Emma - Targeting DNA methyltransferases in non-small-cell lung cancer

Al-Yozbaki, M., Jabre, I., Syed, N. and Wilson, C.

Review:

Targeting DNA methyltransferases in Non-small-cell lung cancer

Minnatallah Al-Yozbaki[a], Ibtissam Jabre[b], Naeem H. Syed[a], and Cornelia M. Wilson[a,c]

[a] Canterbury Christ Church University, School of Human and Life Sciences, Life Sciences Industry Liaison Lab, Sandwich, UK.

[b] Dept. of Microbial Sciences, School of Biosciences and Medicine, Faculty of Health and Medical Sciences, University of Surrey, Guildford, GU2 7XH, UK.

[c] University of Liverpool, Institute of Translation Medicine, Dept of Molecular & Clinical Cancer Medicine

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Abstract

Despite the advances in treatment using chemotherapy or targeted therapies, due to static survival rates, non-small cell lung cancer (NSCLC) is the major cause of cancer-related deaths worldwide. Epigenetic-based therapies have been developed for NSCLC by targeting DNA methyltransferases (DNMTs) and histone-modifying enzymes. However, treatment using single epigenetic agents on solid tumours has been inadequate; whereas, treatment with a combination of DNMTs inhibitors with chemotherapy and immunotherapy has shown great promise. Dietary sources of phytochemicals could also inhibit DNMTs and cancer stem cells, representing a novel and promising way to prevent and treat cancer. Herein, we will discuss the different DNMTs, DNA methylation profiling in NSCLC as well as current demethylating agents in ongoing clinical trials. Therefore, providing a concise overview of future developments in the field of epigenetic therapy in NSCLC.

Significance: Combined approaches of epigenetic therapy have the potential to modify the entire cancer epigenome and improve the overall survival rate of lung cancer. This review summarizes the role of DNA methyltransferases and the clinical benefits of altering DNA methylation in the tumour, with a focus on current epigenetic developments in the clinic.

Introduction

Lung cancer is currently the leading cause of cancer-related death worldwide. Lung cancer has a high incidence at ~17% combined with a poor survival rate (~5-years) [1, 2]. Approximately, ~20% are small cell carcinoma (SCLC) and ~80% are non-small cell carcinoma (NSCLC) including adenocarcinoma (AD), squamous cell carcinoma (SCC), and large cell carcinoma (LCC) subtypes. The risk factors for lung cancer has been proposed to be driven by an accumulation of genetic mutations or alterations, often combined with factors present in the environment. As well as tumour suppressor genes (TSGs) and oncogenes, the expression of tumour progenitor genes may be disrupted by epigenetic alteration during early stages of cancer progression [3]. The tumour progenitor genes assist in controlling a number of biological processes, the progression of disease and cancer spread by metastasis [4]. These genes play a crucial role in epigenetic alteration that involves chromatin compaction and nuclear architecture. The overall survival (OS) rate in the last five years has remained at ~15% despite the improvements by research and development into novel therapies [5, 6]. The future development of therapies using an epigenetic approach such as DNA methyltransferase inhibitors (DNMTi) and histone-modifying drugs may provide a new route in treatment and would potentially contribute to better OS. These small molecule inhibitors have already been tested in the clinic on their own or in combination with chemotherapy drugs. The idea of using combination therapy allows the cancer cells to be exposed first to the epigenetic drug thus priming the cancer cells to the chemotherapy [7, 8].

DNA methylation is a process that facilitates the transfer of methyl groups to the 5’ site of a cytosine in a cytosine-guanine (CpG) dinucleotide. The latter are frequently found in high density areas, termed CpG islands, which are located in more than half of the human gene promoters. The function of this modification, in combination with histone

modifications, facilitates the inhibition of RNA polymerase to bind to this region and thus silence the affected gene [9, 10].

Preventing abnormal DNA methylation as that occurring in cancer cells could provide a protective mechanism allowing the cells to recover and facilitate the efficacy of treatment such as chemotherapeutics. Herein, we will discuss the current published and ongoing clinical trials utilizing epigenetic therapy in NSCLC.

DNA methylation

DNA methylation is the major type of epigenetic modification that plays a key role in regulating gene expression, mammalian developmental stages, genomic imprinting and in cancer progression [11-13]. DNA methyltransferases (DNMTs) facilitate the transfer of methyl to cytosine using the methyl donor S-adenosyl methionine (SAM) (Figure 1). In cancer cells, there are two types of DNA methylation abnormalities; hypo- and hyper-methylation. Hypermethylation can result in the silencing of TSGs or genes that are involved in cancer progression such as metastasis, invasion, and immune response of T-cell recognition [14, 15]. Global genome hypomethylation which is typical of aging cells, is a hallmark of cancer [16] and is associated with genomic instability [17].

DNA methyltransferases

Mammals have four active members of DNMTs called DNMT1, DNMT3A, DNMT3B and DNMT3L; the latter being an accessory protein in the DNA methylation process [18]. The DNMTs are a group of proteins that share a similar amino acid sequence and are highly conserved. The N-terminus is composed of a regulatory domain and the C-terminus has the catalytic domain [19]. In lung cancer, multiple epigenetic modifications, including DNA methylation, result from chronic carcinogen exposure

such as smoking and radon gas [20]. DNMTs upregulation has been reported in smokers with NSCLC, being associated with silencing of TSGs such as FHIT, CDKN2A and RARβ [21, 22]. The reactive oxygen species (ROS) could be produced during exposure to cigarette smoke caus localized inflammation in the airways [23]. There is a fine balance between oxidants and antioxidants termed "redox homeostasis" that's critical for a healthy response in all organisms including mammals. Excessive oxidative stress results in the targeting of transcriptional repressors and thus an increase in DNA methylation. The biological consequences lead to chromosomal changes, mutations etc. that are important processes contributing to carcinogenesis [24].

DNMT1

The DNMT1 is the main maintenance methyltransferase responsible for copying methylation patterns to the nascent DNA strand after DNA replication [25, 26]. DNMT1 is found associated with DNA replication forks in the S phase of the cell cycle, which methylate's the newly synthesized DNA [27, 28]. The N-terminal region is composed of 621 amino acids (aa) that are not required for DNMT1 activity but play a role in distinguishing between hemi-methylated and unmethylated DNA [29] (Figure 2). The C-terminal region is composed of 500 aa that contains the active centre and displays conservation between C5 DNMTs from eukaryotes and prokaryotes. All DNMTs catalytic domains display a shared core structure called the "AdoMet-dependent methyltransferase". This region is required for cofactor binding (motifs I and X) and substrate catalysis (motifs IV, VI and VIII). The target recognition domain located between motifs VIII and IX plays a role in DNA recognition and specificity. The DNMT1 is the main DNMTs directed towards the replication foci. There exists three nuclear localization signal (NLS) sequences located to the N-terminus which

contribute to the maintenance of methylation and facilitate the enzyme to be close to the nuclear replication site including the replication foci targeting sequence, the poly bromo homology domain and the proliferating cell nuclear antigen (PCNA) binding domain. PCNA is involved in DNA replication and its interaction with DNMT1 could facilitate the newly synthesized daughter strands to be remethylated prior to them being packaged into chromatin. DNMT1 closely binds to the replication machinery thus allowing DNMT1 to associate with newly replicated and histone-free DNA [30]. It is suggested that p21, cell-cycle regulator can block DNMT-PCNA interacting. DNA damage activates p21 protein which could regulate DNMT-PCNA association. In addition, p21 could down regulate DNMT1 expression. Deletion of both alleles of DNMT1 in mice demonstrates lethality on day E9 of development [31]. There are other cell cycle regulators that can interact with DNMT1 such as retinoblastoma gene product (Rb). Rb can bind to DNMT1 and block the methytransferase activity of DNMT1 during DNA replication. Downregulation of Rb may allow DNMT1 to freely move in the genome and thus could lead to abnormal *de novo* methylation of CpG [32, 33]. Poly (ADP-ribose) polymerase 1 (PARP1) assists in repairing single-strand DNA breaks. Blocking the action of PARP1 prevents the repair of single-strand breaks and leads to double strand breaks (DSB). Interestingly, poly (ADP-ribose) polymerase 1 (PARP1) has been shown to interact with the promoter of DNMT1 and has a role in protecting CpG island methylation and limiting the silencing at transcription [34]. In other studies, this interaction of PARP1 and DNMT1 regulates the activity of DNMT1 [35, 36]. In subsequent studies, PARPi and DNMTi were combined to target BRCA proficient triple negative breast cancer and AML resulting in enhanced binding of PARP1 in chromatin and retaining PARP1 and DNMT1 at double strand breaks (DSB) [37].

TRDMT1 (DNMT2)

TRDMT1 formally known as DNMT2 is the shortest of all the mammalian DNMT. It is composed of the C-terminal region lacking the regulatory N-terminal domain (Figure 2). It is thought that TRDMT is involved in recognizing DNA damage, mutation repair and DNA recombination [38]. While TRDMT is not considered to be a DNA methylase, it can methylate cytosine-38 of the small transfer RNA (tRNA) as opposed to DNA [39, 40].

DNMT3A , -3B, L

The DNMT3 family (DNMT3A and DNMT3B) were first cloned and characterized back in 1998 [41]. There appears to be high conservation regarding amino acid sequence between different organisms including human and mouse DNMT3 display high similarity (~95%) along with maize, Arabidopsis thaliana, and zebrafish [41-43]. The DNMT3A and DNMT3B are unable to distinguish between hemi-methylated and unmethylated CpG sites and therefore are unable to copy patterns of methylation or contribute to methylation maintenance [44], these enzymes are suggested to function as *de novo methyltransferases* and remain distributed in the nucleus [44]. Mouse knockouts of the DNMT3A die early at around four weeks while DNMT3B knockout results in embryonic lethality between E14.5 to E18.5 [44], and reduces the embryonic skeletal growth at E14.5 and E18.5 [45]. Expression levels of DNMT3A and DNMT3B are higher at early embryonic stage than after differentiation and in somatic tissues at the adult stage. This is not surprising as the embryonic stage is when the major *de novo methylation* events are likely to occur. However, in cancer the DNMT3s are overexpressed in tumour cells [46]. A number of studies have highlighted the relation

of DNMT3B overexpression in various cancers [47-50]. DNMT3B has been demonstrated to play a pivotal role in *de novo hypermethylation* of promoter CpG islands, which maybe an important process for the down regulation of TSGs in cancer cells [46].

DNMT3L is another member belonging to the same family of DNMT3s has been shown to have a regulatory function during *de novo methylation* [51]. DNMT3L is highly conserved to DNMT3A and DNMT3B apart from motifs necessary for DNMT activity at the C-terminus are lacking [51].

Some reports indicate that in human colon cancer cells the association of DNMT1 and DNMT3B could be important in regards to the maintenance of DNA methylation patterns [51]. Recent studies have shown that DNMT3A and DNMT3B are interacting together with nucleosomes that contain methylated DNA [51]. Interestingly, the levels of DNMT3A and DNMT3B are elevated in many different cancers [52], this could be responsible for hypermethylation of promoter CpG-rich regions of particular TSGs found in cancer [53].

DNA methylation: global and gene-specific aberration in cancer cells

DNA methylation is crucial for gene regulation of tissue and developmental stages [54, 55]. This biological process has been demonstrated to increase with aging, and also when cell lines are maintained in culture [56, 57]. Several studies have shown that both global DNA hypomethylation and local hypermethylation appear during cancer progression [58]. This epigenetic abnormality usually occurs as a nonrandom event and is specific to the type of tumour [14]. The DNA methylation status and patterns of chromatin are aberrantly altered in cancer cells. A majority of cancers display both reduced methylation of depleted CpG regions in areas where all the CpG dinucleotides

would be methylated, and elevated levels of methylation of CpG islands within promoter regions of a gene [59]. In addition, methylation in cancer is usually specific to certain promoters in the tumour [60]. A reduction in DNA methylation status in human cancer could have several effects on tumourigenesis. The first could be lowered transcriptional repression of dormant regions of the genome which may lead to an insertion of viral genes, or alteration of imprinting leading to biallelic gene expression [61]. Secondly, reduced methylation in cancer could have a major impact on the stability of the chromosomes [62, 63].

DNA methylation has been indicated to act as an initiator of cancer and progression through inactivation of genes [64, 65]. These epigenetic disorders are often attributed to a dysfunction of DNMT [12, 18]. DMNTs play a pivotal role in the maintenance of chromosomal homeostasis due to their catalytic role and ability to block gene transcription [66]. Dysfunction of DMNTs has a significant consequence on the DNA and histone modifications leading to instability of the genome, inactivation of genes and chromatin remodeling. Thus, the genome of cancer cells shows global hypomethylation and regional hypermethylation to specific areas [67]. Furthermore, epigenetic dysfunction could be a result of cross talk between DNMTs and chromatin regulators such as transcriptional co-suppressors and histone methyltransferases [68-71].

In cancer cells, DNA hypomethylation was the first described type of epigenetic abnormality [14]. Indeed, deletion or reduced expression levels of DNMT1 has been shown to cause global hypomethylation of the genome and instability of chromosomes [62]. In acute myeloid leukemia (AML) presented with DNMT3A mutations, HOXB cluster was demonstrated to contain hypomethylated CpG islands (CGIs) [72]. These studies have given some insights into the role of aberrant DNMTs on global

hypomethylation in tumorigenesis. Further studies are required to understand the mechanisms that control global genome hypomethylation in cancer progression [11].

In healthy cells, DNA methylation appear in dinucleotides with fewer CpG, while CpG rich areas are unmethylated [66, 69] (Figure 3). Advanced stages of cancer are associated with a global change in methylation status where hypermethylation of the promoter region of a number of genes is elevated [73]. Thus, this type of methylation leads to the silencing of specific TSGs that are pivotal to cancer progression (Figure 3). Aberrant CGI hypermethylation is a key epigenetic characteristic of cancer where hypermethylation of TSGs is mostly implicated [11, 74, 75]. Regulation of gene expression can occur due to interaction between DMNT1 and noncoding RNAs that affects the DNA methylation level of the methylation-sensitive gene CCAAT/enhancer-binding protein alpha (CEBPA) [76].

Many teams around the world have studied the role of abnormal TSG methylation in respect to cancer progression. In most studies, the DNMTs are indicated as a driving force in these studies. For example, in breast cancer, DNMT3B is overexpressed and is attributed to the hypermethylation of BRCA1 promoter [77]. In knockout DNMT3 mice, hypomethylation is reduced in conserved domains (canyons) containing transcription factors [78]. The canyon related genes such as HOX are elevated with DNMT3A mutation in AML [78].

A number of studies are discovering that non-TSGs are becoming largely methylated during an early stage in cancer. It has been reported for an AML profile study of DNMT3A mutations that a majority of hypermethylated cytosines are found in intergenic regions and gene bodies [79]. Likewise, transgenic mice with DNMT3A mutation have higher levels of hypermethylation in intergenic regions and gene bodies of lymphocyte developmental genes e.g. Gata3 and Notch1 [80]. Also, gene body

methylation by DNMT3B could promote transcription and could be a novel therapeutic target for cancer [81]. DNA hydroxy-methylation could play a role in cancer, as in glioma, a 5-hydroxymethyl cytosine (5hmC), which is produced from 5-methylcytosine (5mC) with the help of ten-eleven translocation (Tet) enzyme is a novel non-canonical aberrant DNA hyper-methylation type [82]. Both DNA hypermethylation, along with aberrant expression of non-coding RNAs (ncRNA) play a role in developing lung cancer [83].

DNA methylation profile in lung tumour/biofluids

The major cause of lung cancer is smoking-related which is driven by molecular changes in the tumour tissue. The genetic changes alter between different types of lung cancer i.e. SCLC and NSCLC but minor changes are observed between subtypes such as SCC and AD [84]. Smoking is mostly responsible for the epigenetic aberration causing the downregulation of the p16 gene in NSCLC [85]. Different types of cancers display in their tumour tissue a unique signature of aberrant methylation [86]. Early studies investigated the methylation profile of neuroendocrine tumours of SCC and AD, indicating that there was no significant difference in the methylation profile between the SCLC and NSCLC groups, while the methylation of the TSG RAS association domain family 1A (RASSF1A) was much higher in tumours of SCLC than in NSCLC [87-90]. The methylation of RASSF1A was also linked to earlier recurrence at stages I and II of NSCLC [91]. In contrast, the occurrence of p16, CDH13 and APC methylation was elevated in NSCLC compared to carcinoid tumours. In addition, the expression and methylation status of the TSG, p16/INK4A has a more favourable prognostic role in cancer [92-94]. Aberrant hypermethylation has been observed in many other promoters such as APC, RARβ, CDKN2A, MGMT, MLH and MSH2 and many others in lung

cancer which are detailed in Table 1 [59, 95-99]. In addition, a number of chromatin modifications can be used as prognostic markers. A study by Moran and colleagues compared the methylation pattern of about 104 genes known to have altered methylation status in NSCLCs to its corresponding non-tumour controls [100]. Moreover, they studied the prognostic correlations in NSCLC patients and found that calcitonin related polypeptide alpha (CALCA) and matrix metalloproteinase (MMP-2) hypermethylation are associated with poor prognosis, compared to RASSF1 hypermethylation that confers a good prognosis [100]. Another study analysed the profile of the expression level of the promoter, along with the DNA methylation status and they found upregulated/hypomethylated promoters such as myeloma overexpressed (MYEOV), which is overexpressed in NSCLC. MYEOV knockdown showed a decrease in cell proliferation, invasion and increase in apoptosis [101]. In addition, several histone gene loci are abnormally hypermethylated in lung cancer, and histone genes methylation can be used as an early detection biomarker in the samples of bronchoalveolar fluid (BALF) [102]

Dietary phytochemicals effect on epigenetic mechanisms

Despite the continuous work on developing new anti-cancer drugs, phytochemicals that affect cellular ROS have been paid limited attention. However, maintenance of the redox homeostasis is by antioxidant and pro-oxidant compounds intake. Depending on the concentration and cellular microenvironment, dietary phytochemicals such as polyphenols can exhibit both antioxidant and pro-oxidant activities [103, 104]. Dietary phytochemicals or phytonutrients also named secondary plant metabolites that have the ability to fight the disease if taken in effective concentration [105]. They are found in fruits, vegetables, and products derived from plants, for example, tea, wine, and spices.

According to different studies, regular intake of these fruits, vegetables, or supplements that have phytochemicals may be useful in preventing or controlling different types of cancer [106-111]. Also, dietary phytochemicals could modulate the epigenetic mechanisms including DNA methylation, histone modification, and non-coding RNAs [112-114]. Genistein, Resveratrol, Polyphenols, and different types of fatty acids are an example of nutrients that have positive effects on health; they are further studied as protective factors against cancer, cardiovascular disease and obesity through understanding their epigenetic mechanisms [115].

DNA methylation is one of the epigenetic mechanisms that depends on methyl donors availability. S-Adenosylmethionine (SAM) is the known methyl group donor synthesised in the methionine cycle which depends on various diet-derived precusors, such as methionine, folate, choline, betaine, vitamin B2, B6, and B12 that enter the cycle and end up with SAM generation. Thus, deficiency of methyl donors results in low SAM synthesis and alternation of DNA methylation pattern [116, 117]. Netherlands Cohort Study on diet and cancer demonstrated that severe famine during childhood and adolescence is correlated to a lower risk of colorectal cancer through modulating the methylation status of cancer-related genes [118]. Also, famine during prenatal period is associated with low DNA methylation status of insulin-like growth factor 2 (IGF2) gene compared to control group, illustrating that early stages of fetal development have an effect on maintaining the epigenetic marks [119]. In addition, diet could affect histone modification, such as acetylation, deacetylation, and methylation that have an impact on the intiation and development of cancer [120]. A study by Wolff and colleagues found that feeding pregnant female mice with a diet rich in methyl donor resulted in producing offspring with a high percentage of wild-type color in coat compared to controls that had a normal diet by affecting the chromation structure and

transcription through epigenetic modifications [121]. However, the connection between the environment (diet) and biological effect (regulation of transcription) is mediated through histone post-translational modifications [122]. On the other hand, dysregulation of miRNA expression promotes cancer intiation through modulating cell death, proliferation, migration and invasion [123]. Different studies illustrated the effect of plant-derived foods and bioactive compounds, for example curcumin, sulforaphane, genistein and others in modulating oncogenic and tumour suppressive miRNAs expression [124-127].

Many studies have investigated the effect of phytochemicals on epigenetic mechanisms in lung cancer. A study by Taniguchi and colleagues examined the effect of (−)-epigallocatechin gallate (EGCG) which is the major polyphenol compound of green tea on both artificial and spontaneous lung metastasis and they found that the number of lung nodule had decreased depending on the amount of EGCG solution (0.05% or 0.1%) that was administered perorally [128]. A cohort-based study showed the association between the dietary factors such as leafy green vegetables, folate, and multivitamins and the DNA metylation status of cells derived from aerodigestive tract of both smokers and former smokers [129]. This study explained the use of combination therapy of TRAIL and naringenin on TRAIL-resistant NSCLC A549 cells to induce apoptosis [130]. Other studies explaining the effect of bioactive ingredients on cancers are summarised in Table 2. Further studies of the effects of phytochemicals on epigenetic mechanisms could provide new biomarkers for cancer prevention and alternative therapeutic approach in cancer.

Current development of demethylating agents for lung cancer treatment

Aberrant DNA methylation of cancer cells plays a pivotal role in cancer progression

which is evident from early stages in the disease. Novel applications are being developed to address the improvement of lung cancer treatment which now includes epigenetic therapy (Figure 4).

Currently, there are several drugs specifically targeting epigenetic alterations that have been approved by the US Food and Drug Administration (FDA). These include the demethylating agents 5-azacytidine (myelodysplastic syndrome treatment) which is subcutaneously injected at a dose of 75 mg/m^2/day for 7 days and then repeated every 28 days; the dose can be decreased to 50 mg/m^2 for haematological toxicity or increased to 100 mg/m^2 in case of no response after two cycles. Thrombocytopenia and leukopenia were among haematological toxicity, along with extramedullary toxicity such as pneumonia, arthralgia, diarrhoea, and irritation at the site of injection which was uncommon [131]. Different doses of decitabine (myelodysplastic syndrome and AML treatment) were used for the treatment of myelodysplastic syndrome arranged in three cycles to achieve the best efficiencies, while decitabine dosage for AML is 25 mg/m^2 for 1-4 days and repeated every 4 weeks as a cycle [132]. World Health Organisation toxicity grade was used to evaluate decitabine toxicity which included infection, liver toxicity nausea, vomiting, cardiovascular effects, and mucositis [132], histone deacetylases inhibitors (HDACi) for T-cell lymphoma are romidepsin (FDA dose 14 mg/m^2, 4 h infusion on 1, 8, 15 days of 28-day cycle) requires monitoring for patients' thrombocytopenia, neutropenia, lymphopenia, and anaemia during treatment while vorinostat (phase II trials maximum tolerated dose 400 mg/day) results in gastrointestinal symptoms, fatigue, and thrombocytopenia as common side effects and thrombosis is the most serious event [133, 134]. Belinostat used for peripheral T-cell lymphoma as an intravenous infusion of 100 mg/m^2/day over 30 minutes on days 1 - 5 of a 21 day cycle with common side effects including nausea, vomiting, fatigue,

anaemia while serious side effects are pneumonia, thrombocytopenia, and multi-organ failure [135] and Panobinostat for multiple myeloma is given as three doses per week of an initial dose schedule of 20 mg once every other day of weeks and 2 of each 21-day cycle for 8 cycles, which can be adjusted depending on the type of toxicity developed in the patient such as thrombocytopenia, anaemia, diarrhoea, nausea and vomiting [136].

For lung cancer, there are some drugs currently in clinical trial (phase I/II) that are being tested in refractory patients with metastatic NSCLC (Table 3). The results showed that a combination of epigenetic treatment with low dose of DNA methylation inhibitor, azacytidine (CC-486) (phase II dose 40 mg/m^2/day) and the histone deacetylase inhibitors, entinostat (7 mg fixed dose) may improve survival in advanced NSCLC with common side effects such as reactions at the site of injection, gastrointestinal disturbances, hyperglycaemia, and haematological side effects [137]. Combined treatment of the epigenetic drugs and chemotherapy for NSCLC has been trialed in the clinic. Combinational treatment with paclitaxel and carboplatin with a HDAC inhibitor, vorinostat or placebo trialed in phase II. These trials indicate that vorinostat promotes the treatment with paclitaxel and carboplatin in advanced lung cancer patients [138]. Due to lack of efficacy, phase III trial of this combination was terminated [139]. *In vitro* studies have shown that DNA methylation can be inhibited by 5-aza-2'deoxycytidine which sensitizes lung cancer cells to the EGFR inhibitor, gefitinib [140]. Treatment with gefitinib is efficient in patients presenting with EGFR mutations, while a majority of tumour cells are resistant to this treatment. The EGFR gene can be hypermethylated and thus silenced in numerous cell lines. Treating with 5-aza-2'deoxycytidine restores EGFR expression and thus, enables the cells to respond to gefitinib treatment [140]. In a late stage NSCLC study, 5-aza-2'deoxycytidine treatment revealed improved survival

times by re-expression of TSG silenced while haematopoietic toxicity is the major side effect [141].

A number of clinical trials are currently on-going for NSCLC including azacitidine combined with pembrolizumab and pembrolizumab alone in advanced NSCLC [142]. Other trials are studying combined nab-paclitaxel with azacitidine as a second line for advanced NSCLC [143]. A new epigenetic drug called RRx-001 can inhibit HDACs, DNMT1 and DNMT3a expression. This drug is currently being tested in combination with platinum doublet chemotherapy treatment [144]. In another trial (phase I), azacytidine is being tested as an inhaled product in NSCLC [145]. A few studies have reported that PARPi enhances the formation of DSBs with radiosensitisation in lung cancer models [146, 147]. A preclinical trial has recently utilized DNMTi to sensitize NSCLC cells to PARP inhibition and radiotherapy by a mechanism resulting in aberration DNA damage repair [148].

Zebularine is another member of nucleoside DNMT inhibitor family, that showed good results *in vitro* experiments regarding potency and considered as a promising agent to be used for future clinical trials [149]. Zebularine inhibits DNMT by the formation of tight covalent complexes between DNMT protein and zebularine-substituted DNA [150]. At acidic and neutral pH, zebularine is highly stable, which is unlike azacytidine and decitabine. It is administered by frequent dosing or continuous intravenous infusion to maintain prolonged DNMT inhibition [151]. Combinations of zebularine with other therapeutic agents such as chemotherapy, immunotherapy or radiotherapy could be the future medical use of this drug as it is a promising drug candidate [149].

Technological advancements and HTS drug discovery

The availability of 3D DNMTs, medicinal chemistry, drug repurposing and *in silico* methods has helped develop drug candidates or probes that target DNMTs. Due to the different side effects caused by DNMTi, low specificity prompts the need to develop more potent inhibitors through experimental and computational strategies. Different approaches such as molecular docking, virtual screening, pharmacophore modelling, and molecular modelling have been used to understand the activity of the known compounds and help design novel DNMTi. Structure-based approaches suggest that long scaffolds molecules can target DNMTs catalytic and cofactor-binding sites; while screening of compound database by *in silico* studies has led to the discovery of active compounds [152]. Moreover, DNMTs crystallographic structures have contributed to the lead compounds optimisation that is based on the target structure. In addition, Computer-aided drug repurposing and computational nutri-epigenomics are considered as synergistic approaches. Designing of master key epigenetic compounds would be the most effective approach to proceed with epi-drugs into the clinic and help in illustrating the epigenetic mechanisms through the development of epi-probes that target DNMTs [153].

A recent study has illustrated the pilot drug combination (DC) high throughput screening (HTS) of 45 pairwise 4 x 4 drug combination matrix that is generated from 10 test compounds and arrayed onto 3 x 384-well growth inhibition assays for six patient-derived melanoma cell lines by using the pairwise drug combinations between dasatinib and melanoma approved drugs (dabrafenib, vemurafenib, or trametinib). They found that these DCs synergistically inhibited cell growth, activated apoptosis, and increased cell death of mouse melanoma cell lines independent of their drug resistance phenotypes. The *in vitro* studies pave the way for further investigation of

DCs of melanoma combination therapies to improve the outcome and/or to prevent or delay the development of disease resistance [154]. Another study explained the use of structure-activity relationship (SAR) to test the anticancer activity of caged xanthones and their derivatives against various cancer cell lines as a model, such as A549, HepG2, and U251. In this study, the compounds were selected and docked to find the potential target and their metabolites were assessed by pharmacokinetics. After that, the lead compound was selected for further analysis like gene ontology, signalling pathway maps, metabolic networks and identification of off-target sites [155].

Conclusion and future perspective

The incidence of lung cancer is associated with aging and the use of tobacco products negating cardiovascular and pulmonary comorbidities. Dysregulation of DNA methylation is a hallmark of many cancers including lung cancer. DNMTs targeted therapy is an emerging treatment of diseases controlled by epigenetic changes. Currently, there are two DNMTi, azacytidine and decitabine approved for treating patients affected by MDS and AML while others are being trialled in multiple types of cancers including lung cancer. Many efforts are being made to improve the efficacy through combining DNMTis with other therapies. Recent studies on PARPi and DNMTi combination with radiotherapy are promising and could provide a new avenue of treatment especially for patients that are unable to tolerate platinum-based chemoradiotherapy. Another avenue of treatment not well explored to date would be the use of antisense technology to downregulate DNMT in lung cancer. A few studies have explored the effect of using DNMT1 siRNA on lung cancer [156, 157]. Researchers have developed epigenetic tools from the bacterial immune system, CRISPR/Cas9 system [158, 159]. A number of studies have reported the use of CRISPR/Cas9 editing tools to alter DNA methylation and gene expression [160, 161]. These same tools could be developed to specifically target and modulate DNMT's in lung cancer. Currently, lung cancer is the major cause of cancer death worldwide. The patients and relatives affected by this terrible disease rely on the scientific community to make the discoveries moving quickly from bench to bedside to deliver and improve outcome. The development and a greater understanding of epigenetic therapies will lead the way to solve this endeavour.

Figures

Figure 1 DNA methylation. Cytosine is converted to 5'-methyl-cytosine catalyzed by DNMTs and SAM that donates the methyl group. SAM, S-adenosyl methionine.

Figure 2 Structure of the known DNA methyltransferases (DNMT's) and DNMT-like proteins. The N-terminal domain is composed of a motif interacting with proteins or DNA essential in the regulatory function. The C-terminal domain is composed of a methytransferase region necessary for the catalytic function. Other important regions such as the nuclear localization sequence (NLS), replication foci targeting (RFT), zinc binding region of DNMT1 or the cysteine-rich PHD (plant homeodomain) region located to DNMT3A/3B.

Figure 3 DNA methylation pattern in normal and cancer cells. CpG islands in promoter region are unmethylated in normal cells, whereas they are either hyper- or hypo- methylated in cancer cells. In gene bodies, the CpG islands are rarely methylated in normal cells, while they are abnormally methylated in cancer cells, leading to silencing of TSGs or genes that are involved in cancer or genomic instability due to activation of transcription of several incorrect regions. 1, 2, 3 exons of the gene; X, transcription inactivation; DNMTs, DNA methylatransferase.

Figure 4 Targeting epigenetic alterations. DNA methylation, histone acetylation, and methylation represent the common epigenetic alterations related to gene silencing. H1, histone 1 or linker histone 1 that binds around the DNA entry and exit sites and responsible for maintaining the nucleosome's structure. DNMTs, HATs, HMTs, and

HDACs are the enzymes responsible for transferring or removing the chemical groups from DNA or histone resulting in DNA methylation, histone acetylation, histone methylation, and histone deacetylase, respectively. Inhibition of these enzymes stops epigenetic alterations resulting in gene silencing and used for the treatment of different cancers. While binding proteins are responsible for recognising the modified histone or methyl-CpG island. DNMTs, DNA methyltransferases; HMTs, histone methyltransferases; HATs, histone acetylases; HDACs, histone deacetylases.

Table 1 Genes hypermethylated in lung cancer.

Table 2 A summary of studies exploring the effect of bioactive ingredients on cancers.

Table 3 DNA methyl transferase inhibitors and combined therapies undergoing clinical trials for NSCLC.

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Table 1 Genes hypermethylated in lung cancer

Mechanism	Target gene	Gene function	References
Apoptosis	DAPK1	Pro-apoptotic	[162-164]
	TNFRSF6	TNF-receptor family, mediates apoptosis, SCLC	[165]
	DR4, DR5	TNF-receptor family, mediates apoptosis, SCLC	[165]
	CASP8	Apoptosis effector, SCLC	[165]
	CXCL14	Pro-apoptosis, cell cycle arrest	[166]
	DCL1	Apoptosis, cell growth and cell adhesion	[167]
	FHIT	Apoptosis, transcriptional activator	[168-171]
Cell cycle	P16	CDK4/6 inhibitor in cell cycle arrest at G1/S checkpoint	[59, 85, 172, 173]
	RASSF1A	Cell cycle regulation, and ras-induced apoptosis	[164, 174, 175]
	PTEN	AKT/mTOR negative regulator and cell cycle	[176]
	CDKN2A	Cyclin-dependent kinase inhibitor; cell cycle arrest	[164, 167]
	Reprimo	p53-mediated cell cycle arrest	[177]
	CCNA1	Cell cycle regulator	[178]
	P14 (ARF)	p53 and cell cycle regulator	[179]
DNA repair	MGMT	DNA repair, removes alkyl from O6 position of guanine	[164, 180, 181]
	hMLH1	DNA repair	[95]
	MSH2	DNA repair	[95]
	CYGB	ROS scavenger	[182]
	OTUD4	DNA damage repair pathways (GG-NER and alkylation damage repair pathway)	[183]
Cell adhesion and invasion	CDH1	Cell-cell adhesion, cell motility inhibitor, invasion and metastasis	[164, 184]
	CDH13	Regulates cell proliferation	[184]
	TSLC1	Cell-cell adhesion	[185]
	DAL-1	Cell-cell contact	[186]
	DCL1	Apoptosis, cell growth and cell adhesion	[167]
	MMP2	Degradation of extracellular matrix	[181]
	TIMP3	Tissue inhibitor of metalloproteinase 3; metastasis	[167]
	NISCH	Cytoskeleton organization and cell migration	[187]
	KIF1A	Kinesin family; microtubule transport	[187]
	CTSZ	cysteine cathepsin protease family; Cell invasion	[178]
	LOX	Monoamine oxidase lysyl oxidase; Cell invasion	[178, 188]
Transcription Regulation	APC	Negative regulator of Wnt pathway and β-catenin	[96, 189]
	RARβ-2	Cell growth and differentiation	[97, 164]
	SHOX2	Regulator of transcription, cell growth and differentiation	[190]
	RUNX3	Transcription factor, TSG and pro-apoptotic	[174, 191]
	BHLHB4	Regulator of transcription; differentiation	[181]
	BLU	Transcription repressor	[175, 181, 192]
	HOXA	Homeobox transcription factors; differentiation	[193, 194]
	TCF21	Differentiation	[195]
	BRMS1	Transcriptional repressor of NF-κB; pro-apoptotic; prognostic NSCLC	[196, 197]
	BNC1	Regulator of rRNA transcription	[178, 198]
	TBX-2	Cell development and differentiation	[199]
Signalling and Wnt pathway	DKK3	Wnt pathway antagonist	[181, 200]
	SFRP1	Wnt pathway antagonist	[201-203]
	WIF1	Wnt Pathway antagonist	[203, 204]
	OGDHL	AKT-Dependent Signaling and NF-κB Function	[205]
	DOK1	Role in mitogenic signaling	[206]

Table 2 A summary of studies exploring the effect of bioactive ingredients on cancers.

Bioactive compound	Epigenetic functions	Reference
(-)-epigallocatechin 3-gallate (EGCG)	Inhibitor of DNMT1 and HDAC	[207]
Genistein	Inhibitor of DNMT1	[208]
Apigenin	Inhibitor of DNMT1, DNMT3a, DNMT3b, HDAC1, HDAC3	[209]
Delphinidin	Inhibitor of DNMT, HAT, HDAC-3	[210, 211]
Kaempferol	Inhibitor of DNMT3a, DNMT3b, HDAC1	[212, 213]
Naringenin	Inhibitor of DNMT1, DNMT3a, DNMT3b, HDAC1	[214, 215]
Quercetin	Inhibitor of DNMT1, HDAC. Down-regulation of histone demethylation	[216-218]
Xanthohumol	Inhibitor of DNMT and HDAC	[219-221]
Resveratrol	Inhibitor of DNMT3a, DNMT3b. SIRT1, SIRT2, and SIRT3 activity modulated (up- and downregulated). HAT inhibitor regulation of histone phosphorylation	[222, 223]
Luteolin	Inhibitor of DNMT, HDAC	[224]
Magnolol	Reduced the protein expression levels of HDAC2, HDAC3, and HDAC8	[225]
Polyphenol Mixture (PM)	Decreased the protein expression levels of HDAC3	[225]

Table 3 DNA methyl transferase inhibitors and combined therapies undergoing clinical trials for NSCLC.

Drug	Mechanism of action	Clinical trial	Phase
Adjunctive therapy			
Decitabine	Phosphorylated form is incorporated into DNA and inhibits methyltransferase 1	NCT00019825	I
Azacitidine	Inhibition of DNA methyltransferase activity to transfer methyl groups to hemimethylated DNA	NCT01281124	II
Combined with chemotherapy			
Azacitidine, Entinostat, and Nivolumab		NCT01928576	II
5-azacitidine and Romidepsin		NCT01537744	I
5-Fluoro-2-Deoxycytidine and Tetrahydrouridine		NCT00978250	II
CC-486 with MK-3475		NCT02546986	II
CC-486 with nab-paclitaxel		NCT02250326	II
Nivolumab with decitabine		NCT02664181	II
Tetrahydrouridine-decibatine (THU-DAC) with pembrolizumab		NCT03233724	I/II
Combined with epigenetic therapies			
Decitabine and Valproic Acid		NCT00084981	I
CC-486 and Vidaza		NCT02223052	I
Vorinostat and Gefitinib		NCT01027676	I
Panobinostat with Sorafenib		NCT01005797	I
Vorinostat and Bortezomib		NCT00798720	II

Figure 1

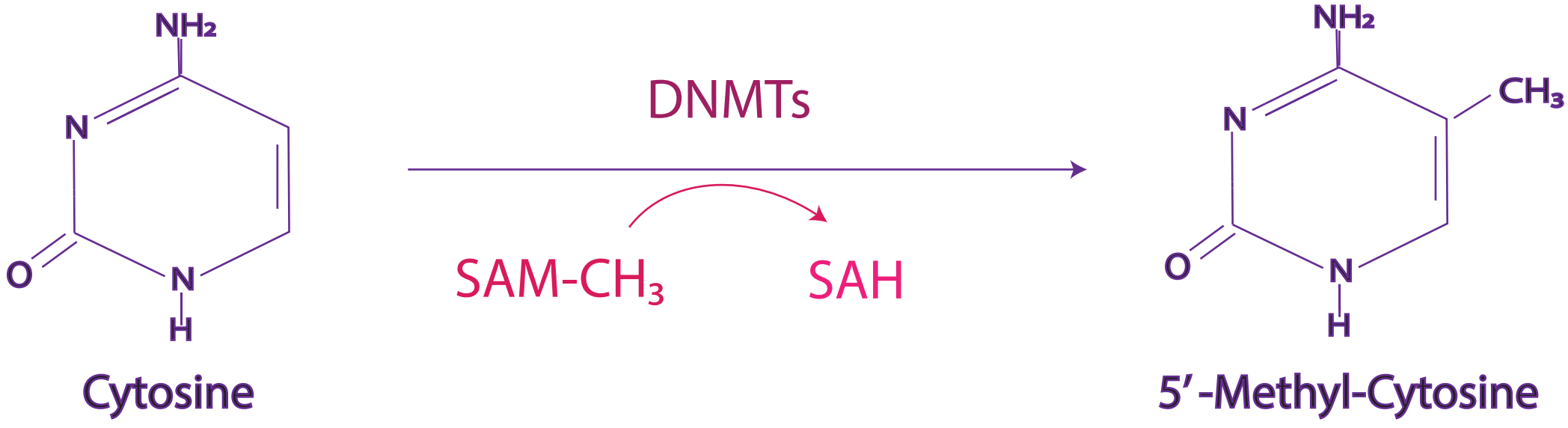

NH2
N
O
N
H
DNMTs
SAM-CH3
SAH
NH2
CH3
N
O
N
H
Cytosine
5’-Methyl-Cytosine

Figure 2

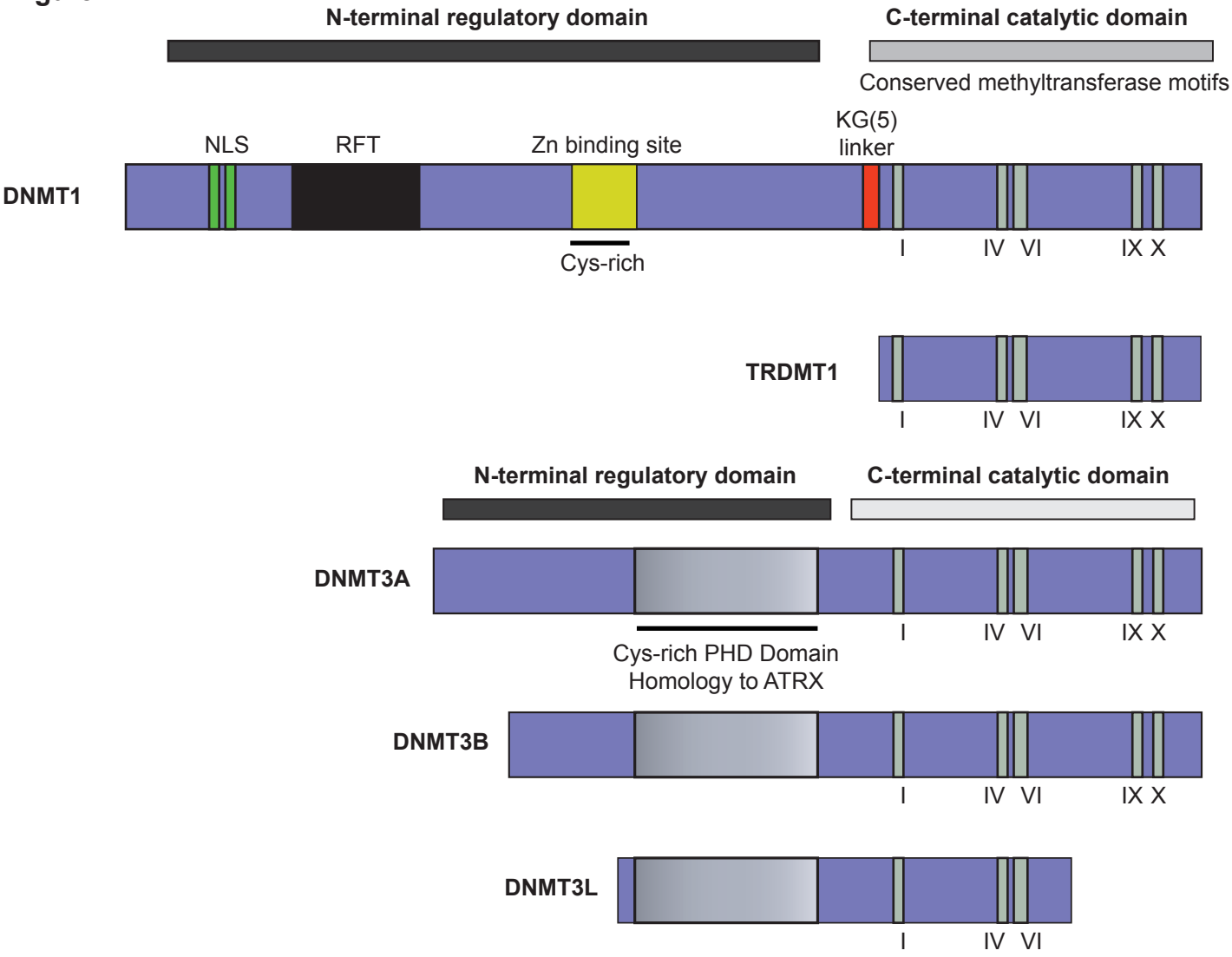

N-terminal regulatory domain
C-terminal catalytic domain
Conserved methyltransferase motifs
NLS
RFT
Zn binding site
KG(5) linker
DNMT1
Cys-rich
I
IV
VI
IX
X
TRDMT1
N-terminal regulatory domain
C-terminal catalytic domain
DNMT3A
Cys-rich PHD Domain
Homology to ATRX
DNMT3B
DNMT3L

Figure 3

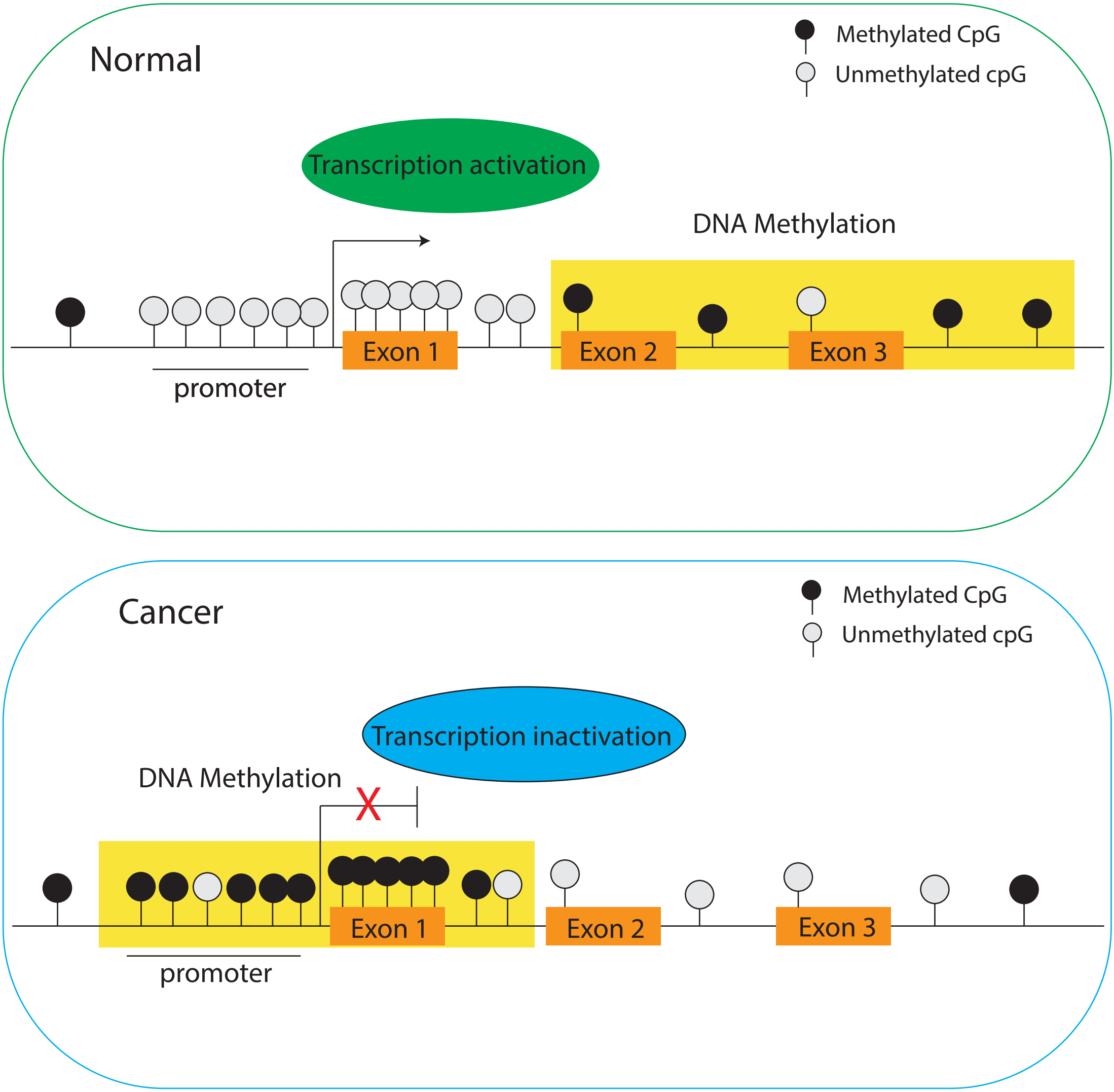
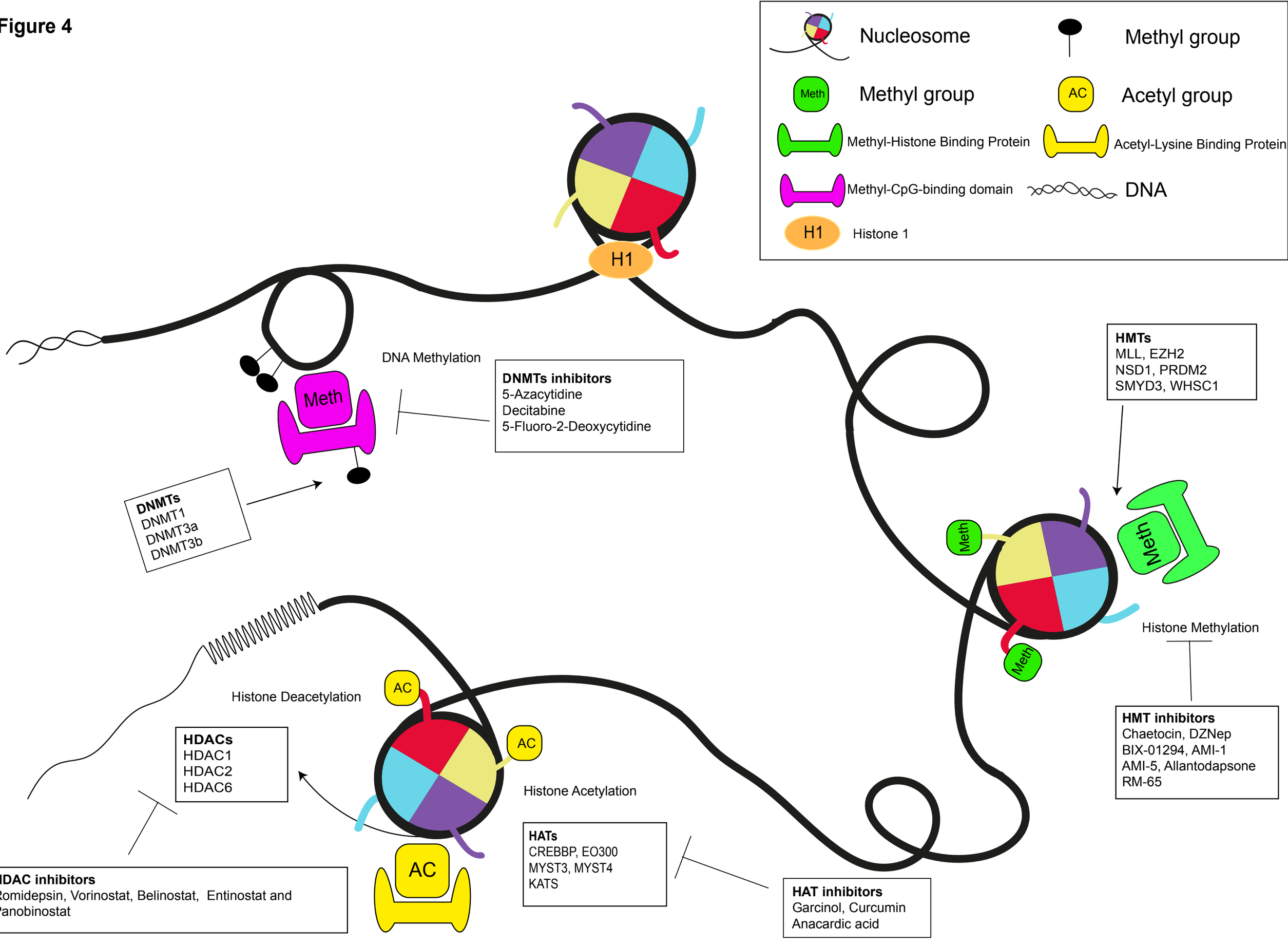

Normal
Methylated CpG
Unmethylated cpG
Transcription activation
DNA Methylation
Exon 1
Exon 2
Exon 3
promoter
Cancer
Methylated CpG
Unmethylated cpG
Transcription inactivation
DNA Methylation
Exon 1
Exon 2
Exon 3
promoter

Figure 4


Nucleosome
Methyl group
Meth
Methyl group
AC
Acetyl group
Methyl-Histone Binding Protein
Acetyl-Lysine Binding Protein
Methyl-CpG-binding domain
DNA
H1
Histone 1
H1
DNA Methylation
Meth
DNMTs inhibitors
5-Azacytidine
Decitabine
5-Fluoro-2-Deoxycytidine
DNMTs
DNMT1
DNMT3a
DNMT3b
HMTs
MLL, EZH2
NSD1, PRDM2
SMYD3, WHSC1
Meth
Meth
Meth
Histone Methylation
HMT inhibitors
Chaetocin, DZNep
BIX-01294, AMI-1
AMI-5, Allantodapsone
RM-65
AC
Histone Deacetylation
HDACs
HDAC1
HDAC2
HDAC6
AC
AC
Histone Acetylation
HATs
CREBBP, EO300
MYST3, MYST4
KATS
HDAC inhibitors
Romidepsin, Vorinostat, Belinostat, Entinostat and Panobinostat
HAT inhibitors
Garcinol, Curcumin
Anacardic acid