Acta Biochimica Polonica

Vol. 53 No. 2/2006, 245–256

on-line at: www.actabp.pl

Review

Mammalian DNA methyltransferases

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Received: 28 December, 2005; revised: 17 February, 2006; accepted: 24 February, 2006
available on-line: 03 April, 2006

DNA methylation is an epigenetic process affecting gene expression and chromatin organization. It can heritably silence or activate transcription of genes without any change in their nucleotide sequences, and for a long time was not recognized as an important regulatory mechanism. However, during the recent years it has been shown that improper methylation, especially hypermethylation of promoter regions, is observed in nearly all steps of tumorigenesis. Aberrant methylation is also the cause of several major pathologies including developmental disorders involving chromosome instabilities and mental retardation. A great progress has been made in our understanding of the enzymatic machinery involved in establishing and maintaining methylation patterns. This allowed for the development of new diagnostic tools and epigenetic treatment therapies. The new approaches hold a great potential; several inhibitors of DNA methyltransferases have already shown very promising therapeutic effects.

Keywords: DNA methylation, DNA methyltransferases

INTRODUCTION

A cell can be characterized by its gene expression pattern. One of the key components regulating gene expression are transcription factors (TF). By recognizing and binding to specific nucleotide sequences in gene promoter regions they can trigger activation or repression of transcription and often promote other regulatory events such as changes in the structure of chromatin (Cosma *et al.*, 1999).

Apart from transcription factors there are other mechanisms regulating gene expression. During different phases of a cell's life it is essential not only to activate/repress genes but also to restrict certain parts of the genome in a semi-stable manner. One of the mechanisms that can stably inactivate genes is DNA methylation (Robertson & Wolffe, 2000). Methylated sequences are not recognized by transcription factors, which prevents expression of corresponding genes (Fig. 1). DNA methylation is also associated with heterochromatin formation, making parts of the genome unavailable (Li *et al.*, 1993; Bird, 2002). This epigenetic process has been shown to play a major role during cell development and also after differentiation to a specialized cell type. Aberrant gene expression patterns linked to DNA methylation have been shown to cause many defects and diseases including development disorders and cancer (Robertson & Wolffe, 2000; Jones & Baylin, 2002; Esteller, 2005).

BIOCHEMISTRY OF METHYLATION

DNA methylation refers to biochemical addition of the methyl group to nucleotide bases. There are three known types of base methylation: adenine can be modified at the N-6 position (in bacteria and also in most eukaryotes) (Baniushin, 2005); cytosine can be modified at N-4 position (mostly thermophilic bacteria) (Gromova & Khoroshaev, 2003) and at the C-5 position. The latter modification is the most

Abbreviations: AdoMet, S-adenosylmethionine; BAH, bromo-adjacent homology domain; CR, cysteine-rich region; DMAP, DNA methyltransferase associated protein; DNMT, DNA methyltransferase; DNMT3L, DNMT3-like; EGCG, (K)-epigallocatechin-3-gallate; ES, embryonic stem; HDAC, histon deacetylase; ICF, immunodeficiency, centromeric instability and facial anomalies; NLS, nuclear localization signal; PBD, DNA-binding region; PCNA, proliferating cell nuclear antigen; RB, retinoblasotma; RFTD, replication foci targeting domain; SAH, *S*-adenosyl L-homocysteine; SAR, structure-activity relationship; TF, transcription factor; TRD, target recognition domain; Xist, X-inactive specific transcript.

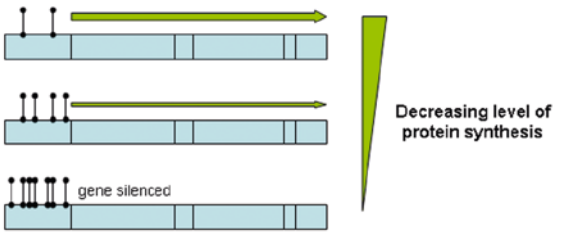

Figure 1. Epimutations silence gene expression.
Methylation changes the transcription level of a gene. Proceeding cumulative promoter methylation decreases the level of protein expression; in the final stage promoter hypermethylation silences the gene altogether.

common form of DNA alteration among all organisms (Bestor, 2000). In this review we will concentrate on the C-5 modification, referring to it as cytosine methylation.

Cytosine methylation is catalyzed by various methyltransferase enzymes (DNMT). In eukaryotes there are three DNMT families (1, 2 and 3). DNMT1 and DNMT3 proteins comprise two domains: the N-terminal "regulatory" domain and C-terminal "catalytic" domain; in contrast DNMT2 has only the "catalytic" domain. Prokaryotic methyltransferases are similar in size to DNMT2 and less complex than the other DNMT family members. They have been easier to study and also to crystallize. Their three dimensional structure helped to understand the base flipping phenomenon (base swinging out of the helix into an extrahelical position) and the catalytic mechanism of DNA methylation.

All methyltransferases utilize a common catalytic mechanism and employ AdoMet cofactor (*S*-adenosylmethionine) as the source of the methyl group (Eden *et al.*, 2003; Villar-Garea *et al.*, 2003). The reaction is started when cytosine is flipped out of DNA and inserted into the binding pocket of a methyltransferase enzyme (Fig. 2). In the active site the catalytic cysteine thiolate forms a transition state intermediate with the carbon-6 of the cytosine's ring. This creates a reactive 4–5 enamine which attacks the methyl group provided by the cofactor. After the methyl group transfer to the C-5 position of the cytosine ring proton abstraction from the 5 position causes reformation of the 5,6 double bond and the release of the enzyme by β-elimination (Santi *et al.*, 1983; Bestor & Verdine, 1994).

In mammals, cytosine methylation takes place almost exclusively at cytosines located 5′ to guanine (CpG, cytosine-phospho-guanine) (Singal & Ginder, 1999). These CpG dinucleotides are underrepresented in the human genome, their occurrence being 5–10 fold lower than statistically expected. The CpG dinucleotides are also unevenly distributed; in mammalian genomes they are clustered in so called CpG islands — short regions of 0.5 to 4 kb in length

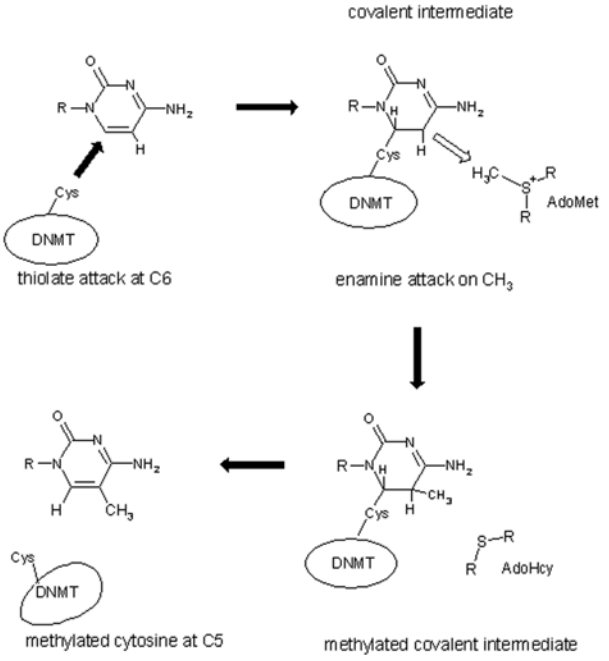

Figure 2. Reaction catalyzed by C5-methyltransferases.

having a rich (60–70%) CG content. Over 50% of all dinucleotides in these islands are CpG, compared to the rest of the genome where the CpG content is ≤ 20% (Bird, 2002).

CpG islands are recognized as an important regulatory mechanism for gene expression (Li, 2002). About 50% of CpG islands are located in promoter regions and around transcription start sites of mainly housekeeping genes. Most of promoter-associated CpG islands are unmethylated in normal cells (with the exception of inactive chromosome X in females). The switch from unmethylated to methylated CpG islands is recognized as an essential contributor to gene silencing. It has been shown that aberrant promoter methylation (hypermethylation) is associated with a loss of gene function similar to that caused by mutations (Robertson & Wolffe, 2000; Jones & Baylin, 2002). Genomic hypermethylation has a great impact on the cell function; the inactivation of key genes/pathways affects very diverse processes like aging, heart diseases (Post *et al.*, 1999), development disorders (Li, 2002) and also tumorigenesis (Jones & Laird, 1999; Jones & Baylin, 2002; Robertson, 2005).

SIMILARITIES AND DIFFERENCES AMONG MEMBERS OF THE METHYLTRANSFERASE FAMILIES

Eukaryotic methyltransferases (beside DNMT2) are built from two domains, the N-terminal and the C-terminal domain (Fig. 4, in the next chapter). The N-terminal domain, referred to as "regulatory", differs among DNMT sub-families and

contains various interaction regions, e.g. the DNA binding region (PBD) or nuclear localization signal region (NLS). The C-terminal domain comprises highly conserved motifs responsible for the enzyme's catalytic activity. All enzymes that methylate the C-5 position of the cytosine ring use the same mechanism of enzymatic reaction described in the previous chapter and they all have highly conserved residues that form the active site pocket. Cytosine methyltransferases have ten conserved, characteristic sequence motifs; six of them are present in nearly all cytosine methyltransferases from bacteria through fungi, plants to mammals (Santi *et al.*, 1983; Posfai *et al.*, 1989). The motifs are usually described regarding their function (Lauster *et al.*, 1989): motifs I to III create the cofactor binding pocket; motif IV provides the proline-cysteine dipeptide that forms the thiolate initiating the methyl group transfer reaction; motifs VI, VIII and X form the binding pocket for the substrate–cytosine; and finally motifs V and VII are responsible for fold preservation of the target recognition domain (Fig. 3).

The DNMT enzymes are distantly related to each other and probably diverged early in eukaryotic evolution. The genes are well conserved among eukaryotes whose genomes are methylated (i.e. mammals, *Arabidopsis thaliana*, *Xenopus laevis*). In mammals DNA methylation patterns are established and maintained by at least five methyltransferase enzymes: DNMT1, DNMT2, DNMT3a, DNMT3b, DNMT3L (Bestor, 2000; Li, 2002). They all regulate distinct processes but as more studies reveal there is a considerable level of cooperation and functional overlap among them. The idea that different methylation activities are performed by different methylation enzymes seems to be far too simplistic (Lyko *et al.*, 1999; Freitag & Selker, 2005).

DNMT3 FAMILY; *DE NOVO* METHYLATION AND MORE

The mammalian genome encodes two cytosine methyltransferases of the DNMT3 family, DNMT3a and DNMT3b (Fig. 4). Both enzymes are generally regarded as *de novo* DNA methyltransferases, though it has been recently proposed that they may also play a role in the maintenance of methylation (Liang *et al.*, 2002; Hsieh, 2005). It is during the early developmental stages that DNMT3a and DNMT3b are highly expressed and most of the *de novo* methylation occurs at that time (Okano *et al.*, 1998; 1999). Both enzymes are necessary for proper development of mammalian embryos by establishing new methylation patterns and are required, especially DNMT3b, for methylation of specific genomic regions such as pericentromeric repetitive sequences and CpG islands on the inactive X chromosome. Numerous developmental aberrations and diseases are associated with defective or absent DNMT3a or DNMT3b enzymes (Okano *et al.*, 1999; Robertson, 2005). Furthermore, DNMT3b appears to be specialized for methylation of particular parts of the genome – inactivation of DNMT3b in ICF syndrome causes demethylation only at specific CpG islands on the inactive X chromosome (Bestor, 2000).

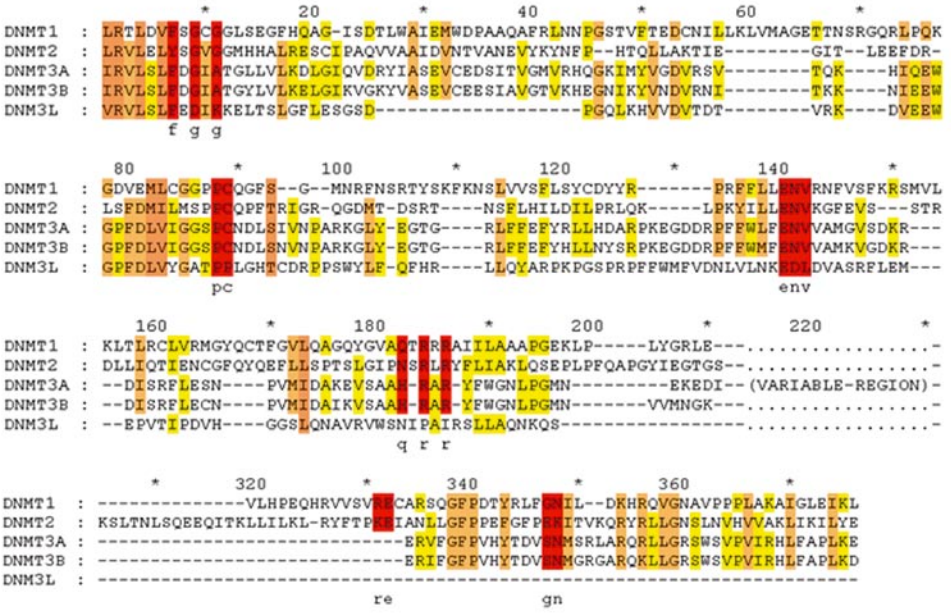

Figure 3. Multiple alignment of human methyltransferases.
The six highly conserved structural motifs are shown in red and their consensus is given in small letters below the alignment. Identical or similar residues are colored yellow to orange. The sequences aligned here correspond to the C-terminal parts of the proteins. There is also a "variable region" of about 50–80 residues not shown which is thought to be responsible for DNA recognition and is different in sequence and structure in all proteins.

Enzymes from the DNMT3 family show no preference for hemimethylated over fully unmethylated DNA substrates. Additionally, DNMT3a shows methylation of non-CpG sites both *in vitro* and *in vivo* (Okano *et al.*, 1998; Ramsahoye *et al.*, 2000). These biochemical features reflect the *de novo* methylation function of the DNMT3 family.

It has been proposed that *de novo* methyltransferases may also participate in the maintenance of methylation by restoring methylation at sites missed by DNMT1 during replication (Liang *et al.*, 2002; Hsieh, 2005). A possible cooperation between these enzymes may exists as a DNMT1-deficient cancer cell line showed little loss of DNA methylation compared to a more dramatic loss of DNA methylation observed when both DNMT1 and DNMT3b were knocked out from that cell line (Rhee *et al.*, 2000; 2002).

Recently it was suggested that the DNMT3 family contains one more member — a third homolog called DNMT3L (DNA methyltransferase 3-like) (Goll & Bestor, 2004). This protein is expressed specifically in germ cells (Aapola *et al.*, 2000). DNMT3L is related to DNMT3A and DNMT3B in both the N- and C-terminal domains, and retains the cysteine-rich domain but lacks the PWWP domain (suggested to be involved in protein–protein interactions) and the ATRX domain (involved in histone deacetylase interactions) (Slater *et al.*, 2003). The protein has not been shown to possess methyltransferase activity. However, DNMT3L is essential for establishment of a subset of methylation patterns in both male and female germ cells by functioning as a regulatory factor (Bourc'his *et al.*, 2001).

THE ENIGMATIC DNMT2 PROTEINS

The most strongly conserved of all known cytosine methyltransferases are enzymes belonging to the DNMT2 family. DNMT2 is also the most widely distributed one; its homologues are present even in species that are believed not to methylate DNA (i.e. *Schizosaccharomyces pombe, Caenorhabditis elegans*). In a number of species DNMT2 is even the only DNA methyltransferase homologue. These enzymes are also the most enigmatic ones, as their function or substrate are still under question (Hermann *et al.*, 2004).

Human DNMT2 is a relatively small protein of 391 amino acids which lacks the large N-terminal recognition domains present in the DNMT1 and DNMT3 families (Fig. 4). The lack of the N-terminal part makes DNMT2 quite a unique methyltransferase, which more closely resembles the bacterial ones.

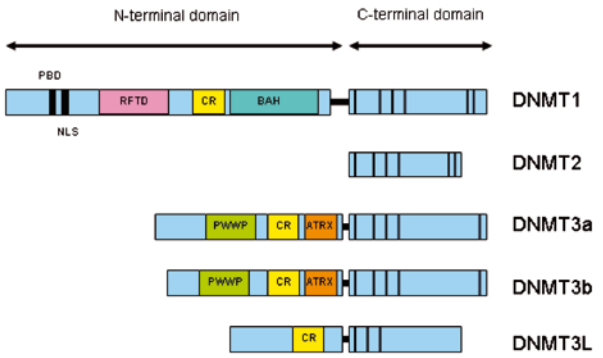

Figure 4. Domain arrangement of human methyltransferase family members.
In the N-terminal part the sub-domain organization is shown: PCNA binding site (PBD), nuclear localization signal region (NLS), replication foci targeting domain (RFTD), bromo-adjacent homology domains (BAH) and cysteine rich region (CR). Additionally PWWP domain (involved in protein–protein interactions) and the ATRX domain (involved in histone deacetylase interactions) is also shown. N- and C-terminal domains are linked with Gly-Lys dipeptides. In the C-terminal part the highly conserved motifs are shown as black thick lines.

The strong sequence conservation of all 10 catalytic motifs suggests that DNMT2 should be a DNA cytosine methyltransferase. So far it is uncertain whether this enzyme is an active cytosine methyltransferase. Some sensitive mechanism-based assays failed to detect any DNA methyltransferase activity of DNMT2 (Okano *et al.*, 1998; Yoder & Bestor, 1998; Dong *et al.*, 2001). On the other hand, two independent assay systems show that the purified Dnmt2 protein has weak DNA methyltransferase activity (Hermann *et al.*, 2003) and there are also results demonstrating that Dnmt2 is both necessary and sufficient for DNA methylation in *Drosophila* suggesting a CpT/A-specific DNA methyltransferase activity for Dnmt2 proteins (Kunert *et al.*, 2003).

The function of DNMT2 has also been addressed by genetic studies. DNMT2 is ubiquitously expressed in most human and mouse adult tissues (Yoder & Bestor, 1998; Goll & Bestor, 2004). Their expression patterns are very similar to those of DNMT1. In fact organisms that contain members of the DNMT1 and DNMT3 families always contain DNMT2. Nevertheless embryonic stem (ES) cells homozygous for disruption of DNMT2 do not show methylation abnormalities and are of normal phenotype (Okano *et al.*, 1998; Yoder & Bestor, 1998).

It has been suggested that DNMT2 homologues could play some role in centromere function. Centromere structure and function is conserved among species that have a DNMT2 homolog and is quite different from that in organisms which lack them (Bestor, 2000; Hermann *et al.*, 2004).

Recently Timothy Bestor's group has shown a different function of the DNMT2 enzyme (Goll *et al.*, 2006). They have found that purified DNMT2 enzyme from human and *Drosophila melanogaster* can methylate cytosine in $tRNA^{Asp}$ and not in DNA. These finding would rather place DNMT2 among RNA methyltransferases, despite the strict conservation of the DNMT catalytic motifs. Although the newly discovered function of DNMT2 may explain some of the questions asked it also raises new ones; namely what is the purpose of this modification and why it is so crucial that the enzyme is so strongly conserved among such divergent taxa as bacteria, plants and mammals? So far, despite numerous attempts, DNMT2 remains an enigmatic enzyme and continues to be a great challenge for the community.

DNMT1: (NOT ONLY) MAINTENANCE METHYLATION

The first eukaryotic DNA methyltransferase that was purified and cloned was named DNMT1. Initially its *de novo* methylation ability was identified (Bestor *et al.*, 1988), later it was discovered it has a 5–30 fold preference for hemimethylated DNA, depending on the nucleotide sequence (Yoder *et al.*, 1997). This preference caused the DNMT1 enzyme family to be recognized as the "maintenance methyltransferase".

The term "methylation maintenance" refers to a process that reproduces parental DNA methylation pattern into the daughter DNA strand. This process provides heritability to genomic methylation patterns which are copied during DNA replication. Indeed, DNMT1 is closely associated with the DNA replication machinery and has a preference for new CpG sequences whose complement has a methyl group attached.

Also, it is worth noting that the specific activity of DNMT1 on unmethylated DNA substrates is greater than that of DNMT3a and DNMT3b, which are recognized as *de novo* DNA methyltransferases (Okano *et al.*, 1998; Hsieh, 2005). Whether this activity is inhibited by some unknown cellular factors that enforce only or mostly maintenance methylation or whether DNMT1 also has *de novo* methylation abilities *in vivo* remains a mystery.

Eukaryotic (cytosine-5) DNA methyltransferase 1 (DNMT1) is a protein about three times larger than its prokaryotic counterpart. It was proposed to result from a fusion between three genes, one of them being an ancestral prokaryotic methyltransferase (Margot *et al.*, 2000). The enzyme is built of two domains; the N-terminal region functioning as the DNA-binding and regulatory domain, and the C-terminal part recognized as the catalytic domain (Fig. 4). Both domains are linked by a short stretch of repeated Gly-Lys dipeptides. This sequence has some similarity to the N-terminal tail of histone H4 and may be a site of posttranslational modifications.

One of the main functions of the N-terminal domain is to facilitate interactions with numerous proteins and to recognize specific DNA sequences. The DNA recognizing and binding region called target recognition domain (TRD) is located at the beginning of the domain (in human DNMT1 residues about 122–417) (Araujo *et al.*, 2001). It is also in proximity to various protein binding sites, especially close to the PCNA binding site (PBD). The N-terminal domain can interact with numerous proteins like PCNA (proliferating cell nuclear antigen, an auxiliary factor for DNA replication and repair), RB (retinoblastoma tumour suppressor gene product), HDAC1 and 2 (histone deacetylases), and DMAP1 (DNA methyltransferase associated protein-1) (Chuang *et al.*, 1997; Robertson *et al.*, 2000; Rountree *et al.*, 2000; Goll & Bestor, 2004). These interactions can establish a link between DNA methylation, histone deacetylation and sequence-specific DNA binding activity, as well as a growth–regulatory pathway that is disrupted in nearly all cancer cells.

In the N-terminal domain a replication foci targeting domain (RFTD) is also present. This region contains a sequence required for import of DNMT1 into nuclei, as well as a sequence required for association with the replication foci (structures in which DNA synthesis occurs within mammalian nuclei) (Liu *et al.*, 1998; Goll & Bestor, 2004). Near the center of the N-terminal domain there is a cysteine-rich region with several zinc binding sites. The function of this cysteine-rich region is currently investigated; it is present in all mammalian cytosine methyltransferases, methyl-binding proteins, CpG binding proteins and various proteins affecting cytosine methylation (Chuang *et al.*, 1996; Goll & Bestor, 2004).

At the end of the N-terminal domain of mammalian DNMT1 two bromo-adjacent homology (BAH) domains are present. These regions are found in the origin recognition complex proteins and in other proteins involved in chromatin regulation (Callebaut *et al.*, 1999). It has been proposed that the BAH domains could function as protein–protein interaction sites.

The C-terminal part of DNMT1 functions as the catalytic domain. If isolated, the C-terminal part is catalytically inactive despite the presence of all the highly conserved sequence motifs typical for active methyltransferases (Zimmermann *et al.*, 1997; Fatemi *et al.*, 2001). It is only active when at least a large part of the N-terminal domain is also present. This might implicate that during evolution the catalytic activity of DNMT1 has become dependent upon the regulatory domain.

The role of DNMT1 in mammalian cells has been thoroughly investigated with most of the experiments done in mice or other murine embryonic stem cells. The loss of function alleles of *DNMT1* produce several major changes in the cell. The most visible one is severe demethylation of the genome (both in mice and ES cells) although methylation is still present at a 5% level compared to the wild type (Lei *et al.*, 1996). Among other changes one can see a modest (about 10-fold) increase in mutation rates in ES cells (Chen *et al.*, 1998) and a small defect in mismatch repair system (Guo *et al.*, 2004). Another important finding is that ES cells that lack DNMT1 grow normally in the undifferentiated state but when induced to differentiate they die because of apoptosis (Li *et al.*, 1992; Robertson, 2005). Apoptosis is also the cause of death of embryos lacking *DNMT1*.

Other changes include inactivation of all X chromosomes in mutant embryos due to the demethylation and activation of *Xist* (X-inactive specific transcript) (Panning & Jaenisch, 1996), and biallelic expression of most imprinted genes in homozygous embryos. Some imprinted genes are expressed from both alleles, whereas others are expressed from neither (Li *et al.*, 1993; Goll & Bestor, 2004). Finally, loss of *DNMT1* causes the demethylation and very high levels of expression of transposons – *Dnmt1* it is the only gene known to be required for the repression of transposons in mammalian somatic cells.

On the other hand, overexpression of DNMT1 in transgenic animals causes *de novo* methylation of normally unmethylated genes, especially at imprinted loci. This *de novo* methylation was lethal when the level of expression was more than a few-fold increased (Biniszkiewicz *et al.*, 2002). These experiments suggest *de novo* activity, implicating that DNMT1 does not function exclusively as a maintenance cytosine methyltransferase.

DISEASES ASSOCIATED WITH DEFECTIVE METHYLATION

Alterations in DNA methylation patterns can cause changes in gene transcription patterns and can also promote mutational events (Jones & Baylin, 2002; Robertson, 2005). Aberrant changes in the expression pattern of genes caused by cytosine methylation are called epigenetic mutations, or epimutations. Epimutations are usually associated with promoter hypermethylation — extensive methylation of CpG dinucleotides in the gene promoter region. It has been shown that promoter hypermethylation causes gene inactivation and is associated with many diseases including cancers and neurodevelopmental disorders like ICF (immunodeficiency, centromeric instability and facial anomalies), Rett and FraX (fagile X) syndromes, and genomic imprinting deficiencies (Jones & Baylin, 2002).

Aberrant DNA methylation patterns also play an essential role in the development of cancer. Cancer cells show an overall decrease in the level of genomic cytosine methylation, mainly in centromeric satellite and other repeated sequences (Ehrlich, 2002). This genome hypomethylation is believed to be linked to genome instability resulting in a variety of chromosomal effects and tumorigenesis (Eden *et al.*, 2003). In addition to losing methylation at satellite sequences, cancer cells acquire methylation in normally unmethylated promoter regions.

In many cancer types one can see tumour suppressor genes epigenetically silenced by hypermethylation (Baylin & Bestor, 2002; Robertson, 2005). In this respect, epimutations can be functionally equivalent to genetic mutations because they result in the loss of gene functions and, due to the maintenance methyltransferase activity, can be inherited between cell generations. However there is also a distinct difference between classic mutations and epimutations (Fig. 1). Gene silencing by promoter hypermethylation is usually a gradual process that causes, unlike mutations, gradual decrease in the level of gene transcription. The process involves spreading of methylation from heavily methylated regions into the flanking CpG sites (Baylin & Herman, 2000; Freitag & Selker, 2005). Hypermethylation is therefore a progressive process that can finally cause complete gene silencing.

In human cancers promoter hypermethylation is at least as common as mutations causing disruption of tumour suppressor genes (Ushijima, 2005). There is also a growing list of genes important for tumorigenesis that are frequently epigenetically silenced but very seldom mutated in certain cancers. Among them are *MGMT* — encoding a DNA repair protein, *MTS1* (*p16*) and *MTS2* (*p15*) — encoding cell cycle regulators, *RASSF1A* — encoding a protein that binds to the *RAS* oncogene, and *MLH1* — encoding a mismatch repair protein (Herman *et al.*, 1997; Dammann *et al.*, 2000; Esteller *et al.*, 2000; Jones & Baylin, 2002). Promoter hypermethylation is usually the only mechanism responsible for the loss of function of these genes.

There are also genes that may be both mutated and epigenetically silenced. Among them are such important genes as *TP53 (p53)* and *BRCA1*. In the case of the latter the gene was thought to be important only for familial type of breast cancer. However, methylation studies showed that 10–15% of all non-familial breast cancers have a hypermethylated *BRCA1* promoter. Furthermore expression profiles of breast cancers with hypermethylated *BRCA1* are identical to those in familial breast cancers and dis-

tinct from other breast cancer types (van't Veer *et al.*, 2002).

There are various examples of tumours that have one mutated allele and the other one hypermethylated (Herman *et al.*, 1998; Myohanen *et al.*, 1998; Grady *et al.*, 2000). Interestingly, hypermethylation seems to be present only on the wild type allele, not the mutated one (Esteller *et al.*, 2001). This is especially visible in the familial type of cancers when the mutated gene is inherited and hypermethylation is recognized as the second inactivating change leading to tumorigenesis.

In many genes promoter hypermethylation seems to precede genetic changes by occurring in pre-malignant types of neoplasia that do not yet have gene mutations (Esteller *et al.*, 2001; Kim *et al.*, 2005). It is also clear that epimutations can affect mutational rates. 5-Methylcytosine is itself mutagenic; it can undergo spontaneous hydrolytic deamination to cause a C→T transition mutation. Methylation that occurs in the transcribed region of a gene increases susceptibility to spontaneous mutation. The mutation rate caused by deamination is further increased by environmental factors like UV exposure (skin cancers) and the tobacco carcinogen benzo(*a*)pyrene diol epoxide (Rideout *et al.*, 1990; Yoon *et al.*, 2001).

DNMT1, *DNMT3A* and *DNMT3B* are all slightly overexpressed in many types of tumour cells (Robertson & Wolffe, 2000; Robertson, 2005). It has been shown that modest overexpression of *Dnmt1* in mice can promote cellular transformation (Wu *et al.*, 1993; Bakin & Curran, 1999). On the other hand, inactivation of *Dnmt1* in a mouse model of gastrointestinal cancer decreases the development of gastrointestinal tumours. Also a mice model (predisposed to colon neoplasia) with mutated *Dnmt1*, or treated with the demethylating drug 5-aza-2′-deoxycytidine, showed reduced number of colonic polyps (Laird *et al.*, 1995; Hermann *et al.*, 2004).

REVERSIBILITY AS A TREATMENT OPPORTUNITY

Unlike mutagenic events, epigenetic mutations can be reverted. Demethylation of aberrantly silenced genes can restore gene expression and function. This effect has been observed both by demethylation of specific genes (Herman *et al.*, 1998; Brueckner *et al.*, 2005) and by global genomic demethylation (Soengas *et al.*, 2001). Reduced activity of human DNMT1 has been shown to cause a significant reduction in genomic DNA methylation levels (Robert *et al.*, 2003). It has been shown that the reduction of DNMT1 activity causes a significant decrease in the global DNA methylation level in mice (Li *et al.*, 1992) and that DNMT1 is necessary to maintain aberrant CpG islands methylation in human cancer cells. Because DNMT1 is also essential for the maintenance of epimutations (Robert *et al.*, 2003), the enzyme has become a primary target for drug development and experimental cancer therapy.

A specific inhibitor of DNA cytosine C5-methyltransferases would be useful for studying genomic imprinting, X-chromosome inactivation, carcinogenesis, and regulation of tissue-specific gene expression — these physiological phenomena appear to be regulated through DNA methylation in promoter sequences.

So far, the most widely known methylation inhibitors are cytosine nucleoside analogs: 5-azacytidine and its derivative 5-aza-2′-deoxycytidine (Fig. 5). Both compounds have been used in the majority of methylation inhibition experiments and in a large number of clinical trials, resulting in recent approval for 5-azacytidine (Vidaza) in the treatment of myelodysplastic syndrome.

Unfortunately the use of these compounds faces several problems, namely their low stability in aqueous solutions and high toxicity (Lyko & Brown,

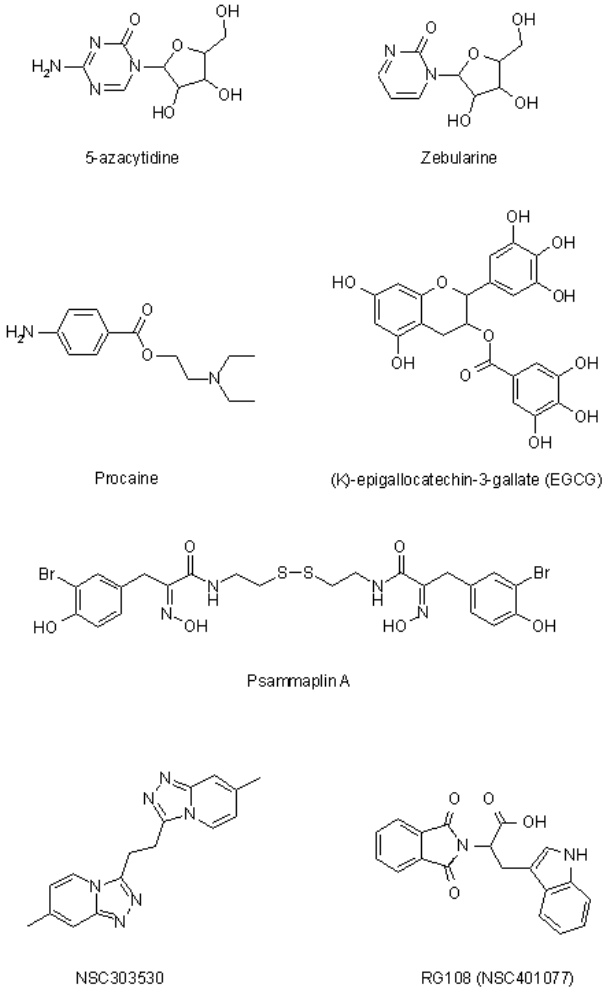

Figure 5. Known inhibitors of DNA methylation.
The first two compounds (5-azacytidine and zebularine) are mechanism-dependent inhibitors of DNMT enzymes.

2005). Cytosine derivatives become incorporated into nucleic acids and act as suicidal substrates. The enzyme initiates catalysis and a covalently bound protein-DNA intermediate is formed. However, the modified pyrimidine ring of the compounds blocks the completion of the methyl-group transfer reaction, and the enzyme thus remains permanently bound to DNA (Juttermann *et al.*, 1994). Bound methyltransferases become depleted from the nucleus and subsequent DNA replication over several cell generations results in a passive demethylation of the genome. However, the DNA-methyltransferase adducts are believed to be responsible for the highly toxic effect these cytidine derivatives have on mammalian cells (Jackson-Grusby *et al.*, 1997). This feature is the main drawback for the use of cytidine derivatives and greatly limits their potential as chemotherapeutic agents.

In the recent years there has been a race to find novel small-molecule compounds that could inhibit DNMTs in cells (Szyf, 2005). Some of the more promising compounds are listed in Fig. 5. Among the compounds tested are procaine and its derivative procainamide (Fig. 5), an approved antiarrhythmic drug and a local anesthetic, having a demethylating activity leading to reexpression of silenced tumour suppressor genes in cancer cells (Villar-Garea *et al.*, 2003). Both compounds bind to CpG-rich sequences but do not become incorporated into DNA. This might disturb the interactions between DNMTs and their target sequences, but direct methyltransferase inhibition was not shown.

Another molecule with demethylating activity is (K)-epigallocatechin-3-gallate (EGCG) (Fig. 5), the main poly-phenol compound in green tea. Cancer cells treated with EGCG showed reduced DNA methylation and increased transcription of tumuor suppressor genes (Fang *et al.*, 2003). Unfortunately, EGCG influences a wide variety of cellular processes which downgrades its specificity. Again, no direct methyltransferase inhibition has been demonstrated.

Inhibition of methyltransferase and histone deacetylase activity has been shown with psammaplins (Fig. 5), compounds derived from the marine sponge *Pseudoceratina purpurea* (Pina *et al.*, 2003). These leads could be promising as they might enable a combinatorial inhibition of two epigenetic pathways. But psammaplins also inhibit other enzymes involved in DNA metabolism like topoisomerase II (Kim *et al.*, 1999) and are therefore not specific for epigenetic modifier proteins.

Recently, two new small-molecule compounds, NSC401077 (RG108) and NSC303530 (Fig. 5), were shown to inhibit DNA methyltransferase activity both *in vitro* and *in vivo* (Brueckner *et al.*, 2005; Siedlecki *et al.*, 2006). The chemical structures of these compounds are similar to each other. Both compounds contain two indene-based heterocycles linked by a two-carbon chain and show high similarities in the arrangement of hydrogen bond donors, acceptors and hydrophobic features. A considerable specificity in the interaction between RG108 and a DNA methyltransferase has been suggested by *in silico* SAR (structure activity relationship) experiments. They revealed a critical role of the carboxyl-group of RG108 in its interaction with the active site. A synthesized derivative that lacks the central carboxyl-group has been tested in an *in vitro* assay and failed, as expected, to inhibit DNA methylation and demethylate genomic DNA of HCT116 cells (Brueckner *et al.*, 2005). Cell line experiments have also shown that the new compounds do not integrate with DNA and strongly suggesting a mechanism-independent enzyme inhibition.

A strong demethylation effect, structural specificity and direct methyltransferase inhibition without formation of covalent adducts renders these two compounds as potent methylation inhibitors, without the drawbacks of the two widely used cytosine derivatives. They provide a new insight into the design of methylation inhibitors and may help in development of new epigenetic treatments.

THREE DIMENSIONAL STRUCTURES

The search for novel DNA methyltransferase inhibitors has been held back by the fact that the three-dimensional structures of the most relevant DNA methyltransferases have not been resolved yet. Currently, X-ray diffraction data are available only for two bacterial methyltransferases, M.*Hae*III (Reinisch *et al.*, 1995) and M.*Hha*I (Klimasauskas *et al.*, 1994), as well as for the human DNA methyltransferase DNMT2 (Dong *et al.*, 2001).

The methyltransferase structures show structural conservation of the catalytic domain (Dong *et al.*, 2001). The crystal structures are essentially superimposable over the C-terminal domain, showing all the conserved motifs in a correct orientation in the active sites (Fig. 6). The structure of the M.*Hha*I enzyme crystallized with DNA and the *S*-adenosyl L-homocysteine (SAH) cofactor allowed elucidation of the catalytic mechanism of cytosine methylation. M.*Hae*III is crystallized only with DNA and the DNMT2 protein only with the SAH cofactor.

The available structural data for M.*Hha*I, M.*Hae*III and DNMT2 permits a homology modelling approach for structural analyses of the catalytic domain of human DNMT1 enzyme (Siedlecki *et al.*, 2003). The overall sequence homology to both prokaryotic and eukaryotic proteins is low, close to 20%. Nevertheless, based on a set of highly con-

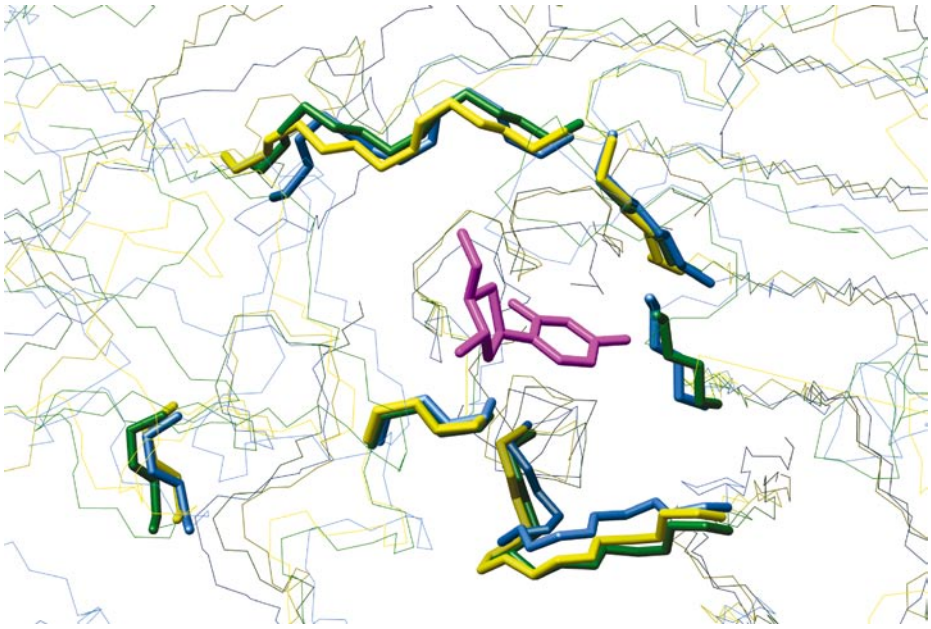

Figure 6. Structural conservation of the active sites of human and bacterial methyltransferases.
Superimposed crystal structures of M.*Hha*I (green), human DNMT2 (yellow) and human DNMT1 model (blue) with deoxycytidine (magenta). The enzymes are shown in backbone representation, bold parts highlight conserved motifs: from top to bottom QxRxR, ENV, PC, GN, RE and FxGxG. The binding pockets show high structural conservation of residues interacting with deoxycytidine and cofactor (not shown).

served blocks of amino acids in the catalytic region (shared by most known (cytosine-5) DNA methyltransferases) an informative homology model of the DNMT1 C-teminal domain can be built.

Initial position of the methyl-donor *S*-adenosylmethionine (AdoMet) in the model's active site was based on the M.*Hha*I (*MHT) and M.*Hae*III (1DCT) structures. The model-cofactor was subjected to a multi-step minimization procedure which allowed relaxation of various improper restrains but prevented major changes to the backbone conformation. The cytosine nucleoside was inserted into the binding pocket using simulated annealing which permited both the ligand and the enzyme to adjust to each other during binding. Flexibility of the active site is crucial for a homology model which not necessarily represents active/open conformation.

This model demonstrated a significant conformational preservation of the catalytic site also revealing a number of unique structural features (Siedlecki *et al.*, 2003). These are: (1) changes to the local environment by substitutions e.g. His1459 of DNMT1 is substituted with alanine at the corresponding positions in both M.*Hha*I and M.*Hae*III (2) changes to side chains conformations e.g. Arg1310 and Arg1312 have different conformations compared to corresponding arginine side chains in the two bacterial methylases (these differences influence the binding of the deoxyribose ring of the cytosine ligand and by creating a "tighter" cavity); (3) differences in the model were also observed for the charge of the active site defined as a 6 Å sphere around cytosine nucleoside (the charge of this space was three times higher in the DNMT1 catalytic site than in the bacterial enzymes and in human DNMT2).

CONCLUSIONS

Knowledge of the DNA methylation patterns has now become an increasingly important medicinal tool, used for early diagnosis and identification of cancer patients for specific treatment (Villar-Garea *et al.*, 2003; Robertson, 2005). DNA methylation has also become an important target for development of new therapeutics (Lyko & Brown, 2005). The opportunity lies in the natural reversibility of DNA methylation. Methylation patterns and thus epimutations must be actively maintained by methyltransferases in order to be sustained and inherited between cell generations. Targeting this enzymatic machinery by pharmacological inhibition could correct expression of epigenetically silenced tumour suppressor genes. Following of this concept may provide insight into the regulative role of methylation in gene expression, and provide new opportunities for epigenetic therapy and cancer treatment.

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