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Review

DNA Methylation as a Target for Drug Design

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DNA methylation is essential for normal embryonic development. Distinctive genomic methylation patterns must be formed and maintained with high fidelity to ensure the inactivities of specific promoters during development. The mutagenic and epigenetic aspects of DNA methylation are especially interesting because they may lead to the inactivation of genes which are involved in human carcinogenesis. The mutagenicity of 5-Methylcytosine (5mC) and the role of promoter hypermethylation in gene silencing, particularly in cancer, suggest a clinical significance for the design of novel DNA methylation inhibitors which may be utilized to reverse the effects of DNA methylation.

KEY WORDS: DNA methylation; 5-Methylcytosine; cancer; 5-Aza-2′-deoxycytidine.

INTRODUCTION

Genomic Methylation Patterns in Normal Cells

DNA methylation is an epigenetic mechanism which can directly alter the function of genes without changing the genetic code. The heritability of DNA methylation patterns and the influence of these patterns on gene expression, mutagenesis, and tumorigenesis have made this process a focus of interest in the fields of embryology and cancer biology. The presence of 5mC in DNA is necessary for embryonic development most likely due to its roles in transcriptional silencing, X-chromosome inactivation, and genomic imprinting. Cytosine methylation normally occurs at CpG dinucleotides which are represented at a lower than expected frequency in the eukaryotic genome, with the exception of regions of the genome known as CpG islands which have the statistically expected frequency of CpGs. These regions also remain unmethylated in the germline and rarely become methylated in normal somatic cells (1–2). Tissue-specific methylation patterns are established during embryonic development in a regulated fashion which entails demethylation and *de novo* methylation activities which are maintained during subsequent cell divisions through the action of (cytosine-5)-DNA methyltransferase.

Cytosine Methylation by DNA Methyltransferase

The eukaryotic (cytosine-5)-DNA methyltransferase catalyzes cytosine methylation at position 5 of the pyrimidine ring immediately after DNA synthesis. The fundamental chemistry of cytosine methylation was first proposed by Santi and colleagues (3), and the enzymatic mechanism has been further characterized in more recent studies (4–7). Although various prokaryotic and eukaryotic (cytosine-5)-DNA methyltransferases exhibit unique properties, such as, sequence-specificities, they share the following methyl-transfer reaction mechanism in common: First, a target cytosine is recognized and placed into the catalytic pocket of the methyltransferase enzyme. Next, the enzyme forms a covalent bond (via a conserved cysteine residue in its catalytic domain) at position 6 (C6) of cytosine. Formation of this bond activates the carbon at position 5 (C5) of cytosine and catalyzes the transfer of a methyl group from the cofactor S-Adenosylmethionine (AdoMet). Finally, a proton is abstract-ed from C5, and the enzyme dissociates from C6 as AdoMet is converted to S-Adenosylhomocysteine (AdoHcy).

Maintenance DNA methylation entails the addition of a methyl group to an unmodified cytosine within a hemimethylated CpG palindrome. In contrast, *de novo* methylation entails methylation at a CpG opposite an unmethylated CpG. Studies have revealed that eukaryotic DNA methyltransferases prefer hemimethylated target CpGs over unmethylated CpGs (7–9), and this distinguishes them from prokaryotic enzymes which do not discriminate strongly between hemi- and un-methylated target sequences. It has been proposed that the eukaryotic (cytosine-5)-DNA methyltransferase is capable of both maintenance and *de novo* methylase activities, however, an opposing theory suggests that an independent enzyme catalyzes *de novo* methylation. Recent evidence for this latter theory has been demonstrated by Lei *et al.* (10) who have shown that embryonic stem cells with a homozygously deleted methyltransferase gene (*Dnmt*) can *de novo* methylate both endogenous and exogenous DNA substrates *in vivo*. Nevertheless, the prevailing uncertainty regarding the existence of a eukaryotic "*de novo* methylase" has motivated intense research in this area.

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ABBREVIATIONS: 5-Methylcytosine; 5mC; S-Adenosylmethionine; AdoMet; S-Adenosylhomocysteine; AdoHcy; 5-Azacytidine; 5-Aza-CR; 5-Aza-2′-deoxycytidine; 5-Aza-CdR; Pseudoisocytidine; ΨICR; benzo-[a]-pyrene diol epoxide; BPDE: 1-β-D-Arabinofuranosylcytosine; ARA-C.

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Molecular biologists are also interested in determining whether a eukaryotic "demethylase" enzyme exists which actively demethylates DNA sequences. DNA demethylation is an active process catalyzed by a distinct DNA demethylase enzyme according to one theory, however, a contradictory theory proposes that demethylation occurs passively during site-specific events like gene transcription or DNA replication (11). Unfortunately, an enzyme which catalyzes CpG demethylation has not yet been cloned. The existence of independent enzymes responsible for either demethylation or *de novo* methylation is of biological significance because it may elucidate how tissue-specific methylation patterns are established during development. Identification and characterization of these activities may explain why organized genome-wide demethylation and *de novo* methylation events have been observed in mammalian cells during embryonic development (12–14).

DNA Methylation in Cancer

DNA methylation patterns change during tumorigenesis, resulting in global genomic hypomethylation and regional CpG island hypermethylation. These phenomena exist in addition to increased mutations at CpGs, therefore both the mutagenic and epigenetic effects of DNA methylation are believed to contribute to tumorigenesis. For example, 5mC represents an endogenous mutagen because it spontaneously deaminates to thymine (3), and this may explain why CpGs are mutational hotspots in the human germline (15). Since this phenomenon may exclusively explain the high frequency of transition mutations at target cytosines of methyltransferase in human cancer, it has been proposed that the DNA methyltransferase itself may also contribute to this phenomenon (16). With regard to the epigenetic effects of DNA methylation, the transcriptional inactivation of tumor suppressor genes via *de novo* methylation of CpG islands within their upstream regulatory sequences is believed to occur during the development of numerous cancers. Recent studies also suggest a role for DNA methylation in genetic instability (17). Investigating the normal functions of DNA methylation will facilitate the identification of abnormalities in this process which mediate the initiation and progression of human cancer. The mutagenic and epigenetic roles of DNA methylation in human cancer, and the therapeutic and pharmacological potential of DNA methylation inhibitors will be discussed in this review.

DNA METHYLATION ERRORS IN CANCER

The Role of DNA Methylation in Mutation

The eukaryotic DNA methyltransferase catalyzes maintenance methylation by recognizing and methylating hemimethylated CpG dinucleotide palindromes which are generated upon replication of fully methylated sequences. The propensity for target cytosines recognized by DNA methyltransferases to mutate at higher frequencies than other cytosines in the genome suggests that DNA methylation may contribute to mutagenesis. In fact, an estimated 31% of germline mutations which lead to genetic disorders can be attributed to 5mC $\rightarrow$ T transitions at CpG dinucleotides mediated by cytosine methylation (15–18). Whether the high frequency of C $\rightarrow$ T transitions in eukaryotic cells is attributed to 5mC deamination or to methyltransferase enzyme-mediated C $\rightarrow$ U $\rightarrow$ T mutations, however, still needs to be clarified.

The deamination of 5mC to thymine generates a G:T mismatch which is recognized and repaired by G/T mismatch thymine DNA glycosylase (19). If this enzyme is not 100% efficient, a C $\rightarrow$ T transition mutation is generated after one cell division. Such transition mutation may also arise via the spontaneous deamination of cytosine to uracil, generating a G:U mismatch which is recognized and repaired by uracil DNA glycosylase. An accumulation of G:U or G:T mismatches which escape repair by the appropriate uracil- (or thymine) DNA glycosylases will increase the frequencies of C $\rightarrow$ U $\rightarrow$ T (or C $\rightarrow$ T) transition mutations in the genome. The existence of C $\rightarrow$ T transition mutations may also be enhanced by the DNA methyltransferase enzyme which may block the repair of G:U mismatches by uracil DNA glycosylase. For example, we have demonstrated that the bacterial DNA methyltransferase *Hha*I exhibits a high affinity for target sites which contain a G:U mismatch which effectively blocks repair by uracil DNA glycosylase (20, 21). The bacterial DNA methyltransferase *Hpa*II with a mutation introduced into its AdoMet-binding domain was also shown to function as a mutator enzyme, facilitating cytosine deamination to uracil (22).

Additional studies have also confirmed that DNA methyltransferase may mediate 5mC$\rightarrow$T transition mutations at CpG dinucleotides under specific conditions which include increased DNA methyltransferase expression or decreased cellular AdoMet levels (20–22). Likewise, several bacterial (cytosine-5)-DNA methyltransferases are able to enzymatically induce C$\rightarrow$U or 5mC $\rightarrow$ T transition mutations *in vitro* when the cofactor AdoMet or the reaction product AdoHcy are limiting (23–25). The absence of the cofactor allows access of solvent water into the enzyme's catalytic center and the formation of an unstable enzyme-dihydrocytidine intermediate which readily undergoes hydrolytic deamination to uracil. The pathways of spontaneous hydrolytic deamination of 5mC and methyltransferase-mediated cytosine deamination are each illustrated in Figure 1.

It is not known whether eukaryotic DNA methyltransferases mediate a similar reaction or whether AdoMet-limiting conditions may exist in mammalian cells, which might lead to enzyme-mediated cytosine deaminations (26–27). Nevertheless, studies have shown that dietary methyl-deficiency in rats decreases AdoMet levels and increases AdoHcy levels various tissues (28). If such fluctuations in AdoMet and AdoHcy levels normally exist in mammalian cells, a decrease in AdoMet levels may lead to increased C $\rightarrow$ T transition mutations. This phenomenon provides a possible biochemical explanation for the contribution of methyl-deficiency to carcinogenesis. In fact, supporting evidence from other studies shows that methyl-donor starvation leads to DNA hypomethylation and increases the risk for liver and colon tumors (29–30). The influence of AdoMet-deficiency on the frequency of C $\rightarrow$ T transition mutations is also implicated by observations that enzyme-mediated cytosine deamination can be increased by cofactor analogs such as Sinefungin and 5′-amino-5′-deoxyadenosine which compete with AdoMet and AdoHcy for enzyme binding *in vitro* and inhibit DNA methylation (31). In conclusion, 5-Azacytidine (5-Aza-CR) has also been proposed to increase the rate of mutations at the target cytosine of DNA methyltransferase *in vivo* by covalently trapping the enzyme, facilitating C:G $\rightarrow$ G:C transversion mutations (32). Since all (cytosine-5)-DNA methyltransferases are believed to

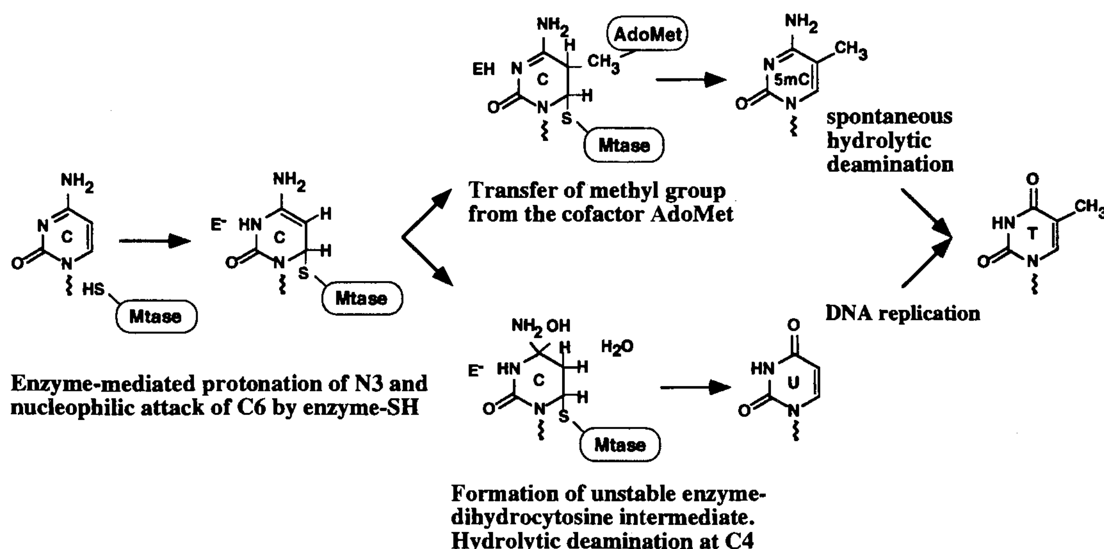

Fig. 1. The pathways of spontaneous hydrolytic deamination of 5mC (top) and methyltransferase-mediated cytosine deamination (bottom).

share a common reaction mechanism (21,33–35), eukaryotic methyltransferase enzymes may also contribute to the high frequency of transition mutations at CpG dinucleotides much like the enzymes in prokaryotic systems. It remains unclear, however, whether the high frequency of C → T transition mutations in eukaryotic cells can be explained entirely by spontaneous deamination of 5mC to T, or whether enzyme-mediated deamination mediates C → U → T mutations which also mediates mutagenesis at CpG sites.

Mutations in Tumor Suppressor Genes

Recent studies have also shown that CpG dinucleotides are hotspots for inactivating mutations in tumor suppressor genes (36). The classification of mutations which occur most frequently in certain tumor suppressor genes may elucidate which carcinogens and/or mutagens are responsible for the induction of specific cancers. The distribution of intragenic mutations in the p53 gene has been extensively studied, especially since this gene is mutated in more than 50% of solid human tumors (37). Results showed that an estimated 24% of *p53* point mutations identified in all tumors studied so far were C → T transitions at CpGs, suggesting that DNA methylation may contribute to these mutations (38). Subsequent studies demonstrated that mutational hotspots at CpGs were actually methylated in all tumor and normal specimens investigated (36,38–39). Additional studies by Denissenko *et al.* (40–41) involved the mapping of benzo[a]pyrene diol epoxide (BPDE) adducts along the p53 gene in plasmid constructs, and interesting results showed that BPDE adducts selectively occurred at guanines in CpGs of codons 157, 248, and 273 which are mutational hot spots in lung cancer. Adduct formation at CpGs (which may be methylated) may thus determine the *p53* mutational spectrum in lung cancer and further associates a distinct chemical carcinogen with a specific cancer. Methylated CpGs may not only represent endogenous mutagens which facilitate 5mC ↔ T transition mutations, but also potential hot spots for DNA damage via the selective formation of guanine-BPDE adducts.

Combined analyses of the *p53* mutational spectrum in various cancers demonstrated that 5mC deamination is more common in some cancers (including bladder, breast, and colon) than in lung cancer (42). In fact, exogenous substances and oxygen radicals may contribute to *p53* point mutations in lung cancer. The spectrum of mutations in the p53 gene has also been compared to that in the *p16* (CDKN2/MTS1) tumor suppressor which is mutated or deleted in both the germline and in primary tumors (39,43). Results showed that C ↔ T transition mutations at CpG sites varied dramatically for each gene, which might be due to the fact that the p16 gene (unlike *p53*) is not methylated in the germline. A model was therefore proposed in which C ↔ T transitions in a methylated p53 gene may occur more frequently during embryonic development than during tumorigenesis. In contrast, C ↔ T transitions in the predominantly unmethylated p16 gene were proposed to occur much more frequently in certain tumors than in the germline, possibly arising from abnormal methylation of the gene during tumorigenesis (42). DNA methylation may have alternative roles in tumorigenesis by facilitating transition mutations at CpGs either in the germline or in somatic cells, which varies among different genes. Comparing the broad mutational spectra of additional growth regulatory genes in the germline and tumor tissues may reveal early genetic events leading to specific cancers.

The Epigenetic Effects of DNA Methylation

The mechanisms which alter methylation patterns during tumorigenesis are still undefined. DNA methylation changes which have been observed in cancer include a genomewide decrease in 5mC content (44) and a localized hypermethylation of CpG islands (45). CpG islands which normally remain unmethylated in somatic cells are often associated with the promoters and/or coding regions of genes, and abnormal methylation of CpG islands within promoter sequences may result in gene inactivation. The mechanisms by which aberrant methylation patterns may inhibit gene expression are currently under investigation. For example, the effects of cytosine methylation

on gene transcription have been investigated *in vitro* utilizing methylated reporter gene constructs in cell free extracts, demonstrating that CpG methylation interfered with gene transcription (46–47). CpG island methylation within gene promoters may hinder transcription by attracting various repressor proteins or Methylated DNA Binding Proteins (MDBPs) which compete for methylated DNA sequences and prevent transcription factor binding (Fig. 2) (48–49). CpG methylation may also directly inhibit binding of transcription factors to promoter sequences, preventing transcription (Fig. 2) (49). Furthermore, the disruption of local chromatin structure by DNA methylation changes has also been proposed to perturb protein-DNA interactions and interfere with gene transcription (50–51). Current studies focus on defining the potentially causal role of CpG island hypermethylation in gene silencing.

Transcriptional repression via hypermethylation of promoter sequences suggests an alternative means for the inactivation of tumor suppressor genes in cancer, in addition to point mutations or gene deletions. DNA methyltransferase levels have been shown to increase in various cancers (52), possibly explaining the increased *de novo* methylation of CpG islands often observed during tumorigenesis. Enzymes which mediate either *de novo* methylation or demethylation may be responsible for the methylation changes often observed in cancer, but such activities have not been defined in eukaryotes. Numerous investigations have demonstrated that CpG islands within promoter sequences of growth regulatory genes undergo *de novo* methylation in both primary tumors and cancer-derived cell lines (Table I). For example, abnormal methylation of CpG islands of tumor suppressor genes in cancer have been reported for the Rb gene in retinoblastomas (53–54), the VHL gene in sporadic renal cell carcinomas (55), the estrogen receptor gene in breast cancers (56), the p15 (INK4B/MTS2) gene in leukemias (63), the p16 gene in human tumor cell lines (65–66), and the H19 gene in Wilms' tumor (69). Random methylation errors associated with tumorigenesis may occur which lead to the *de novo* methylation of tumor suppressor genes containing CpG islands in their promoters, resulting in decreased expression. Cells which accumulate these errors may further be selected for once they begin dividing rapidly, leading to tumor progression (73–74). Since studies have shown that frequent CpG island *de novo* methylation is associated with the immortalization of cell lines (75–76), methylation errors are believed to occur early during the neoplastic process. In agreement with Knudson's two-hit hypothesis (77), mutation or deletion of one tumor suppressor gene allele cou-

Table I. CpG Island Methylation in Tumor Cells

CpG island	Cell Type	Reference
Rb	Retinoblastoma	(53, 54)
VHL	Renal Cell Cancers	(55)
Estrogen Receptor	Various Cancers	(56–60)
E-Cadherin	Various Cancers	(61)
Myf3	Breast Cancer	(62)
p15	Leukemias	(63)
p16	Various Cancers	(64–68)
H19	Wilms' Tumor	(69)
Endothelin B Receptor	Prostate Cancer Cell Lines	(70)
Bcr-abl	Chronic Myelogenous Leukemia	(71)
BRCA1	Breast Cancer	(72)

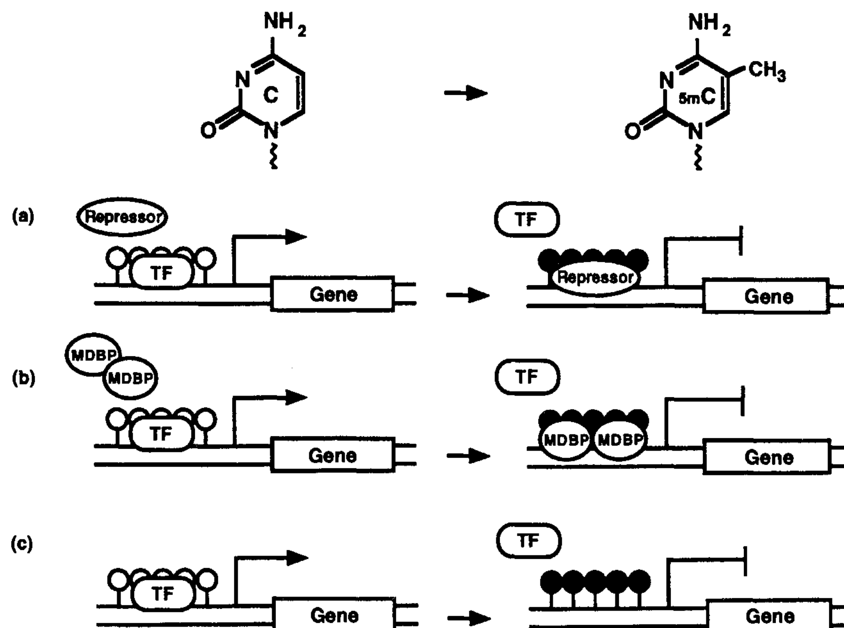

Fig. 2. Models for the effects of promoter CpG island methylation on transcription factor binding and gene transcription. Repressor proteins (a) or Methylated DNA Binding Proteins (MDBPs) (b) may compete with transcription factors for binding of methylated sequences, thus preventing transcription. CpG island methylation may also repel transcription factors (c) which normally bind unmethylated promoter sequences, again preventing transcription. Open circles represent unmethylated CpGs, and filled circles represent methylated CpGs.

pled with hypermethylation-based inactivation of the other allele may completely inactivate the gene and facilitate cell proliferation.

Recent studies have raised new questions regarding the role of methylation defects in gross chromosomal imbalances in cancer in which alterations in DNA methylation patterns have been proposed to contribute to genetic instability (17,78). Forms of genetic instability associated with tumorigenesis include point mutations, deletions, insertions, and the loss or gain of entire chromosomes. A recent study by Lengauer and colleagues (17) showed that exogenously introduced retroviral genes, which were not expressed, were *de novo* methylated in colorectal cell lines deficient in mismatch repair. In contrast, lines proficient in mismatch repair expressed the exogenous genes which did not become methylated, and these cells were termed "methylation deficient". The authors suggest that a "methylation defect" (defined by the inability to methylate exogenous substrates) may mediate the loss and gain of entire chromosomes, thus contributing to genomic instability in cancer. Since it has been shown that demethylation is often associated with chromosomal abnormalities such as chromosomal segregation and translocation (79–80), it is possible that these abnormalities exist in "methylation deficient" cells. The association between genetic instability and DNA methylation drawn from this study again illustrate the multiple roles DNA methylation may play during carcinogenesis.

DNA METHYLATION INHIBITORS

The diverse roles of DNA methylation in cancer have encouraged the search for therapeutic agents which inhibit DNA methylation. DNA methylation inhibitors may be utilized to reverse the effects of methylation, including the reduction of mutations at methylated CpGs and the reactivation of genes suppressed by hypermethylation. In fact, many studies of tumor suppressor genes silenced by hypermethylation have demonstrated that cytidine analogs can reactivate these genes *in vitro*. The cytidine analogs are potent, mechanism-based inhibitors of DNA methylation which are popular both clinically and experimentally. Additional agents which inhibit DNA methylation have also been investigated (Table II), however most of them have been tested only in experimental studies. Unfortunately, many of these agents exhibit nonspecific effects by inhibiting enzymes besides DNA methyltransferase, suggesting the need for new strategies to reduce genomic methylation levels. The following discussion describes the cytidine analogs in greater detail because their pharmacokinetics have been defined and because they have been studied extensively in clinical trials.

Mechanism-Based Inhibition of DNA Methyltransferases by Nucleoside Analogs

The nucleoside analog 5-Aza-CR was first synthesized in 1963 as a cancer chemotherapeutic agent (104), and clinical and

Table II. Inhibitors, Target Enzymes, and Mechanisms that Lead to Inhibition of DNA Methylation

Target enzyme	Mechanism of inhibition	Inhibitors (example)
(cytosine-5)-DNA-Methyltransferase	nucleotide analogs as mechanisms-based inhibitors of Mtase	5-azacytidine (5-Aza-CR) (81), 5-azadeoxycytidine (5-Aza-CdR)(81), 5-Fluoro-2′-deoxycytidine (81), Pseudoisocytidine (81), Pyrimidinone (82)
	reduction of mRNA of Mtase	antisense mRNA (83) and oligonucleotides (84)
	competitive inhibition	AdoHcy (85), Sinefungin and analogs (31, 86–88), 5′-deoxy-5′-S-isobutyl-adenosine (SIBA) (87), 5′-methylthio-5′-deoxyadenosine (MTA) (31, 89, 90)
Dihydrofolate Reductase	reduction of AdoMet synthesis	Methotrexate (91, 92) and analogs (93)
AdoMet Synthase	reduction of AdoMet synthesis	Ethionine (90, 94, 95), L-cis-AMB (90), Cycloleucine (90)
AdoHcy Hydrolase	AdoHcy accumulation	Neplanocin A (93), 3-deazaneplanocin (93), 4′-thioadenosine (96), 3-deaza-aristeromycin (97)
Ornithine Decarboxylase	AdoMet accumulation	Difluoromethylornithine (DFMO) (98)
Methylthioadenosine-Phosphorylase (MTAP)	MTA accumulation	Difluoromethylthioadenosine (DFMTA) (90)
Spermidine Synthase	chromatin structure	S-methyl-5′-Methylthioadenosine (MTA), L-cisAMB, AdoDATO, MGBG (93, 99, 100)
Glutathione-S-Transferase	reduction of AdoMet synthesis	(101)
Others	various mechanisms	Sodium Butyrate (90), Procainamide (102), Phenobarbital (103)

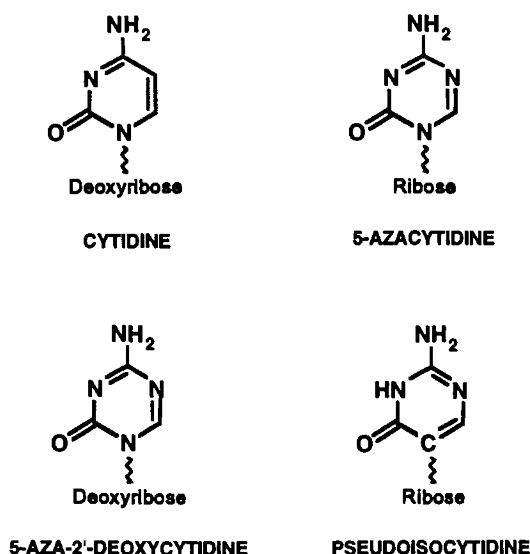

Fig. 3. Structures of cytidine and its analogs: 5-Azacytidine, 5-Aza-2′-deoxycytidine, and Pseudoisocytidine. Modified from Jones and Taylor (81).

preclinical trials with this agent began in the United States in 1970. Since 5-Aza-CR and its deoxy analog 5-Aza-CdR were shown to be chemically unstable and susceptible to hydrolysis in neutral or basic solutions (105), Pseudoisocytidine (ΨICR) (with a more stable ring system) was subsequently developed (106). The structures of cytidine and these analogs are illustrated in Figure 3. The cytosine rings of 5-Aza-CdR, 5-Aza-CR, and ΨICR all contain nitrogen atom substitutions in position 5. Clinical studies with the cytidine analogs demonstrated that nausea, vomiting, and leukopenia are their dose-limiting toxicities, and ΨICR are all similar because they exhibited the most extreme side effects in patients. Therefore, most subsequent studies have utilized either 5-Aza-CR or 5-Aza-CdR for their antitumor activities in patients with leukemia (107–109). The nucleoside analogs were introduced into studies of DNA methylation in biological systems once they were shown to inhibit DNA methylation (81).

5-Aza-CR and 5-Aza-CdR inhibit DNA methylation by reducing the biochemical activity of DNA methyltransferase via the formation of a covalent complex with this enzyme (Fig. 4) (110–111). The nature of the interaction between the DNA methyltransferase and its target DNA is currently being elucidated, in fact, it has been shown that HhaI cyt-5-DNA methyltransferase actually flips its target base out of the DNA while it methylates its substrate (112). The capacities for 5-Aza-CR and 5-Aza-CdR to covalently trap the DNA methyltransferase may explain why substitution of only 5% of incorporated cytosines in substrate DNA with 5-Aza-CdR reduced DNA methylation levels in excess of 80% (110–113). Drug-induced inhibition of DNA methyltransferase results in the formation of hypomethylated DNA, a state which is heritable for subsequent cell divisions following drug treatment.

Studies have demonstrated that 5-Aza-CdR (or 5-Aza-CR) must be phosphorylated to its nucleotide form by deoxycytidine kinase (or uridine-cytidine kinase) and subsequently incorporated into replicating DNA to inhibit DNA methyltransferase (114–116). Since its activity is limited to organs undergoing

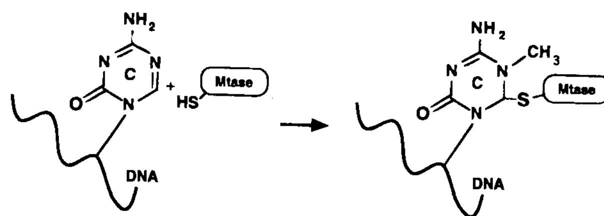

Fig. 4. Mechanism of inhibition of the eukaryotic DNA methyltransferase by 5-Aza-2′-deoxycytidine-containing DNA in the presence of AdoMet. This mechanism was revised by Gabbara and Bhagwat (115).

rapid cellular proliferation, the toxicity of 5-Aza-CdR is restricted to cells entering S-phase because it incorporates into replicating DNA (117). Earlier studies of the cytotoxic effects of 5-Aza-CdR also showed that 5-Aza-CdR did not impair DNA, RNA, or protein synthesis at concentrations which produced 50% cell kill *in vitro* (118). The cytotoxic effects of 5-Aza-CdR *in vitro* therefore do not result from the immediate inhibition of macromolecular synthesis. This idea is consistent with the hypothesis that the toxicity of 5-Aza-CdR may arise from the covalent trapping and cellular depletion of the DNA methyltransferase (119). In contrast, 5-Aza-CR incorporates into RNA while inhibiting protein synthesis (120) although approximately 10% of 5-Aza-CR is converted into 5-Aza-CdR and incorporated into DNA (121). Unlike 5-Aza-CdR, 5-Aza-CR may exhibit additional toxicity in organs with lower proliferative activities because it is not S-phase specific as it incorporates into RNA (122). In conclusion, 5-Aza-CdR is a more potent inhibitor of DNA methylation than its ribo-analog.

Although 5-Aza-CR and 5-Aza-CdR significantly reduce genomic DNA methylation levels and represent promising antitumor agents for the treatment of cancers which arise from methylation errors, these agents are both cytotoxic and mutagenic *in vitro* and *in vivo*. Earlier studies by Michalowsky and Jones (123) entailed the establishment of cell lines which were either sensitive or resistant to the effects of 5-Aza-CdR. Experimental evidence showed that the cell lines more sensitive to 5-Aza-CdR had a greater total cellular 5mC content which resulted in the formation of more hemimethylated CpG sites, favoring nuclear protein binding. This increased sensitivity may be due to the increased binding of DNA methyltransferase to 5-Aza-CdR-containing DNA. More recent studies have also shown that both embryonic stem cells and embryos with reduced DNA methyltransferase levels are more resistant to the toxic effects of 5-Aza-CdR (119), further suggesting DNA methyltransferase levels may mediate the cytotoxicity of 5-Aza-CdR. Despite the evidence that 5-Aza-CdR reduces intestinal neoplasia in Min mice (which harbor a cancer predisposing mutation) (124), these phenomena suggest the need for less toxic agents which target the DNA methylation machinery for the treatment of cancer.

Numerous studies have also shown that altering DNA methylation patterns with either 5-Aza-CR or 5-Aza-CdR can profoundly change the differentiation state of cell lines (153). For example, 5-Aza-CR induces the formation of functional fat, muscle, and cartilage cells from mouse embryonic cells in culture (125). The induction of new cell types and the inhibition of DNA methylation were also dependent on the concentrations of 5-Aza-CR or 5-Aza-CR used. Finally, these same cytidine

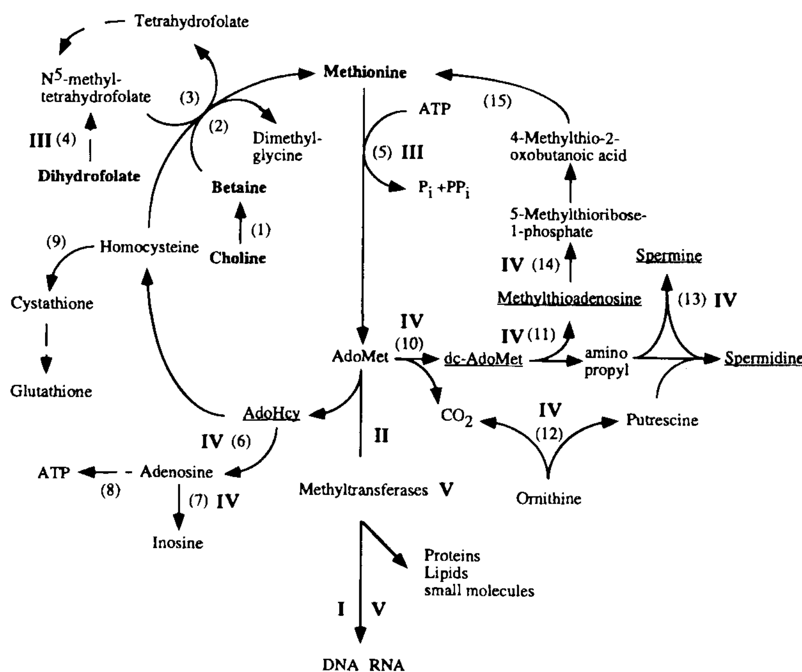

Fig. 5. The metabolism of S-Adenosylmethionine and the targets affecting DNA methylation. Interference with the enzymes (1)–(15) involved in the metabolism of AdoMet can affect DNA methylation either by limiting AdoMet synthesis or by favoring the accumulation of metabolites with inhibitory activity (underlined). Enzymes are: (1) choline dehydrogenase, (2) betaine-homocysteine methyltransferase, (3) methionine synthase, (4) dihydrofolate reductase, (5) AdoMet synthase, (6) AdoHcy hydrolase, (7) adenosine deaminase, (8) adenosine kinase, (9) cystathione β-synthase, (10) AdoMet decarboxylase, (11) spermidine synthase, (12) ornithine decarboxylase, (13) spermine synthase, (14) methylthioadenosine phosphorylase (MTAP), (15) transaminases. **I–V**: The targets of inhibitors affecting DNA methylation as listed in Table II. **Bold:** Insufficient supply of choline, folate, vitamine B12 and methionine can result in hypomethylation of genomic DNA. Underlined: Accumulation of AdoHcy, dc-AdoMet, MTA, spermine and spermidine can result in hypomethylation of genomic DNA. Adopted from Zingg and Jones (138).

analogs with cytosine ring substitutions in position 5 (Fig. 3) induced similiar developmental changes in mouse embryonic cells, again correlating with their abilities to inhibit DNA methylation (81). Since DNA methylation ultimately functions to suppress gene expression in eukaryotic cells, it was proposed that alterations in DNA methylation patterns repress cell lineage-determining regulatory genes which modulate specific pathways of cellular differentiation in leukemic cells. In fact, 5-Aza-CdR was shown to induce *in vitro* differentiation of myeloid leukemic cell lines (126) and to exhibit antileukemic effects in patients with myeloid leukemia in phase I trials (127–128).

Inhibition of DNA Methyltransferases By Targeting Metabolic Pathways of S-Adenosylmethionine

Additional compounds which inhibit DNA methylation are listed in Table II. Many of these agents inhibit DNA methylation as competitive inhibitors of AdoMet, inhibitors of AdoMet synthesis, or inhibitors of AdoHcy metabolism to target AdoMet-dependent methyltransferases. Inhibition of methyl-transfer and decarboxylation reactions by analogs and metabolites of AdoMet has been demonstrated in numerous studies (86,129–130), and evidence for the role of aberrant AdoMet metabolism in carcinogenesis has recently motivated the design of new agents which target the cofactor AdoMet. Figure 5 illustrates the multiple pathways of AdoMet metabolism which represent potential targets for the inhibition of DNA methylation. Numerous possibilities exist which can alter AdoMet metabolism. Unfortunately, interference with AdoMet metabolism may yield nonspecific effects on other cellular methyl-transfer reactions which require AdoMet, including spermidine and spermine synthesis (131) which require AdoMet as the single aminopropyl-donor utilized (Fig. 5). The clinical utility of such compounds which interfere with all AdoMet-dependent methyl-transfer reactions is therefore limited. The following approaches to inhibit DNA methylation by interference with AdoMet-dependent methyltransferases will be described in this section: (i) inhibition of AdoMet biosynthesis, (ii) competitive inhibitions of AdoMet-dependent methyltransferases, and (iii) inhibition of AdoHcy metabolism.

One approach to indirectly reduce genomic DNA methylation levels is to inhibit AdoMet synthesis via alterations of AdoMet synthase (132). Inhibition of this enzyme should decrease intracellular AdoMet levels and mediate the inhibition of all AdoMet-dependent enzymes, particularly DNA methyltransferase. The absence of the methyl-donor AdoMet will ultimately prevent DNA methylation. Analogs of L-Methionine

have been investigated as potential inhibitors of AdoMet synthase (133–135), and the most potent inhibitors of AdoMet synthase were shown to effectively decrease AdoMet levels and increase L-Methionine levels *in vivo* (136). Unfortunately, the utilization of L-Methionine analogs as inhibitors of AdoMet synthase is discouraged because AdoMet synthase has a high structural-specificity for methionine, and inhibition of AdoMet synthase may ultimately inhibit all AdoMet-dependent enzymes and exhibit cytotoxic effects.

S-Adenosylmethionine analogs have been investigated as competitive inhibitors of AdoMet-dependent methyltransferases to inhibit DNA methylation (86,137). For example, Sinefungin and its derivatives represent effective competitors of AdoMet-dependent methyltransferases (31,137–138). In our laboratory we have demonstrated that these agents not only compete with AdoMet for binding free enzyme, but also inhibit the methyltransfer reaction catalyzed by DNA methyltransferase (31). Experiments to determine if Sinefungin or its derivatives inhibit DNA methylation by changing the affinities of methyltransferase-cofactor complexes for their target sequences have also been performed (31), and these agents were shown to inhibit DNA methylation by perturbing AdoMet interactions with the methyltransferase instead of preventing methyltransferase binding to target sequences. The high specificity of AdoMet-dependent methyltransferase has unfortunately restricted the development of additional AdoMet analogs, and the use of AdoMet analogs for the exclusive inhibition of DNA methylation has been discouraged because these compounds may exhibit nonspecific effects on other enzymes which require AdoMet for their activities, including AdoMet decarboxylase and AdoHcy hydrolase (87–88).

The AdoHcy regulatory mechanism has also been investigated for the design of methyltransferase inhibitors. Approaches which lead to the intracellular accumulation of AdoHcy (via alteration of AdoHcy metabolism) should result in the negative-feedback inhibition of AdoMet-dependent methyltransferases. This can be achieved via the inhibition of AdoHcy hydrolase which catalyzes the reversible hydrolysis of AdoHcy to adenosine and homocysteine via an oxidative-reduction mechanism (139). Inhibitors of AdoHcy hydrolase are listed in Table II. Various structural analogs of AdoHcy have been investigated for their abilities to inhibit AdoMet-dependent methyltransferases. If the AdoHcy binding sites of different methyltransferases are unique, systematic alterations of the structure AdoHcy may generate an agent which is specific for AdoMet-dependent DNA methyltransferases. In conclusion, novel agents to inhibit DNA methylation should be developed which are chemically stable and specific for the DNA methyltransferase even prior to its interaction with DNA.

CLINICAL AND PHARMACOLOGICAL IMPLICATIONS OF DNA METHYLATION INHIBITORS

Clinical Studies of 5-Aza-2′-Deoxycytidine

The numerous roles of DNA methylation in cancer renders this process a potential target for therapeutic intervention, and further characterizing the epigenetic aspects of DNA methylation will facilitate the development of new agents which may reverse a disease state by reactivating genes abnormally silenced by hypermethylation. The thorough investigations of 5-Aza-CR and 5-Aza-CdR in previous clinical trials should also facilitate the approval of new studies which use these agents to specifically target DNA methylation in solid tumors, particularly since these agents are effective in the treatment of leukemia. Unfortunately, the mutagenic, cytotoxic, and chemically-unstable properties of cytidine analogs discourage their widespread clinical use, and patients with methylation defects within specific cancer-predisposing genes must be pre-screened and pre-selected before therapies with demethylating agents can be guaranteed practical and effective.

Tumor suppressor genes which become inactivated in cancer via *de novo* methylation suggest a specific use for demethylating agents to target tumor cells which have acquired abnormal methylation patterns. Studies of tumor suppressor genes which are hypermethylated in certain tumors show that they can be reactivated by 5-Aza-CR or 5-Aza-CdR, thus justifying the renewed interest in DNA methylation as a potential target for drug design. We have recently shown that 5-Aza-CdR suppresses cellular growth in numerous human tumor cell lines after recovery from the toxic effects of this agent, and that this phenomenon was often associated with reactivation of the *p16* growth regulatory gene (140). These observations suggest the potential usage of demethylating agents during cancer chemotherapy to possibly reactivate dormant growth regulatory genes (either alone or in combination with other undefined genes) silenced by *de novo* methylation in solid tumors to restore growth control to rapidly proliferating cells (Fig. 6). 5-Aza-CdR-mediated growth suppression and gene activation were also heritable for numerous cell population doublings following 5-Aza-CdR treatment (80,140), suggesting that demethylating agents may have long-term therapeutic effects on patients.

Rivard and Momparler (108) reported some of the earliest clinical studies of 5-Aza-CdR as an antileukemic drug. These studies among others were designed to establish which concentrations of 5-Aza-CdR were least cytotoxic, yet clinically effective (108–109,141–142). Preliminary results showed that 5-Aza-CdR significantly reduced the levels of circulating blasts in pediatric leukemia patients, while systematic dose increases frequently induced complete remissions (142). Consequently, more clinical trials were initiated to focus on the cytotoxicity and clinical pharmacology of this agent in the treatment of leukemia. These studies altogether demonstrated that 5-Aza-CdR is an effective antineoplastic agent in the treatment of acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL). The mechanism of 5-Aza-CdR's antileukemic activity is not clearly defined, but it is believed to entail the reactivation of repressed genes by inhibiting DNA methylation in leukemic cells which regulate cellular differentiation, senescence, and apoptosis. Although 5-Aza-CdR represents a potent antileukemic agent, new approaches to improve its clinical potency are still being investigated. Optimization of dose schedules and working concentrations of 5-Aza-CdR relies on: (i) the drug's cellular metabolism (via activation or inactivation by cellular enzymes), (ii) the drug's pharmacokinetic properties, and (iii) the drug's cell cycle specificity.

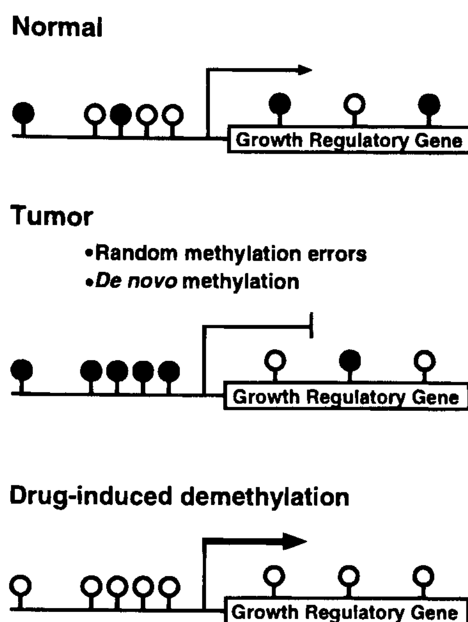

Fig. 6. Models for the acquisition of aberrant DNA methylation patterns in tumor cells and the drug-induced demethylation of hypermethylated promoter CpG islands. CpG island hypermethylation within gene promoters may inhibit the transcription of growth regulatory genes and lead to increased cell proliferation. Demethylating agents may be utilized to reverse these effects and reactive dormant growth regulatory genes, possibly restoring growth control to tumor cells. Open circles represent unmethylated CpGs, and filled circles represent methylated CpGs.

Pharmacological Studies of 5-Aza-2′-Deoxycytidine

Understanding the cellular metabolism of 5-Aza-CdR is essential for determining which drug concentrations should be utilized during therapy. For example, fluctuations in the intracellular pools of deoxynucleotides may influence 5-Aza-CdR metabolism (143–144). Cytidine deaminase modulates the metabolism of deoxynucleotides by regulating intracellular dCTP and dTTP levels, resulting in decreased dCTP levels and increased dTTP levels (147). Furthermore, dTTP has been observed to decrease intracellular dCTP levels via feedback inhibition of both ribonucleotide reductase and dCMP deaminase (146–147). Interest in cytidine deaminase for the purposes of clinical studies resulted from evidence that this enzyme inactivates 5-Aza-CdR, likewise, cells with elevated cytidine deaminase levels may be insensitive to 5-Aza-CdR. Inhibitors of this enzyme are currently under investigation to overcome the problem of drug resistance which varies among patients and may arise from increased cytidine deaminase activity in target cells.

Defining the cellular mechanisms of 5-Aza-CdR activation, much like its inactivation, is also crucial for the optimization of therapeutic drug concentrations. Studies have shown that deoxycytidine kinase phosphorylates 5-Aza-CdR to its active nucleotide form (5-Aza-dCTP) preceding its incorporation into replicating DNA. This explains why cells deficient in deoxycytidine kinase are resistant to 5-Aza-CdR and why increased intracellular dCTP levels influence drug resistance, especially since dCTP is a feedback inhibitor of deoxycytidine kinase (148–149). dCTP also competes with 5-Aza-CdR for DNA polymerase during replication to further decrease its efficacy. In summary, cellular drug resistance to 5-Aza-CdR can be influenced by decreased deoxycytidine kinase levels, increased dCTP levels, or increased cytidine deaminase levels (150).

The pharmacokinetics of 5-Aza-CdR must also be considered when designing dose schedules for the treatment of leukemia because the drug's clinical efficacy and toxicity both depend on plasma concentrations of this agent. Since 5-Aza-CdR has a relatively short half life, continuous i.v. infusions should be utilized to maintain its steady state in the plasma. The steady state plasma concentration should also surpass its minimal cytotoxic concentration. 5-Aza-CdR is most unstable under alkaline conditions and undergoes a rapid and reversible cleavage between positions 1 and 6 of the azacytosine ring (151). Most clinical studies of 5-Aza-CdR have adopted continuous infusion schedules based on its chemically instability, and the first continuous dose schedules were initially modeled after those optimized during leukemia therapy with the related deoxycytidine analog 1-β-D-Arabinofuranosylcytosine (ARA-C) (152).

Studies by Skipper et al (153) demonstrated that the antileukemic activity of ARA-C in murine leukemia models was strictly schedule-dependent. This compound was also a potent antileukemic agent, however 5-Aza-CdR was shown to be more effective in the treatment of mice with L1210 leukemia (150,152,154). This phenomenon may be explained by the different mechanisms of action utilized by these S-phase specific compounds: 5-Aza-CdR inhibits DNA methylation by covalently trapping the DNA methyltransferase, whereas ARA-C directly inhibits DNA replication and blocks cell cycle progression. Finally, the cell cycle profile of the target cell population represents an important parameter during the optimization of drug treatment schedules because cells must be entering S-phase for 5-Aza-CdR to be effective.

Combination Therapy

New approaches are also being investigated to counteract drug resistance to 5-Aza-CdR, and effective combination therapy with 5-Aza-CdR and other antileukemic agents is promising. Since resistance to 5-Aza-CdR may arise from an increased pool of dCTP (which competes with 5-Aza-CdR for the catalytic domain of DNA polymerase) 3-deazauridine (3-DU) and cyclopentyl-cytosine (CPC) may be used to inhibit the activity of CTP synthase because these agents reduce the cellular pools of dCTP (155–156). These agents should therefore enhance the activity of 5-Aza-CdR, especially in combination with inhibitors of cytidine deaminase which help prevent the inactivation of 5-Aza-CdR. The resistance to 5-Aza-CdR therapy observed in some patients may occur due to differential deletion, mutation, and hypermethylation of various tumor suppressor genes in different individuals. The antileukemic capacities of 5-Aza-CdR and 5-Aza-CR may be attributed to the drug-induced hypomethylation of specific genes during future studies, especially as tumor suppressor genes silenced by *de novo* methylation are identified in numerous cancers.

CONCLUSION

Although DNA methylation is essential for normal embryonic development, this process may also contribute to genomic

abnormalities. First, 5mC is intrinsically mutagenic because it can spontaneously deaminate to thymine and second, gene transcription can be inhibited via the abnormal methylation of promoter sequences. These mutagenic and epigenetic phenomena facilitate the inactivation of genes which may lead to carcinogenesis or possibly the onset of other genetic diseases. The design of novel agents which target the DNA methylation machinery should therefore have both therapeutic and pharmacological utility.

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