HDAC inhibitors followed by chemotherapy to sensitize chemotherapy-resistant cells. Chemotherapy can be administered first to debulk the tumor followed by DNMT or HDAC inhibitors to induce differentiation of stem cells. Sequential treatment of DAC and HDAC inhibitors can also be used to induce optimum re-expression of TSG and apoptotic synergy.

TSG and sensitizes tumor cells to chemotherapeutic agents. Pretreatment of solid tumors *in vitro* with DAC sensitizes tumor cells to cisplatin treatment [95]. Also concomitant treatment with 5AC and doxorubicin induces synergistic cytotoxicity in multiple myeloma cells [96]. Sequential treatment of leukemia cells with DAC followed by cytarabine induces synergistic cytotoxicity. Surprisingly, cytarabine inhibits DAC induced global hypomethylation in leukemia cells, probably due to the selective killing of hypomethylated cells by cytarabine [97]. Conversely, sequential treatment with chemotherapy to debulk the tumor followed by DNMT or HDAC inhibitors to restore the differentiation program of chemotherapy resistant tumor-initiating cells is also conceivable. However, this approach is still not validated.

The combination of DNMT inhibitors and HDAC inhibitors is currently under investigation in several clinical trials. Sequential administration of DAC followed by the HDAC inhibitor TSA induces optimal re-expression of densely promoter-methylated genes, which can not be re-expressed by TSA alone [98]. This observation suggests a hierarchical organization of the different epigenetic modifications and incites the sequential use of DNMT inhibitors followed by HDAC inhibitors but not the reverse sequence. Interestingly, the sequential administration of DAC followed by different HDAC inhibitors in leukemia cells induces synergistic re-expression of p21, which lacks promoter CpG methylation [53]. Apoptotic synergy and DNA damage induction are also observed by the same sequential treatment with consequent p21 upregulation in a p53-dependent fashion. This effect highlights the importance of DNA damage as an off-target effect of epigenetic modifiers in regulating gene expression.

## CONCLUSION AND FUTURE DIRECTIONS

Several epigenetic modifiers are currently tested in clinical trials and some are already FDA approved. The mechanism behind the clinical efficacy of 5AC and DAC in hematological malignancies remains elusive. Although epigenetic reversal of DNA methylation is postulated as the mechanism of action, recent reports do not show an association of clinical response with methylation reversal of *CDKN2B* (p15) and other TSG [55, 99]. The diverse biological activities of these compounds may suggest the involvement of mechanisms other than methylation reversal like cytotoxicity, activation of immune response [100, 101] or induction of cellular senescence in the neoplastic clone [102] in mediating their clinical activity. Development of small molecule inhibitors that specifically inhibit different DNMT isotypes is a feasible strategy; however, the *in vivo* antitumor activity of these compounds needs to be demonstrated. Testing lead compounds like RG108 in the recently developed MDS mouse models [103-105] and other leukemia mouse models can predict their efficacy as anticancer agents and initiate the design of new molecular entities that specifically inhibit DNMTs based on structure activity relationship.

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