Trends in Cancer Research
Vol. 12, 2017

Review

DNA methylation and breast cancer: Mechanistic and therapeutic applications

Niaz Mahmood and Shafaat A. Rabbani*

Department of Medicine, McGill University Health Centre, 1001 Décarie Boulevard, Montréal, QC, H4A 3J1,Canada.

ABSTRACT

DNA methylation is an epigenetic mechanism that orchestrates many of the abnormal gene expression changes seen in cancer without altering the actual genomic DNA sequences. Emerging evidence support that aberrant DNA methylation is an archetypal hallmark of cancer, and hypermethylation-mediated inactivation of tumor suppressor genes as well as hypomethylation-mediated activation of prometastatic genes are common attributes of cancer cells. Since methylation of DNA is reversible, targeting the methylome may serve as a suitable anti-cancer strategy. Several epigenetic drugs especially the ones which target the key enzyme in DNA methylation process, the DNA-methyltransferases (DNMTs), have shown promising results in clinical trials. Two DNMT inhibitors, 5-azacytidine (Vidaza®) and 5-aza-2′-deoxycytidine (Decitabine, Dacogen®), have already been approved by the Food and Drug Administration (FDA) for treating several types of cancer. Nevertheless, targeting hypermethylation through the use of DNMT inhibitors can also activate several prometastatic genes apart from the activation of tumor suppressor genes. This may lead to metastasis which is the primary cause of morbidity and mortality associated with solid tumors like breast cancer. So the anti-cancer strategies require a balance between the activation of tumor suppressor genes and repression of prometastatic genes to collectively block tumor growth and metastasis. This review describes some of the common methylation abnormalities seen in promoters of cancer-associated genes, the mechanism of action of various hypermethylation and hypomethylation inhibitors, the potential benefits and challenges of using them as anti-cancer therapeutic agents in general and for patients with breast cancer in particular as monotherapy or in combination settings.

KEYWORDS: hypermethylation, epigenetics, cancer, hypomethylation, anti-cancer drugs, DNMT inhibitors, SAM

*Corresponding author: shafaat.rabbani@mcgill.ca

1. Epigenetics and cancer: An introduction

The identity of a cell is determined by its genetic makeup along with the epigenetic marks which dictate how the genetic information will eventually be read and interpreted [1]. In tumor cells, these genetic and epigenetic codes are often altered in such a way that the information within the genome is read in a completely different manner than the normal cells so as to develop and promote various characteristics associated with cancer. Some of the most common forms of genetic alterations seen in cancer include mutation, amplification, and deletion within the genome while changes in DNA methylation, histone modifications, and microRNAs constitute the three main types of epigenetic alterations found in the cancer genome (Figure 1).

Despite its first identification in the early 1980s [2], the epigenetics of human cancer has greatly been over-shadowed by human cancer genetics. With the passage of time, as our understanding of the epigenetic mechanisms and their role in regulating

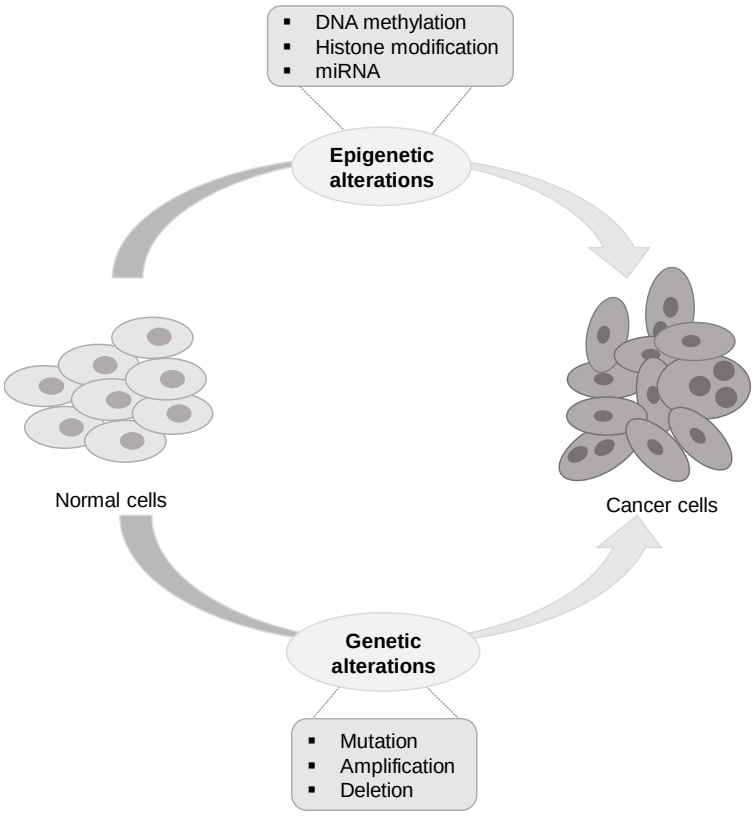

Figure 1. Common genetic and epigenetic aberrations in cancer. Genetic alterations including mutation, amplification, and deletion within the genome as well as epigenetic alterations in DNA methylation, histone modifications and microRNAs change the identity of the cells and a normal cell can become cancerous during the process.

gene expression patterns during the development and progression of cancer became clear, the idea of targeting the epigenome as an anti-cancer strategy became apparent. In this regard, targeting the epigenome gained a great deal of attention over the last two decades. A plethora of epigenetic drugs (Epi-drugs) has been shown to be effective as anti-cancer agents in preclinical and clinical settings with a number of them being already approved for treatment of several types of liquid tumors [3]. Notwithstanding the fact that all three types of epigenetic alterations are important, this review, however, is mainly focused on various aspects of DNA methylation abnormalities seen in cancer and how they are targeted by some of the well-known anti-cancer agents to treat solid tumors like breast cancer.

2. A general overview of DNA methylation

DNA methylation is a type of covalent modification in which methyl (-CH_3) groups are added to specific nucleotides of the genomic DNA (gDNA). In living systems, this reaction is catalyzed by DNA methyltransferase (DNMT) enzymes where universal methyl group donor S-adenosylmethionine (SAM) acts as a cofactor (Figure 2) [4, 5]. The presence of DNA methylation was first reported by Rollin Douglas Hotchkiss in 1948 shortly after the famous trio of Avery–MacLeod–McCarty identified DNA as the genetic material [6, 7]. However, it was during the late 1970s to early 1980s, almost three decades after its initial discovery, several groups reported that DNA methylation is involved in cell differentiation and gene regulation [8, 9]. Since then, a plethora of studies on its role in gene regulation followed and at present DNA methylation is regarded as one of the major mechanisms of gene regulation.

2.1. DNA methylation is evolutionarily primitive

Although DNA methylation is a widespread modification found in bacteria, plants, and

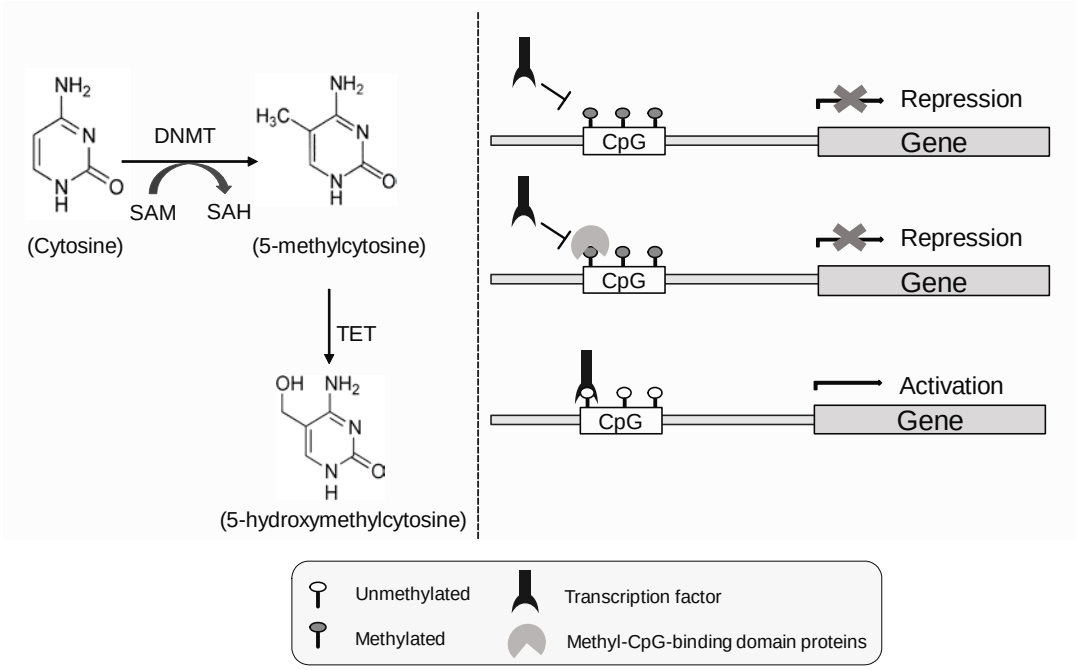

Figure 2. DNA methylation and its role in gene expression. The chemical reaction in the left panel shows the process of methylation and demethylation of cytosine residues. At the molecular level, when cytosine is converted to 5-methylcytosine (5mC) by DNA methyltransferase (DNMT) two things may happen: (i) it may directly inhibit transcription factors (TFs) binding to the CpG islands to repress transcription, or (ii) it may attract the binding of a methyl-CpG-binding protein to methylated CpG islands which recruits other repressive proteins at the site to prevent the access of TFs and thereby suppress transcription. When the methylation mark is removed by TET family of proteins, demethylation takes place. This likely allows the TFs to bind to the CpG sequences and cause activation of gene expression. Here, TET, Ten-eleven translocation (TET) family of proteins; SAM, S-adenosylmethionine; and SAH, S-adenosylhomocysteine.

mammalian species, there is a high degree of phylogenetic variability in the patterns of methylation across species [10]. In prokaryotes, methylation can take place on adenine and cytosine residues of the DNA and play roles in processes like the initiation of DNA replication, DNA repair, cell cycle-coupled transcription and protection against foreign DNA [11]. In mammals, methylation predominantly occurs on the carbon present at the 5th position of cytosine residues (5-mC) within the cytosine-phosphate-guanine (CpG) dinucleotides [12]. In plant and fungal genomes, methylation commonly occurs at the CpH and CpHpH ('H' stands for bases other than 'G') regions [12]. In eukaryotes, DNA methylation plays a role during development, silencing of retroviral elements, maintenance of genome integrity, imprinting, lyonization/X-inactivation and gene expression regulation [5, 13]. Recent evidence suggests that the genome of the nematode worm *Caenorhabditis elegans*, which was previously considered to be devoid of methylation, is actually methylated on the exocyclic -NH_2 groups at the 6th position of the purine ring in adenines (6-mA) [14]. Taken together, this suggests that DNA methylation is indeed an evolutionarily ancient regulatory mechanism [15].

2.2. DNA methylation writers, readers, and erasers: Role in gene regulation

In mammals, there is a specific distribution pattern of the CpG islands in the context of the whole genome where a higher propensity of CpG-rich regions is found near the promoters of up to 70% of the genes [16, 17]. Non-CpG methylation is less prevalent in mammalian species and is primarily present in the embryonic stem cells [18]. Methylation at the CpG islands has been causally linked to transcription regulation, where the promoters of

transcriptionally repressed genes are CpG-methylated and the promoters of transcriptionally activated genes are unmethylated at the CpG islands (Figure 2) [19].

There are three major DNMTs that catalyze the transfer of $-CH_3$ groups in humans which include DNMT1, DNMT3a and DNMT3b [20-23]. S-adenosylmethionine (SAM) acts as the $-CH_3$ group donor for all three enzymes [4]. DNMT1 is called 'maintenance methyltransferase' because of its preference for hemimethylated (newly synthesized) DNA *in vitro*, and it mainly plays a role in the methylation of the newly synthesized strand of DNA during cell division [24]. On the other hand, DNMT3a and DNMT3b have the ability to transfer the methyl moiety to both unmethylated and methylated DNA at an equal rate and are therefore called '*de novo* methyltransferases' [25]. The DNMTs are called 'writers' of methylation as they are the ones that copy the $-CH_3$ group on the cytosine residues of a CpG dinucleotide. In general, methylation at the CpG island may either directly interfere with the binding of transcription factors at the regulatory site to cause transcriptional repression [26], or attract the binding of methyl-CpG-binding proteins which subsequently recruits different types of histone deacetylase (HDAC) complexes and chromatin remodeling factors that causes chromatin compaction ultimately leading to gene repression (Figure 2) [27]. These proteins can sense or read the methylation at the CpG site and therefore they are known as the 'readers' of methylation. Three families of methyl-CpG-binding proteins are found in vertebrates. These include methyl-CpG binding domain (MBD) proteins (MBD1; MBD2; MBD3; MBD4; methyl-CpG binding protein 2, MeCP2), Kaiso family proteins (Kaiso; zinc finger and BTB domain containing 4, ZBTB4; zinc finger and BTB domain containing 38, ZBTB38) and SRA domain proteins (ubiquitin-like containing PHD and ring finger domains 1, UHRF1; ubiquitin-like containing PHD and ring finger domains 2, UHRF2) [13, 28]. There are some enzymes like ten-eleven translocation (TET) methylcytosine dioxygenase family, activation-induced cytidine deaminase (AID), and thymine DNA glycosylase (TDG) that have been shown to be involved in the demethylation of DNA in a direct or indirect manner [29-33]. Since these enzymes can remove the $-CH_3$ group from the cytosine residues of a CpG dinucleotide, they are called the 'erasers' of methylation. When the $-CH_3$ groups are removed, it likely allows the transcription factors to bind to the CpG island and cause activation of gene expression (Figure 2) [34].

3. Breast cancer heterogeneity: A challenge for therapeutic interventions

Breast cancer is a leading cause of mortality in women worldwide [35]. Although a great deal of effort has been made over the years to understand the biology of breast cancer progression and metastasis, its etiology is still not fully understood. One of the main hurdles in breast cancer therapeutics is the high degree of heterogeneity of the breast tumors. This is why the previously hailed 'one size fits all' treatment strategy is largely ineffective in the case of breast cancer. For better prognosis and therapeutic regimens, more specific characterization of the breast tumor is warranted. Hence the idea of using specific biomarkers that act as signatures for different subtypes of breast cancer came into being [36]. The most commonly used biomarkers in the case of breast cancer classification include estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [37]. In clinical settings, these biomarkers are commonly tested by immunohistochemistry (IHC). Furthermore, expression of cell proliferation marker Ki-67 is combined with ER, PR, and HER2 scores to form a better prognostic test called the 'IHC4' [38]. These tests have been proven to be more effective for the prognosis of hormone receptor-positive breast cancers. However, the most aggressive form of breast cancer does not express any of the known hormone receptor markers, and we are yet to identify any potential biomarker for these patients who are more commonly classified as the triple-negative breast cancer subtype. In addition to the protein biomarkers, assays using mRNA-based biomarkers have been developed and used for breast cancer subtyping [39-41].

Since DNA methylation regulates many gene expression programs, it has a profound impact on the prognosis of breast cancer which is dependent on molecular subtypes [42]. A specific pattern of methylation has been correlated with different subtypes of breast cancer [43, 44]. Notably, a higher frequency of DNA methylation is observed

in luminal (ER+) breast tumors compared to non-luminal (ER-) subtypes [43, 44]. These studies suggest that DNA methylation is crucial in the development and progression of distinct breast cancer subtypes [42], and as such, the development of DNA methylation-based biomarkers is gaining interest in the recent years.

4. Aberrant DNA methylation in breast cancer

Accumulating evidence indicate that promoters of genes implicated in critical signaling pathways (for example, cell cycle, apoptosis, DNA repair, cell invasion etc.) are aberrantly methylated in breast cancer. This abnormal gene expression, in turn, dictates the gradual progression of breast cancer cells from less aggressive hormone-responsive phenotype into the more aggressive hormone-independent phenotype [45]. With the advent of high-throughput technologies, there has been an exponential increase in studies related to breast cancer epigenetics [46]. As a result, hundreds of abnormally methylated genes have been identified in breast cancer. Both hypermethylation of tumor suppressor genes and hypomethylation of oncogenes and prometastatic genes are seen in breast cancer [47]. A selected list of some of the important genes frequently hyper or hypomethylated in breast cancer is shown in Table 1.

4.1. Hypomethylation in breast cancer

There are broad genomic regions of hypomethylation in breast tumors [78]. Fang *et al.* through the analysis of cancer methylomes found an association of the hypomethylated CpG islands with a high risk of metastasis and death in breast cancer [79].

Promoter hypomethylation has been observed in several important prometastatic genes (Table 1). This implies that the tumor cells increase the expression of growth factors, cytokines, and proteases that promote invasion and metastasis. Our lab was the first to show that the promoter of urokinase plasminogen activator (*uPA*), a serine protease involved in metastasis, is significantly hypomethylated in highly invasive breast and prostate cancer [49, 50, 80]. We observed an inverse correlation between *uPA* gene expression and hormone sensitivity when primary human mammary epithelial cells (HMEC), hormone-responsive breast cancer cells (MCF-7, ZR-75-1, T-47D, and BT-474) and hormone-insensitive breast cancer cells (Hs578T, BT-549, and MDA-MB-231) were analyzed [50]. The prometastatic *uPA* gene is only expressed in the hormone-insensitive breast cancer cell lines [50]. Further analysis of methylation status revealed that the *uPA* promoter in primary human mammary epithelial cells and hormone-responsive breast cancer cells are methylated at the CpG islands. On the contrary, the same CpG islands were found to be hypomethylated in the highly invasive cell lines (Hs578T, BT-549, and MDA-MB-231). We also observed a similar correlation between *uPA* expression and tumor grade when tumor biopsy samples from

Table 1. Selected list of genes with aberrant methylation in breast cancer.

Biological function	List of genes	Methylation status		Reference
		Hyper	Hypo	
Invasion, metastasis (positive modulators)	*BCSG1*, *CDH3(*P-cadherin), *NAT1*, *uPA*	-	+	[48-52]
Invasion, metastasis (negative modulators)	*CDH1* (E-Cadherin), *CDH13* (H-Cadherin), *CST6*, *SYK*, *TIMP3*	+	-	[53-56]
DNA repair	*ATM*, *BRCA1*, *MGMT*, *MLH1*	+	-	[57-60]
Cell cycle	*AK5*, *CCND2*, *CDH1*, *FOXA2*, *RAD9*, *SFN* (*14-3-3 σ*; *HME1*)	+	-	[61-64]
Apoptosis	*APC*, *BCL-2*, *DAPK*, *DCC*, *HIC1*, *HOXA5*, *TMS1*, *TWIST*	+	-	[55, 62, 63, 65-69]
Cell homeostasis, detoxification	*GSTP1*, *HOXD11*	+	-	[63, 70]
Angiogenesis inhibitors	*SFRP5* (MASPIN), *THBS1*	+	-	[71, 72]
Hormone- and receptor-mediated signaling	*ER*, *HIN-1*, *PR*, *RAR-β2*, *RASSF1A*	+	-	[62, 73-77]

breast cancer patients were analyzed for both promoter methylation and gene expression [50]. We found that hypomethylation at the CpG islands of *uPA* promoter is associated with advanced stage of the disease. With the progression of the disease, the percentage of CpG methylation at the *uPA* promoter decreased. This was a proof of concept that hypomethylation of prometastatic gene plays a major role in breast cancer pathogenesis.

Several other genes involved in the promotion of cell invasion and metastasis (breast cancer-specific gene 1, *BCSG1*; cadherin 3, *CDH3*; N-Acetyltransferase 1, *NAT1*) have also been shown to be hypomethylated in breast cancer [48, 51, 52]. It is known that majority of breast cancer-related deaths occur due to metastasis of the tumor cells, but not for the localized primary tumors per se [81]. In this regard, promoter hypomethylation followed by downstream activation of genes involved in various aspects of metastasis is crucial for better prognosis of breast cancer.

4.2. Hypermethylation in breast cancer

Hypermethylation of hundreds of genes has been reported in breast cancer [82]. Many of these hypermethylated genes are involved in important pathways like DNA damage repair, cell-cycle regulation, apoptosis, cell invasion and metastasis, angiogenesis and hormone signaling (Table 1).

Hypermethylation of estrogen receptor (ER) and progesterone receptor (PR) has been observed in breast cancer and has been proposed to be a mechanism for the development of ER-negative (ER-) breast tumor [83, 84]. Most of the breast carcinomas are ER positive (ER+) in the beginning and subsequently lose the ER to become more aggressive ER- breast cancer. Even though the link between *ER* (also called *ESR1*, estrogen receptor 1) promoter hypermethylation and subsequent decrease in ER expression is evident in the case of breast cancer cell lines [45], similar correlation is not always found in the clinical sample [74, 85]. Hori *et al.* found no association between promoter hypermethylation and expression of ER protein in human breast tumors [85]. On the other hand, Lapidus *et al.* found a correlation between promoter methylation and reduced expression of ER and PR in breast tumors [74, 86]. Another steroid receptor called retinoic acid receptor beta 2 (*RAR-β2*) is hypermethylated in breast cancer [75]. Binding of RAR-β2 with retinoic acid may trigger anti-proliferative signals which can be skipped by the hypermethylation-mediated inactivation of the *RAR-β2* gene [47]. Hypermethylation has also been shown to inhibit tumor suppressors like Ras-association domain family 1 isoform A (*RASSF1A*) [77].

Several genes involved in DNA repair mechanisms are hypermethylated in breast cancer. The loss of DNA repair genes results in genomic instability in the breast cancer genome. Methylation of the CpG islands at the promoter of O-6-methylguanine-DNA methyltransferase (*MGMT*) gene is unique for the tumor tissues and is a predictor of overall survival in cancer [87, 88]. Tserga and colleagues found that aberrant methylation of the promoter of *MGMT* gene has an association with advanced breast tumor grade [58]. Spitzwieser *et al*. found more frequent *MGMT* promoter methylation in patients with breast tumor grade 3 compared to those having tumor grade 2 [89]. MutL homolog 1 (*MLH1*) promoter methylation results in the production of a non-functional protein which impairs the ability of the cells to repair the mismatches occurring during proliferation [90]. Interestingly, pharmacological reversal of methylation using 5-aza-2'deoxycytidine (5-azadC) has been shown to restore the expression of the protein as well as the DNA mismatch repair capacity of the cells in colorectal cancer [91]. Other important DNA-repair genes that are hypermethylated in breast cancer include breast cancer 1 (*BRCA1*) [55] and ataxia-telangiectasia mutated (*ATM*) gene [60].

One of the typical characteristics of tumor cells is to manipulate the cell cycle genes to aid rapid cell growth, proliferation as well to evade cell death. Inactivation of several critical cell cycle regulators and genes involved in apoptosis through hypermethylation is common for the breast cancer cells. In addition, hypermethylation is seen at the promoters of several genes like cadherin 1 (*CDH1*), cadherin 13 (*CDH13*), cystatin 6 (*CST6*), spleen tyrosine kinase (*SYK*), and tissue inhibitor of metalloproteinase 3 (*TIMP3*) that directly or indirectly inhibit tumor cell invasion and metastasis [53-56]. Inactivation of these genes promotes invasion and metastasis of tumor cells.

5. Targeting abnormal DNA methylation as an anti-cancer strategy

In contrast to the genetic changes, DNA methylation changes are potentially reversible by either therapeutic strategies or dietary interventions [92]. This makes DNA methylation an excellent target for anti-cancer therapeutics [93].

5.1. Targeting hypermethylation using DNMT inhibitors

The primary focus of attention for the past two decades has been on the activation of tumor suppressor genes by blocking DNA hypermethylation using DNMT inhibitors. Over the years, many DNMT inhibitors have been developed, and table 2 contains a list of DNMT inhibitors that have been used in many preclinical and clinical studies. Two cytidine analogs 5-azacytidine (Vidaza®) and 5-aza-2'deoxycytidine (Decitabine, Dacogen®) have been approved by the Food and Drug Administration (FDA) as well as the European Medicines Agency (EMA). This review will explain these two hypomethylating agents in detail.

5.1.1. 5-azacytidine and 5-aza-2'deoxycytidine: A historical timeline

5-azacytidine (5-azaC) and 5-aza-2'deoxycytidine (5-azadC) are both hypomethylating agents that are currently approved for treating several specific forms of myelodysplastic syndromes (MDS), acute myeloid leukemia (AML), and chronic myelomonocytic leukemia (CMML) (Figure 3) [94]. These two compounds were first synthesized in Czechoslovakia by Sorm *et al.* in 1964 for use as cytostatic agents [95]. The initial clinical trials to examine their role as anti-cancer agents for liquid cancers started as early as 1967 in Europe followed by 1970 in the United States of America [96].

During the 1970s it was demonstrated that both of these drugs could be incorporated into the DNA, but only 5-azaC could be incorporated into the RNA to cause its disruption and subsequent blockade of protein synthesis [97, 98]. Later on, it was demonstrated that the deoxy derivative 5-azadC is more cytotoxic as well as more potent than 5-azaC [99, 100].

The first clinical trials that used 5-azaC as monotherapy for phase I and II studies in solid tumors commenced during the 1970s [101, 102]. A phase II clinical trial by Weiss *et al.* used a dose of 1.6 mg/kg of 5-azaC daily for a period of ten days and demonstrated an anti-cancer effect in 17% of the breast cancer patients and 21% of the patients having malignant lymphomas [102]. There was a minimal effect of 5-azaC on the other types

Table 2. List of DNMT inhibitors used in cancer.

Class of inhibitors	Compound
Nucleoside analogs	5-azacytidine (Vidaza®) 5-aza-2′deoxycytidine (Decitabine, Dacogen®) Zebularine (dZTP) Guadecitabine (SGI-110) 5-fluoro-2′-deoxycytidine (FdCyd, NSC-48006) 5-azacytidine-5'-elaidate (CP-4200) 2'-Deoxy-N4-[2-(4-nitrophenyl) ethoxycarbonyl]-5-azacytidine (NPEOC-DAC) Fazarabine (Arabinofuranosyl-5-azacytosine) 5,6-Dihydro-5-azacytidine (DHAC; NSC 264880)
Non-nucleoside analogs	RG108 Procaine Procainamide Epigallocatechin-3-Gallate (EGCG) Hydralazine Psammaplin A Mitozantrone Mithramycin A (Plicamycin)
SiRNA	MG98 (antisense oligonucleotide against *DNMT1*)

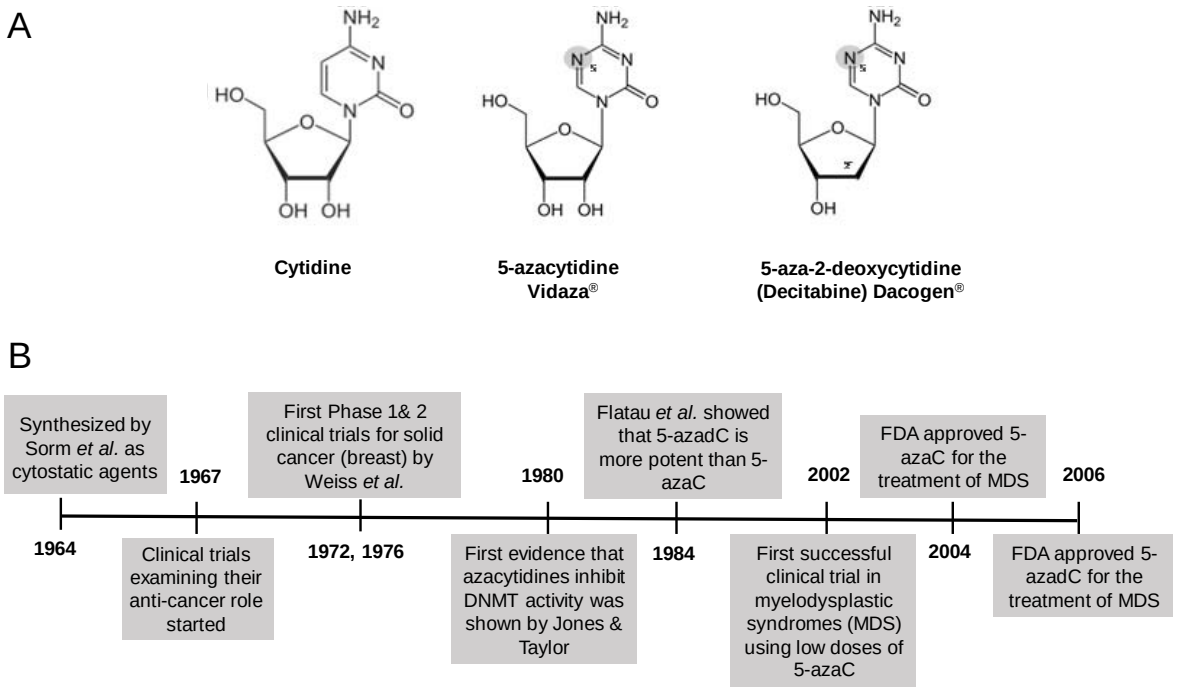

Figure 3. A. Chemical structures of cytidine nucleoside, its analogs 5-azacytidine (5-azaC) and 5-aza-2′deoxycytidine (5-azadC). **B.** A timeline from the discovery until the approval of two well-known azacytidines (5-azaC and 5-azadC).

of solid tumors [102]. Moreover, 5-azaC induced toxic effects like leukopenia, thrombocytopenia, sepsis, and cerebral hemorrhage in patients [101]. These studies demonstrated that these drugs might not be suitable for the treatment of solid tumors as a monotherapy [32]. It should be noted that the dose at which these drugs were used in these studies was too high. So the therapeutic window was smaller.

In a landmark study published in 1980, Jones and Taylor demonstrated that 5-azaC could inhibit DNMT activity [103]. This study also showed the first link between DNA methylation and cellular differentiation and thereby opened the avenue for targeting DNMTs for cancer treatment. They found that prolonged exposure to lower concentrations of the drug led to optimal DNA demethylation and inhibited cell differentiation. On the other hand, the DNMT inhibitors decreased DNA demethylation as well as differentiation when used at higher concentrations [103, 104]. Taken together, these observations renewed the interest in 5-azaC and 5-azadC as anti-cancer therapeutic agents and provided a basis for designing the subsequent clinical trials. The first successful clinical trial of 5-azaC used a low dose (75 mg/m^2) of the drug in patients with myelodysplastic syndromes (MDS) and showed better response over the best available supportive care [105]. This led to the FDA approval of using 5-azaC for the treatment of patients having MDS in 2004. Similarly, a low dose of 5-azadC (15 mg/m^2) given every 8 hours for 3-5 days was effective in MDS patients [106, 107]. In 2006, 5-azadC (Decitabine) was also given the FDA approval for the treatment of patients with MDS. Currently, many clinical trials are going on using these drugs to treat solid tumors.

5.1.2. Mechanism of action of 5-azacytidine and 5-aza-2′deoxycytidine

Two major mechanisms of anti-cancer activity of 5-azaC and 5-azadC have been shown so far. These include (i) demethylation of DNA upon the inhibition of DNMT enzyme which enables the activation of tumor suppressor genes and (ii) cellular cytotoxicity due to the incorporation into DNA (both 5-azaC and 5-azadC) and RNA (5-azaC only) leading to the initiation of DNA damage response [108].

Both 5-azaC and 5-azadC, in their native forms, are prodrugs. Upon cellular uptake by human equilibrative (hENT, SLC29A family) and concentrative nucleoside

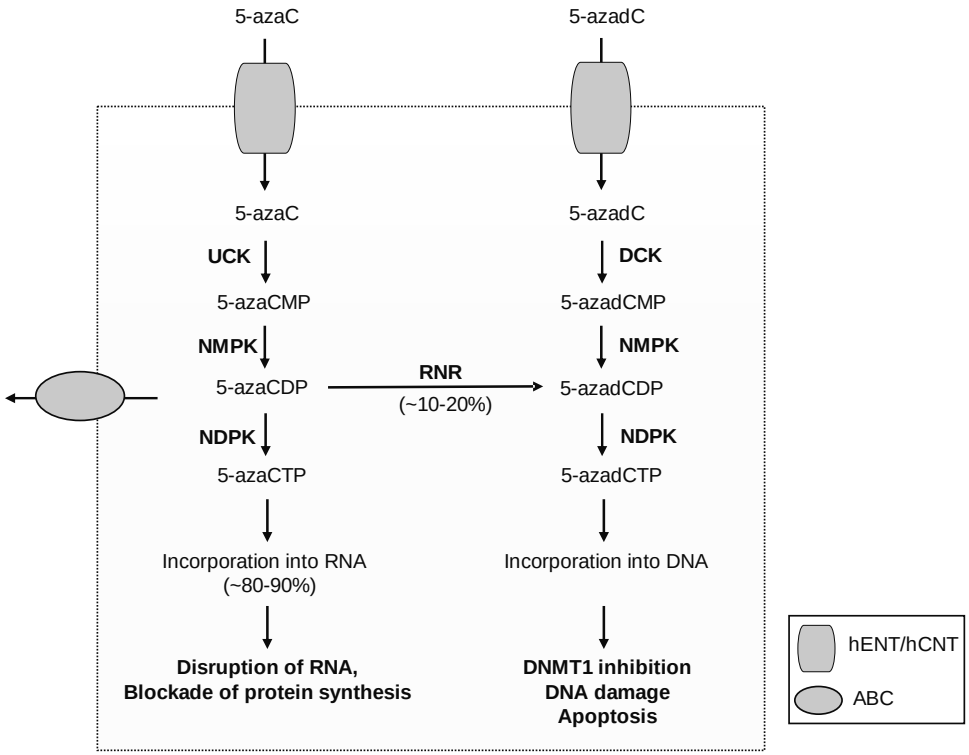

Figure 4. Cellular uptake, metabolism, and mechanism of action of 5-azaC and 5-azadC. Upon cellular uptake by human equilibrative (hENT) and concentrative nucleoside transporters (hCNT) these drugs are activated through a three-step phosphorylation process. The first step is catalyzed by uridine-cytidine kinase (UCK) and deoxycytidine kinase (DCK) for 5-azaC and 5-azadC, respectively. The second step is catalyzed by nucleoside monophosphate kinase (NMPK) for both of these drugs and then a small portion (~10-20%) of 5-azaCDP is converted into 5-azadCDP by the action of ribonucleotide reductase (RNR). The third step is catalyzed by nucleoside diphosphate kinase (NDPK) after which 5-azaCTP and 5-azadCTP are incorporated into the RNA and DNA, respectively. Once incorporated into the nucleic acid strands, these drugs can subsequently mediate the demethylation and cytotoxic effects depending on the dose being administered. ABC transporters play a role in the excretion of these drugs out of the cells.

transporters (hCNT, SLCA28 family), three ATP-dependent phosphorylation steps are needed to convert them to their active forms that can be subsequently incorporated into the DNA and RNA (Figure 4) [108, 109]. The first phosphorylation step to transform the drugs to a monophosphorylated form is catalyzed by the action of uridine-cytidine kinase (UCK) enzyme for 5-azaC and deoxycytidine kinase (DCK) for 5-azadC [97, 108]. The enzymes catalyzing the second and third phosphorylation steps are same for both the drugs. Nucleoside monophosphate kinase (NMPK) and nucleoside diphosphate kinase (NDPK) are the enzymes that catalyze the incorporation of the second and third phosphate groups, respectively to yield active forms of the drugs (5-aza-CTP and 5-aza-dCTP) [108]. Afterward, 5-aza-dCTP is incorporated into the newly synthesized strand of DNA during replication to inhibit DNMT enzyme and cause subsequent DNA damage and apoptosis. However, in the case of 5-azaC, 80-90% are incorporated into the RNA in the form of 5-aza-CTP to cause inhibition of RNA and protein synthesis. Therefore, only 10-20% is available for incorporation into the DNA after the conversion from 5-aza-CDP to 5-aza-dCDP by ribonucleotide reductase (RNR) enzyme (Figure 4) [108]. ATP-binding cassette transporters (ABC family) play a role in transporting both these drugs out of the cells.

5.1.3. Effects of 5-azaC and 5-azadC on breast cancer

As mentioned earlier, both 5-azaC and 5-azadC have been approved for specific types of liquid tumors in the early 2000s. At present, the focus of attention is towards the possibility of using them in solid tumors.

We and others have shown that 5-azaC and 5-azadC can reduce breast cancer growth in both cell lines and mouse models [110, 111]. Treatment with 5-azadC was able to transform the less invasive breast cancer cell lines like MCF-7 and ZR-75-1 into more invasive cells both *in vitro* and *in vivo* [111]. We found that inoculation of MCF-7 cells pretreated with 5-azadC showed significantly reduced tumor growth in mice compared to the control mice inoculated with vehicle-treated MCF-7 cells (Figure 5A) [111]. However, pharmacological inhibition of methylation by 5-azadC also induced the expression of previously quiescent prometastatic (*uPA*, *Heparanase*, C-X-C motif chemokine receptor 4, *CXCR4* and synuclein gamma, *SNCG*) genes involved in tumor cell invasion and metastasis (Figure 5B) [111]. A similar increase in invasiveness and metastasis has also been demonstrated in other types of cancers [112-114]. So careful considerations should be given prior to the use of these drugs for the treatment of cancer.

5.1.4. Combination of 5-azaC and 5-azadC with other therapeutic agents

Both 5-azaC and 5-azadC showed a modest therapeutic effect in solid tumors compared to liquid tumors. However, these drugs can reduce solid tumor growth [111]. Hence, combinations of the DNMT inhibitors with other types of therapeutic agents are now in various phases of clinical trials for breast and other types of solid tumors (source: www.clinicaltrials.gov). A phase II clinical trial of 5-azaC (Vidaza) in combination with a chemotherapeutic agent called Nab-paclitaxel (Abraxane) is ongoing for breast cancer patients. Other types of therapeutic strategies like 5-azadC (Decitabine) in combination with anti-PD-1 antibody, neoadjuvant therapy using pembrolizumab and 5-azadC (Decitabine) before surgery followed by standard chemotherapy are currently in the initial phases of clinical trials with breast cancer patients.

More recently, the combination of DNMT inhibitors with other types of epigenetic drugs like the histone deacetylase (HDAC) inhibitors have gained much interest. It has been demonstrated that a crosstalk exists between DNA methylation and histone acetylation in terms of gene expression [115]. Therefore, the effect of 5-azaC and 5-azadC in combination with different types of HDAC inhibitors has been tested in several studies [116, 117]. Cameron *et al*. first demonstrated the synergistic anti-cancer effect of a combination of DNMT and HDAC inhibitors [116]. Elangovan *et al*. have shown that sodium butyrate (an inhibitor of HDAC) in combination with 5-azadC inhibits tumorigenesis in a mouse model of breast cancer [117].

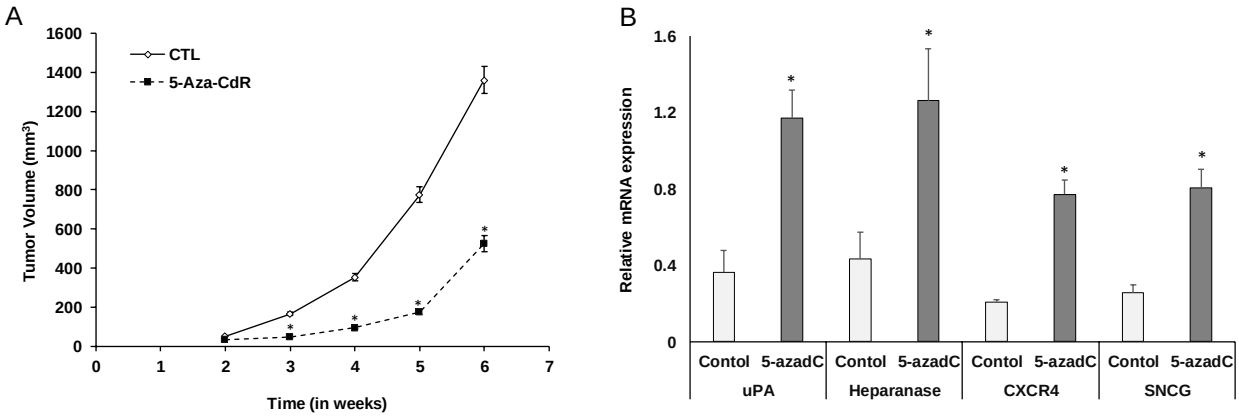

Figure 5. Effect of 5-azadC on tumor growth and metastasis in a xenograft model of breast cancer. A. For this purpose, female Balb C nu/nu mice were inoculated with control MCF-7 cells or MCF-7 cells pretreated with 5 μM 5-azadC and tumor volume was measured weekly for up to six weeks when all the mice were sacrificed. Result shown here represents the mean ± SEM of eight animals in each group and statistical significance is represented by an asterisk (**P* < .05). **B.** Gene expression analysis of several prometastatic genes (*uPA*, *HEPARANASE*, *CXCR4* and *SNCG*) were done from the RNA isolated from the primary tumors. The bar diagram shown in the figure represents the mean ± SEM of tumors from three animals in each group, and statistical significance is represented by an asterisk (**P* < .05). (Adapted and modified from Ateeq *et al.*; Ref. [111])

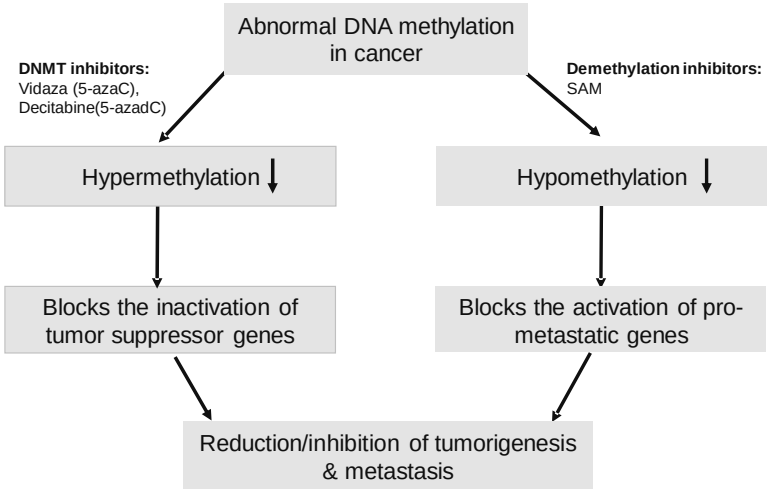

Figure 6. Possible anti-cancer mechanism of the combination therapy using hypomethylating and hypermethylating agents. In cancer genome, hypermethylation and hypomethylation causes the inactivation of tumor suppressor genes and activation of oncogenes, respectively. DNMT inhibitors block hypermethylation and thereby decrease the promoter methylation of tumor suppressor genes seen in cancer to upregulate their expression. Some prometastatic genes have been reported to be upregulated by the action of DNMT inhibitors. On the other hand, SAM can block the activation of oncogenes and proto-oncogenes. Taken together, the combination of these two agents is likely to combat the DNA abnormalities of gene expression seen in cancer.

5.2. Targeting hypomethylation

As shown in table 1, promoters of many prometastatic genes are hypomethylated, and the two approved DNMT inhibitors can reduce tumor growth but also promote the expression of genes involved in metastasis in both cell lines and animal models [111]. Therefore, it stands to reason that targeting of hypomethylation would block tumor cell invasion and metastasis. However, such targeting of hypomethylation is still largely ignored. Currently, there is no approved agent that can be used to block hypomethylation in cancer.

Currently, two approaches have been proposed and used for inhibiting hypomethylation/ demethylation in cancer. The first approach involves the use of universal methyl group donor S-Adenosylmethionine (SAM, also abbreviated as AdoMet) and the second one involves the use of antisense oligonucleotides to block the genes that encode proteins having demethylation activities which mainly provided support for the development of methylating agents as anti-cancer agents [118].

5.2.1. S-Adenosylmethionine (SAM) as an inhibitor of hypomethylation/demethylation

SAM is a naturally occurring molecule mainly synthesized in the liver from the reaction between an essential amino acid called methionine and ATP in the presence of methionine adenosyltransferase (MAT) enzyme [119]. It is ubiquitous in all living cells and plays a role in three important metabolic pathways that include transmethylation, transsulfuration and aminopropylation [120]. SAM is the second most common cofactor in the cells after adenosine triphosphate (ATP) [121], and therefore it has been studied extensively ever since Cantoni first described its chemical structure in 1952 [122]. Research spanning over a few decades provided the rationale for using SAM in clinical conditions like depression, osteoarthritis, fibromyalgia and liver diseases [120], and it is now used as an approved dietary supplement in the USA, Canada, and Europe. SAM is the major methyl group donor in the cytosol of almost every cell [119] and as such is of immense importance in mediating the DNA methylation reaction (Figure 2).

SAM is extremely labile in its native form and degrades rapidly, and therefore stable salts of SAM have been developed over the years. It was not until 1973 that the first commercially available p-toluene sulfate salt of SAM was tested in clinical trials [120]. The pharmacokinetics of exogenously given SAM has been studied by several groups

during the 1970s and 1980s [123-125]. Experimental evidence suggests that exogenous SAM given in rats through oral route can pass the intestinal wall and subsequently increase the level of SAM in the plasma [123]. In humans, a phase I clinical trial aimed to study the antidepressant effect of SAM found its dose-dependent elevation (to a magnitude of 30-50 times higher than the basal levels) in the plasma of males supplemented with 400, 600 and 1000 mg of enteric-coated SAM [126]. Despite such elevation of SAM in plasma, the systemic bioavailability following administration through the oral route remained low. Using Caco-2 cell culture model for enterocyte absorption, McMillan *et al.* found that SAM follows a paracellular transport to move across the intestinal epithelium [127]. They have further inferred that paracellular transportation mode might be a reason for lower systemic bioavailability of SAM. One may still argue that these experiments were done *in vitro* using cultured cells and normal human intestine may have additional membrane transporters that may play some role in the transport of SAM. This is an area that needs to be explored in detail in future. Other studies using radioactive form of SAM found incorporation of ~60% of the radioactivity into the stable pools following excretion through urine and feces [120, 128].

SAM has been effective in treating mood disorders, joint pains, and many other disease conditions. But the therapeutic potential of SAM in cancer was not tested in the first 50 years from its initial discovery by Cantoni.

We were the first to verify that blocking hypomethylation by using SAM would inhibit the expression of prometastatic genes [49]. Through the use of Boyden chamber invasion assay, we found that SAM treatment reduced the invasive capacity of human breast cancer cells MDA-MB-231 [49]. We also showed that SAM treatment caused a dose-dependent reduction in *uPA* gene expression. By using methylation-specific polymerase chain reaction, it was determined that hypomethylated region at the *uPA* promoter in the control MDA-MB-231 cells become methylated in case of the SAM-treated cells. Moreover, immunocompromised mice inoculated with MDA-MB-231 cells pretreated with SAM showed a reduced number of metastatic foci in lung, liver, kidney, and spleen compared to controls suggesting that SAM treatment reduced metastasis *in vivo*. In addition, mice injected with SAM-treated cells also showed a significant reduction in breast tumor growth. This was the first proof of concept study that revealed that SAM-treatment could block breast tumor growth and metastasis. Later studies have demonstrated that SAM can also inhibit metastasis in several other types of cancers [129-131]. When used in combination with 5-azadC *in vitro*, SAM was able to reduce expression of the pro-metastatic genes (*uPA*, *MMP2*) that were upregulated by 5-azadC [132].

SAM, as an anti-cancer agent, can act on DNA hypomethylation at several levels. First, it promotes hypermethylation of prometastatic genes by DNMTs and thereby suppresses their expression [49, 129, 130]. Secondly, it reduces the expression and demethylase activity of Methyl-CpG Binding Domain Protein 2 (*MBD2*) [49]. SAM plays a role in chemoprevention of liver cancer in rat [133]. It was also found that SAM could specifically increase the anti-cancer effect of 5-fluorouracil (5-FU) [134]. Taken together these observations suggest that SAM has the potential to serve as a chemopreventive as well as an anti-cancer and anti-metastatic agent.

In this regard, we propose that combining the demethylation activity of DNMT inhibitors like 5-azaC and 5-azadC with hypermethylating agents like SAM may serve as a true DNA methylation-based anti-cancer therapy to inhibit tumor growth as well as metastasis. This is likely to block the activation of prometastatic genes by the DNMT inhibitors as well as inhibit any possible inactivation of tumor suppressor genes by the hypomethylating agent (Figure 6).

5.2.2. Antisense oligonucleotides as an inhibitor of hypomethylation/demethylation

The second strategy that has been proposed and used to block hypomethylation of metastatic genes involves the inhibition of proteins that cause demethylation. MBD2 is one such protein that has been targeted in this regard. MBD2 is a dual-function protein known to be involved in silencing of methylated genes and also cause demethylation [135], even though the role of MBD2 as a demethylase is controversial [136, 137]. Considering that MBD2 plays a role in demethylation, we have previously targeted it as a means to block hypomethylation/ demethylation of prometastatic genes. We found that

antisense oligonucleotides against *MBD2* reduced tumorigenesis and metastasis in breast and prostate cancer *in vivo* [49, 129]. *MBD2* depletion has also been shown to reduce intestinal tumorigenesis [138].

6. Conclusion and future perspectives

It is now clear that DNA methylation has important implications in breast cancer diagnosis, prognosis as well as therapy. More recently, the role of blood-based DNA methylation markers in the early detection of solid cancer gained much attention even though the evidence is still very limited [139]. This is important because patients diagnosed with breast cancer earlier have a higher survival rate than the patients who are diagnosed at the advanced stage of breast disease [140, 141]. The use of blood-based DNA methylation markers has the potential to change the diagnostics of solid tumors in future allowing early intervention and would, thereby, greatly aid in personalized therapies.

From a therapeutic perspective, there are still lots of challenges in using the two approved DNMT inhibitors 5-azaC and 5-azadC as monotherapy for solid tumors. These drugs are able to reverse the inactivation of tumor suppressor genes seen in cancer and thereby reduce the anticipated tumor growth. But they also induce the expression of prometastatic genes, are less bioavailable and shows toxicity in the cells. Therefore, it stands to reason that other types of DNA-methylation inhibitors are needed to be developed. Zebualrine, another DNMT inhibitor, is showing a promising anti-cancer effect in the case of breast cancer [142]. Zebularine is more stable, has better oral bioavailability and is less toxic than 5-azaC and 5-azadC. Nevertheless, this drug also belongs to the same class of nucleoside analogs and has the potential to raise similar issues as seen with 5-azaC and 5-azadC [118]. The second generation DNMT inhibitors like Guadecitabine (SGI-110) may provide better response in that regard since their chemical structures are quite different from the first generation DNMT inhibitors. However, the new generation DNMT inhibitors need to be tested in case of breast and other solid cancers before coming to any definite conclusion.

The studies related to the anti-cancer effect of SAM in breast cancers are either done *in vitro* or by using pretreated cells implanted into immunocompromised mice. As such, the potential of using SAM as an anti-cancer and anti-metastatic agent has not been demonstrated in a therapeutic setting. We are currently doing preclinical studies using different mouse models to assess the therapeutic potential of using SAM. The results from these studies will provide a rationale for starting clinical trials using SAM as a therapeutic agent in breast and other cancers. It should also be noted that SAM is a pleiotropic molecule [120], and therefore development of target-specific inhibitors of hypomethylation/ demethylation having better stability and bioavailability than SAM is also warranted in future.

Combination treatments of DNMT inhibitors with monoclonal antibodies or chemotherapeutic agents have shown great promise, and therefore they are currently in different phases of clinical trials. We are proposing a combination of DNMT inhibitors with SAM which can be effective against both hyper and hypomethylation. It would also be important to elucidate the possible mechanisms of sensitivity and resistance of these drugs as monotherapy or combination therapy. This, in turn, will pave the way for the development of response-predicting biomarkers.

ACKNOWLEDGMENTS

This work was supported by a grant MOP 130410 from the Canadian Institutes for Health Research (CIHR) to SAR.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

ABBREVIATIONS

5-azaC	:	5-azacytidine
5-azadC	:	5-aza-2′-deoxycytidine
ABC	:	ATP-binding cassette transporters
AID	:	Activation-induced cytidine deaminase
AK5	:	Adenylate kinase 5
APC	:	Adenomatous polyposis coli
ATM	:	Ataxia-telangiectasia mutated
ATP	:	Adenosine triphosphate
BCL-2	:	B-cell lymphoma 2
BCSG1	:	Breast cancer-specific gene 1
BRCA1	:	Breast cancer 1
CCND2	:	Cyclin D2
CDH1	:	Cadherin 1
CDH3	:	Cadherin 3

CDH13 : Cadherin 13
CST6 : Cystatin 6
CXCR4 : C-X-C motif chemokine receptor 4
DAPK : Death associated protein kinase
DCC : Deleted in colorectal carcinoma
DCK : Deoxycytidine kinase
DNMT : DNA-methyltransferases
ER : Estrogen receptor
FOXA2 : Forkhead box A2
GSTP1 : Glutathione S-transferase pi 1
hCNT : Human concentrative nucleoside transporter
HDAC : Histone deacetylase
hENT : Human equilibrative nucleoside transporter
HER2 : Human epidermal growth factor receptor 2
HIC1 : Hypermethylated in cancer 1
HOXA5 : Homeobox A5
HOXD11 : Homeobox D11
MBD : Methyl-CpG binding domain
MBD1 : Methyl-CpG binding domain 1
MBD2 : Methyl-CpG binding domain 2
MBD3 : Methyl-CpG binding domain 3
MBD4 : Methyl-CpG binding domain 4
MeCP2 : Methyl-CpG binding protein 2
MGMT : O-6-methylguanine-DNA methyltransferase
MLH1 : MutL homolog 1
NAT1 : N-Acetyltransferase 1
NDPK : Nucleoside diphosphate kinase
NMPK : Nucleoside monophosphate kinase
PR : Progesterone receptor
RAR-β2 : Retinoic acid receptor beta 2
RASSF1A : Ras-association domain family 1 isoform A
RNR : Ribonucleotide reductase
SAM : S-Adenosylmethionine
SFN : Stratifin
SFRP5 : Secreted frizzled related protein 5
SNCG : Synuclein gamma
SYK : Spleen tyrosine kinase
TDG : Thymine DNA glycosylase
TET : Ten-eleven translocation
THBS1 : Thrombospondin 1
TIMP3 : Tissue inhibitor of metalloproteinase 3
TMS1 : Target of methylation-induced silencing 1
UCK : Uridine-cytidine kinase
UHRF1 : Ubiquitin-like containing PHD and ring finger domains 1
UHRF2 : Ubiquitin-like containing PHD and ring finger domains 2
uPA : Urokinase plasminogen activator
ZBTB4 : Zinc finger and BTB domain containing 4
ZBTB38 : Zinc finger and BTB domain containing 38

REFERENCES

1. Klutstein, M., Nejman, D., Greenfield, R. and Cedar, H. 2016, Cancer Res., 76, 3446-50.
2. Feinberg, A. P. and Vogelstein, B. 1983, Nature, 301, 89-92.
3. Ganesan, A. 2016, ChemMedChem., 11, 1227-41.
4. Zhang, J. and Zheng, Y. G. 2015, ACS Chem. Biol., 11, 583-597.
5. Jeltsch, A. 2002, Chembiochem., 3, 274-293.
6. Avery, O. T., MacLeod, C. M. and McCarty, M. 1944, J. Exp. Med., 79, 137-158.
7. Hotchkiss, R. D. 1948, J. Biol. Chem., 175, 315-332.
8. Holliday, R. and Pugh, J. 1975, Science, 187, 226-232.
9. Compere, S. J. and Palmiter, R. D. 1981, Cell, 25, 233-240.
10. Bird, A. 2002, Genes Dev., 16, 6-21.
11. Sánchez-Romero, M. A., Cota, I. and Casadesús, J. 2015, Curr. Opin. Microbiol., 25, 9-16.
12. Suzuki, M. M. and Bird, A. 2008, Nature Rev. Genet., 9, 465-476.
13. Moore, L. D., Le, T. and Fan, G. 2013, Neuropsychopharmacology, 38, 23-38.
14. Greer, E. L., Blanco, M. A., Gu, L., Sendinc, E., Liu, J., Aristizábal-Corrales, D., Hsu, C.-H., Aravind, L., He, C. and Shi, Y. 2015, Cell, 161, 868-878.
15. Soojin, V. Y. 2012, Genome Biol., 13, 174.
16. Gardiner-Garden, M. and Frommer, M. 1987, J. Mol. Biol., 196, 261-282.
17. Saxonov, S., Berg, P. and Brutlag, D. L. 2006, Proc. Natl. Acad. Sci. USA, 103, 1412-1417.
18. Lister, R., Pelizzola, M., Dowen, R. H., Hawkins, R. D., Hon, G., Tonti-Filippini, J., Nery, J. R., Lee, L., Ye, Z. and Ngo, Q.-M. 2009, Nature, 462, 315-322.

19. Berger, N. A. 2016, Epigenetics, Energy Balance, and Cancer, Springer, 11 (ISBN: 978-3-319-41608-3)
20. Bestor, T. H. 2000, Hum. Mol. Gen., 9, 2395-2402.
21. Li, E., Bestor, T. H. and Jaenisch, R. 1992, Cell, 69, 915-926.
22. Okano, M., Xie, S. and Li, E. 1998, Nature Genet, 19, 219-220.
23. Okano, M., Bell, D. W., Haber, D. A. and Li, E. 1999, Cell, 99, 247-257.
24. Hermann, A., Goyal, R. and Jeltsch, A. 2004, J. Biol. Chem., 279, 48350-48359.
25. Okano, M., Xie, S. and Li, E. 1998, Nat. Genet., 19, 219-20.
26. Tate, P. H. and Bird, A. P. 1993, Curr. Opin. Genetics Dev., 3, 226-231.
27. Nan, X., Ng, H.-H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N. and Bird, A. 1998, Nature, 393, 386-389.
28. Clouaire, T. and Stancheva, I. 2008, Cell Mol. Life Sci., 65, 1509-1522.
29. Kohli, R. M. and Zhang, Y. 2013, Nature, 502, 472-479.
30. Cortellino, S., Xu, J., Sannai, M., Moore, R., Caretti, E., Cigliano, A., Le Coz, M., Devarajan, K., Wessels, A. and Soprano, D. 2011, Cell, 146, 67-79.
31. Cortázar, D., Kunz, C., Selfridge, J., Lettieri, T., Saito, Y., MacDougall, E., Wirz, A., Schuermann, D., Jacobs, A. L. and Siegrist, F. 2011, Nature, 470, 419-423.
32. Cheishvili, D., Boureau, L. and Szyf, M. 2015, Br. J. Pharmacol., 172, 2705-2715.
33. Tahiliani, M., Koh, K. P., Shen, Y., Pastor, W. A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L. M., Liu, D. R. and Aravind, L. 2009, Science, 324, 930-935.
34. Mariani, C. J., Madzo, J., Moen, E. L., Yesilkanal, A. and Godley, L. A. 2013, Cancers, 5, 786-814.
35. DeSantis, C. E., Fedewa, S. A., Goding Sauer, A., Kramer, J. L., Smith, R. A. and Jemal, A. 2016, CA Cancer J. Clin., 66, 31-42.
36. Weigel, M. T. and Dowsett, M. 2010, Endocr. Relat. Cancer, 17, R245-R262.
37. Cornejo, K. M., Kandil, D., Khan, A. and Cosar, E. F. 2014, Arch. Pathol. Lab. Med., 138, 44-56.
38. Cuzick, J., Dowsett, M., Pineda, S., Wale, C., Salter, J., Quinn, E., Zabaglo, L., Mallon, E., Green, A. R. and Ellis, I. O. 2011, J. Clin. Oncol., 29, 4273-4278.
39. Cronin, M., Sangli, C., Liu, M.-L., Pho, M., Dutta, D., Nguyen, A., Jeong, J., Wu, J., Langone, K. C. and Watson, D. 2007, Clin. Chem., 53, 1084-1091.
40. Filipits, M., Rudas, M., Jakesz, R., Dubsky, P., Fitzal, F., Singer, C. F., Dietze, O., Greil, R., Jelen, A. and Sevelda, P. 2011, Clin. Cancer Res., 17, 6012-6020.
41. Toussaint, J., Sieuwerts, A. M., Haibe-Kains, B., Desmedt, C., Rouas, G., Harris, A. L., Larsimont, D., Piccart, M., Foekens, J. A. and Durbecq, V. 2009, BMC Genom., 10, 1.
42. Gyorffy, B., Bottai, G., Fleischer, T., Munkácsy, G., Budczies, J. and Paladini, L. 2016, Int. J. Cancer, 138, 87-97.
43. Kamalakaran, S., Varadan, V., Russnes, H. E. G., Levy, D., Kendall, J., Janevski, A., Riggs, M., Banerjee, N., Synnestvedt, M. and Schlichting, E. 2011, Mol. Oncol., 5, 77-92.
44. Stefansson, O. A., Moran, S., Gomez, A., Sayols, S., Arribas-Jorba, C., Sandoval, J., Hilmarsdottir, H., Olafsdottir, E., Tryggvadottir, L. and Jonasson, J. G. 2015, Mol. Oncol, 9, 555-568.
45. Ferguson, A. T., Lapidus, R. G., Baylin, S. B. and Davidson, N. E. 1995, Cancer Res., 55, 2279-2283.
46. Huang, Y., Nayak, S., Jankowitz, R., Davidson, N. E. and Oesterreich, S. 2011, Breast Cancer Res., 13, 225.
47. Szyf, M., Pakneshan, P. and Rabbani, S. A. 2004, Biochem. Pharmacol., 68, 1187-1197.
48. Kim, S. J., Kang, H.-S., Chang, H. L., Jung, Y. C., Sim, H.-B., Lee, K. S., Ro, J. and Lee, E. S. 2008, Oncol. Rep., 19, 663-668.
49. Pakneshan, P., Szyf, M., Farias-Eisner, R. and Rabbani, S. A. 2004, J. Biol. Chem., 279, 31735-31744.
50. Pakneshan, P., Têtu, B. and Rabbani, S. A. 2004, Clin. Cancer Res., 10, 3035-3041.
51. Paredes, J., Albergaria, A., Oliveira, J. T., Jerónimo, C., Milanezi, F. and Schmitt, F. C. 2005, Clin. Cancer Res., 11, 5869-5877.
52. Gupta, A., Godwin, A. K., Vanderveer, L., Lu, A. and Liu, J. 2003, Cancer Res., 63, 664-673.

53. Toyooka, K. O., Toyooka, S., Virmani, A. K., Sathyanarayana, U. G., Euhus, D. M., Gilcrease, M., Minna, J. D. and Gazdar, A. F. 2001, Cancer Res., 61, 4556-4560.
54. Yuan, Y., Mendez, R., Sahin, A. and Le Dai, J. 2001, Cancer Res., 61, 5558-5561.
55. Esteller, M., Corn, P. G., Baylin, S. B. and Herman, J. G. 2001, Cancer Res., 61, 3225-3229.
56. Rivenbark, A. G., Jones, W. D. and Coleman, W. B. 2006, Lab. Invest., 86, 1233-1242.
57. Esteller, M., Silva, J. M., Dominguez, G., Bonilla, F., Matias-Guiu, X., Lerma, E., Bussaglia, E., Prat, J., Harkes, I. C. and Repasky, E. A. 2000, J. Natl. Cancer Inst., 92, 564-569.
58. Tserga, A., Michalopoulos, N. V., Levidou, G., Korkolopoulou, P., Zografos, G., Patsouris, E. and Saetta, A. A. 2012, Oncol. Rep., 27, 1630.
59. Lotsari, J. E., Gylling, A., Abdel-Rahman, W. M., Nieminen, T. T., Aittomäki, K., Friman, M., Pitkänen, R., Aarnio, M., Järvinen, H. J. and Mecklin, J.-P. 2012, Breast Cancer Res., 14, R90.
60. Vo, Q. N., Kim, W.-J., Cvitanovic, L., Boudreau, D. A., Ginzinger, D. G. and Brown, K. D. 2004, Oncogene, 23, 9432-9437.
61. Cheng, C. K., Chow, L. W., Loo, W. T., Chan, T. K. and Chan, V. 2005, Cancer Res., 65, 8646-8654.
62. Fackler, M. J., McVeigh, M., Evron, E., Garrett, E., Mehrotra, J., Polyak, K., Sukumar, S. and Argani, P. 2003, Int. J. Cancer, 107, 970-975.
63. Miyamoto, K., Fukutomi, T., Akashi-Tanaka, S., Hasegawa, T., Asahara, T., Sugimura, T. and Ushijima, T. 2005, Int. J. Cancer, 116, 407-414.
64. Parrella, P., Poeta, M. L., Gallo, A. P., Prencipe, M., Scintu, M., Apicella, A., Rossiello, R., Liguoro, G., Seripa, D. and Gravina, C. 2004, Clin. Cancer Res., 10, 5349-5354.
65. Jin, Z., Tamura, G., Tsuchiya, T., Sakata, K., Kashiwaba, M., Osakabe, M. and Motoyama, T. 2001, Br. J. Cancer, 85, 69.
66. Fujii, H., Biel, M. A., Zhou, W., Weitzman, S. A., Baylin, S. B. and Gabrielson, E. 1998, Oncogene, 16, 2159-2164.
67. Raman, V., Martensen, S. A., Reisman, D., Evron, E., Odenwald, W. F., Jaffee, E., Marks, J. and Sukumar, S. 2000, Nature, 405, 974-978.
68. Stone, A., Cowley, M. J., Valdes-Mora, F., McCloy, R. A., Sergio, C. M., Gallego-Ortega, D., Caldon, C. E., Ormandy, C. J., Biankin, A. V. and Gee, J. M. 2013, Mol. Cancer Ther., 12, 1874-1885.
69. Conway, K. E., McConnell, B. B., Bowring, C. E., Donald, C. D., Warren, S. T. and Vertino, P. M. 2000, Cancer Res., 60, 6236-6242.
70. Lin, X. and Nelson, W. G. 2003, Cancer Res., 63, 498-504.
71. Suzuki, H., Toyota, M., Caraway, H., Gabrielson, E., Ohmura, T., Fujikane, T., Nishikawa, N., Sogabe, Y., Nojima, M. and Sonoda, T. 2008, Br. J. Cancer, 98, 1147-1156.
72. Li, Q., Ahuja, N., Burger, P. C. and Issa, J.-P. 1999, Oncogene, 18, 3284-3289.
73. Ottaviano, Y. L., Issa, J.-P., Parl, F. F., Smith, H. S., Baylin, S. B. and Davidson, N. E. 1994, Cancer Res., 54, 2552-2555.
74. Lapidus, R. G., Ferguson, A. T., Ottaviano, Y. L., Parl, F. F., Smith, H. S., Weitzman, S. A., Baylin, S. B., Issa, J. and Davidson, N. E. 1996, Clin. Cancer Res., 2, 805-810.
75. Widschwendter, M., Berger, J., Hermann, M., Müller, H. M., Amberger, A., Zeschnigk, M., Widschwendter, A., Abendstein, B., Zeimet, A. G. and Daxenbichler, G. 2000, J. Natl. Cancer Inst., 92, 826-832.
76. Dammann, R., Schagdarsurengin, U., Strunnikova, M., Rastetter, M., Seidel, C., Liu, L., Tommasi, S. and Pfeifer, G. 2003, Histol. Histopathol.,18,665-677.
77. Dammann, R., Yang, G. and Pfeifer, G. P. 2001, Cancer Res., 61, 3105-3109.
78. Hon, G. C., Hawkins, R. D., Caballero, O. L., Lo, C., Lister, R., Pelizzola, M., Valsesia, A., Ye, Z., Kuan, S. and Edsall, L. E. 2012, Genome Res., 22, 246-258.
79. Fang, F., Turcan, S., Rimner, A., Kaufman, A., Giri, D., Morris, L. G., Shen, R., Seshan, V., Mo, Q. and Heguy, A. 2011, Sci. Transl. Med., 3, 75ra25.
80. Pakneshan, P., Xing, R. H. and Rabbani, S. A. 2003, FASEB J., 17, 1081-1088.

81. Weigelt, B., Peterse, J. L. and Van't Veer, L. J. 2005, Nat. Rev. Cancer, 5, 591-602.
82. Hinshelwood, R. A. and Clark, S. J. 2008, J. Mol. Med., 86, 1315-1328.
83. Jovanovic, J., Rønneberg, J. A., Tost, J. and Kristensen, V. 2010, Mol. Oncol., 4, 242-254.
84. Weigel, R. J. 1993, Cancer Res., 53, 3472-3474.
85. Hori, M., Iwasaki, M., Yoshimi, F., Asato, Y. and Ibaraki, M. 1999, Breast Cancer, 6, 79-86.
86. Gaudet, M. M., Campan, M., Figueroa, J. D., Yang, X. R., Lissowska, J., Peplonska, B., Brinton, L. A., Rimm, D. L., Laird, P. W. and Garcia-Closas, M. 2009, Cancer Epidemiol. Biomarkers Prev., 18, 3036-3043.
87. Esteller, M. and Herman, J. G. 2004, Oncogene, 23, 1-8.
88. Esteller, M., Hamilton, S. R., Burger, P. C., Baylin, S. B. and Herman, J. G. 1999, Cancer Res., 59, 793-797.
89. Spitzwieser, M., Holzweber, E., Pfeiler, G., Hacker, S. and Cichna-Markl, M. 2015, Breast Cancer Res., 17, 125.
90. Wajed, S. A., Laird, P. W. and DeMeester, T. R. 2001, Ann. Surg., 234, 10-20.
91. Herman, J. G., Umar, A., Polyak, K., Graff, J. R., Ahuja, N., Issa, J.-P. J., Markowitz, S., Willson, J. K., Hamilton, S. R. and Kinzler, K. W. 1998, Proc. Natl. Acad. Sci. USA, 95, 6870-6875.
92. Szyf, M. 2009, Annu. Rev. Pharmacol. Toxicol., 49, 243-63.
93. Szyf, M. 1994, Trends Pharmacol. Sci., 15, 233-8.,
94. Derissen, E. J., Beijnen, J. H. and Schellens, J. H. 2013, The oncologist, 18, 619-624.
95. Šorm, F., Piskala, A., Čihák, A. and Veselý, J. 1964, Cell Mol. Life Sci., 20, 202-203.
96. Vonhoff, D. D., Slavik, M. and Muggia, F. M. 1976, Ann. Intern. Med., 85, 237-245.
97. Li, L., Olin, E., Buskirk, H. and Reineke, L. 1970, Cancer Res., 30, 2760-2769.
98. Veselý, J. and Čihák, A. 1978, Pharmacol. Ther., 2, 813-840.
99. Flatau, E., Gonzales, F., Michalowsky, L. and Jones, P. 1984, Mol. Cell Biol., 4, 2098-2102.
100. Momparler, R., Rossi, M., Bouchard, J., Vaccaro, C., Momparler, L. and Bartolucci, S. 1984, Mol. Pharmacol., 25, 436-440.
101. Weiss, A., Stambaugh, J., Mastrangelo, M., Laucius, J. and Bellet, R. 1972, Cancer Chemother. Rep., 56, 413-419.
102. Weiss, A., Metter, G., Nealon, T., Keanan, J., Ramirez, G., Swaiminathan, A., Fletcher, W., Moss, S. and Manthei, R. 1976, Cancer Treat Rep., 61, 55-58.
103. Jones, P. A. and Taylor, S. M. 1980, Cell, 20, 85-93.
104. Jones, P. A. and Taylor, S. M. 1981, Nucleic Acids Res., 9, 2933-2947.
105. Silverman, L. R., Demakos, E. P., Peterson, B. L., Kornblith, A. B., Holland, J. C., Odchimar-Reissig, R., Stone, R. M., Nelson, D., Powell, B. L. and DeCastro, C. M. 2002, J. Clin. Oncol., 20, 2429-2440.
106. Lübbert, M., Suciu, S., Baila, L., Rüter, B. H., Platzbecker, U., Giagounidis, A., Selleslag, D., Labar, B., Germing, U. and Salih, H. R. 2011, J. Clin. Oncol., 29, 1987-1996.
107. Kantarjian, H., Oki, Y., Garcia-Manero, G., Huang, X., O'Brien, S., Cortes, J., Faderl, S., Bueso-Ramos, C., Ravandi, F. and Estrov, Z. 2007, Blood, 109, 52-57.
108. Stresemann, C. and Lyko, F. 2008, Int. J. Cancer, 123, 8-13.
109. Rius, M., Stresemann, C., Keller, D., Brom, M., Schirrmacher, E., Keppler, D. and Lyko, F. 2009, Mol. Cancer Ther., 8, 225-231.
110. Thakur, S., Feng, X., Shi, Z. Q., Ganapathy, A., Mishra, M. K., Atadja, P., Morris, D. and Riabowol, K. 2012, PLoS ONE, 7, e43671.
111. Ateeq, B., Unterberger, A., Szyf, M. and Rabbani, S. A. 2008, Neoplasia, 10, 266-278.
112. Sato, N., Maehara, N., Su, G. H. and Goggins, M. 2003, J. Natl. Cancer Inst., 95, 327-330.
113. Habets, G., van der Kammen, R., Scholtes, E. and Collard, J. 1990, Clin. Exp. Metastasis, 8, 567-577.
114. Olsson, L. and Forchhammer, J. 1984, Proc. Natl. Acad. Sci. USA, 81, 3389-3393.
115. Cervoni, N. and Szyf, M. 2001, J. Biol. Chem., 276, 40778-40787.
116. Cameron, E. E., Bachman, K. E., Myöhänen, S., Herman, J. G. and Baylin, S. B. 1999, Nature Genet., 21, 103-107.

117. Elangovan, S., Pathania, R., Ramachandran, S., Ananth, S., Padia, R. N., Srinivas, S. R., Babu, E., Hawthorn, L., Schoenlein, P. V. and Boettger, T. 2013, Mol. Cell Biol., 33, 3920-3935.
118. Szyf, M. 2008, Curr. Oncol., 15, 2-5.
119. Lu, S. C. and Mato, J. M. 2008, J. Gastroenterol. Hepatol., 23, S73-S77.
120. Bottiglieri, T. 2002, Am. J. Clin. Nutr., 76, 1151S-1157S.
121. Mercer, A. C. and Burkart, M. D. 2006, Nat. Chem. Biol., 2, 8-10.
122. Cantoni, G. 1952, J. Am. Chem. Soc., 74, 2942-2943.
123. Stramentinoli, G., Gualano, M. and Galli-Kienle, M. 1979, J. Pharmacol. Exp. Ther., 209, 323-326.
124. Stramentinoli, G. 1987, Am. J. Med., 83, 35-42.
125. Bombardieri, G., Pappalardo, G., Bernardi, L., Barra, D., Di Palma, A. and Castrini, G. 1983, Int. J. Clin. Pharmacol. Ther. Toxicol., 21, 186-188.
126. Bottiglieri, T., Chary, T., Laundy, M., Carney, M., Godfrey, P., Toone, B. and Reynolds, E. 1988, Ala. J. Med. Sci., 25, 296-301.
127. McMillan, J., Walle, U. and Walle, T. 2005, J. Pharm. Pharmacol., 57, 599-605.
128. Giulidori, P., Fellin, M. and Di Padova, C. 1998, Paper presented at the Proceedings of the workshop Methionine Metabolism: Molecular Mechanisms and Clinical Implications Madrid, Spain: Jarpyo Editores.
129. Shukeir, N., Pakneshan, P., Chen, G., Szyf, M. and Rabbani, S. A. 2006, Cancer Res., 66, 9202-9210.
130. Parashar, S., Cheishvili, D., Arakelian, A., Hussain, Z., Tanvir, I., Khan, H. A., Szyf, M. and Rabbani, S. A. 2015, Cancer medicine, 4, 732-744.
131. Hussain, Z., Khan, M., Shahid, M. and Almajhdi, F. 2013, Genet. Mol. Res., 12, 1106-1118.
132. Chik, F., Machnes, Z. and Szyf, M. 2014, Carcinogenesis, 35, 138-144.
133. Pascale, R. M., Simile, M. M., De Miglio, M. R. and Feo, F. 2002, Alcohol, 27, 193-198.
134. Ham, M.-S., Lee, J.-K. and Kim, K.-C. 2013, Mol. Clin. Oncol., 1, 373-378.
135. Bhattacharya, S. K., Ramchandani, S., Cervoni, N. and Szyf, M. 1999, Nature, 397, 579-583.
136. Santos, F., Hendrich, B., Reik, W. and Dean, W. 2002, Dev. Biol., 241, 172-182.
137. Ng, H.-H., Zhang, Y., Hendrich, B., Johnson, C. A., Turner, B. M., Erdjument-Bromage, H., Tempst, P., Reinberg, D. and Bird, A. 1999, Nature Genet, 23, 58-61.
138. Sansom, O. J., Berger, J., Bishop, S. M., Hendrich, B., Bird, A. and Clarke, A. R. 2003, Nature Genet., 34, 145-147.
139. Tang, Q., Cheng, J., Cao, X., Surowy, H. and Burwinkel, B. 2016, Clin. Epigenetics, 8, 115.
140. Etzioni, R., Urban, N., Ramsey, S., McIntosh, M., Schwartz, S., Reid, B., Radich, J., Anderson, G. and Hartwell, L. 2003, Nat. Rev. Cancer, 3, 243-252.
141. Ries, L. A., Harkins, D., Krapcho, M., Mariotto, A., Miller, B. A., Feuer, E. J., Clegg, L. X., Eisner, M., Horner, M.-J. and Howlader, N., 2006, SEER Cancer Statistics Review, 1975-2003.
142. Chen, M., Shabashvili, D., Nawab, A., Yang, S. X., Dyer, L. M., Brown, K. D., Hollingshead, M., Hunter, K. W., Kaye, F. J. and Hochwald, S. N. 2012, Mol. Cancer Ther. 11, 370-382.