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REVIEW

DNA methylation in cancer development, diagnosis and therapy—multiple opportunities for genotoxic agents to act as methylome disruptors or remediators

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The role of DNA methylation and recently discovered hydroxymethylation in the function of the human epigenome is currently one of the hottest topics in the life sciences. Progress in this field of research has been further accelerated by the discovery that alterations in the methylome are not only associated with key functions of cells and organisms, such as development, differentiation and gene expression, but may underlie a number of human diseases, including cancer. This review describes both well established and more recent observations concerning alterations in the methylome, i.e. the global and local distribution of 5-methylcytosines, involved in its normal functions. Then, the changes in DNA methylation pattern seen in cancer cells are discussed in the context of their utilisation in cancer diagnostics and treatment. On this basis, comparisons are made between natural covalent DNA modification and that induced by genotoxic agents, chemical carcinogens and antitumour drugs as regards their impact on epigenetic mechanisms. The available data suggest that DNA damage by genotoxins can mimic epigenetic markers and in consequence disrupt the proper function of the epigenome. On the other hand, the same processes in cancer cells, e.g. DNA demethylation as a result of DNA methyltransferase blocking or the induction of DNA repair by DNA adducts, may restore the activity of hypermethylated anticancer genes. The observed multiple mechanisms by which genotoxic agents directly affect methylome function suggest that chemical carcinogens act primarily as epigenome disruptors, whereas mutations are secondary events that occur at later stages of cancer development when genome-protecting mechanisms have already been deregulated.

Introduction

Chromatin is the term used to describe DNA complexed with proteins (mainly histones) that is found in eukaryotic chromosomes. Two forms of chromatin can be distinguished: a less condensed form, known as euchromatin, and a form tightly compacted throughout the cell cycle—heterochromatin. Euchromatin contains transcriptionally active DNA, rich in genes, relatively weakly associated with histones, which undergoes replication during the early S phase. Genes located in euchromatin may or may not be expressed depending on the cell type and functional needs of the cell. This euchromatin state is controlled by reversible covalent modification of DNA (DNA methylation) and histones (acetylation, methylation, phosphorylation, ubiquitination, etc.) catalysed by enzymes such as DNA methyltransferases (DNMTs), histone acetyltransferases, histone deacetylases (1) and, as recent findings emphasise, by non-coding RNAs and RNA-associated silencing (2,3).

Until very recently, heterochromatin was regarded as transcriptionally inactive DNA, tightly bound to histone proteins. It is located in regions of the genome that undergo replication during the late S phase of the cell cycle and contains relatively few genes, which very rarely undergo expression. Chromatin in the form of heterochromatin is characterised by a low acetylation level of histone tails and a high DNA methylation level. This does not mean, however, that heterochromatin is useless ballast with no function; it has been long recognised as a structure that protects the ends of chromosomes (telomeric regions) and determines the separation of chromosomes to daughter cells during mitosis (centromeric regions) (1). Current investigations carried out in various systems have highlighted the dynamic conversion between different states of chromatin-influencing gene expression. Studies done in mammalian cells for tumour suppressor gene *p15* have revealed that natural antisense RNA may be a trigger for heterochromatin formation and DNA methylation in epigenetic silencing of the sense gene (4). A new role for heterochromatin also emerges following the observation of the transcription of non-coding RNAs from heterochromatic DNA repeats, e.g. centromeric regions. In fission yeast, these RNAs are substrates for the RNA interference pathway and seem to be an important element of gene silencing within the domains coding them (5). The fact that up to 70% of transcripts have antisense partners (6), as well as the broad conservations of RNAi and RNA processing pathways, suggest the widespread occurrence of such regulatory mechanisms.

There is a close connection between DNA methylation, modifications of histone proteins and chromatin structure. It has long been observed that inactivation of chromatin, and therefore the conversion of euchromatin to heterochromatin, is usually preceded by DNA methylation in the promoter regions of some genes, whereas acetylation of histones activates chromatin and DNA demethylation, hence the removal of the factor responsible for the silencing of gene expression (7,8). However, the collection of chemical marks in chromatin is much larger: >100 different post-translational histone modifications have been found, whereas DNA methylation has lost its position of being the sole DNA alteration following the identification of hydroxymethylated cytosines. Also, the number of processes recognised as influencing chromatin

remodelling and function is growing rapidly and, as stated in one review, ' ... researchers are racing to find the biological meaning of these marks ... ' (9).

The sum of natural covalent modifications of chromatin, i.e. DNA and histones, is defined as an epigenome (8). This definition implies that complex organisms have multiple epigenomes as chromatin modifications vary not only between tissues but also change during development and in response to the environment. With its multifactorial impact on chromatin structure and function, the epigenome is thought to be the carrier of an even greater amount of information than the genetic code. In contrast to the genome, it is a dynamic structure subjected to reversible chemical modifications. Gene regulation controlled by epigenetic modifications can lead to either activation or suppression of the genes. A broad look at the processes involved in gene expression has resulted in the development of a new field of research called epigenetics, which includes studies of the chemical alterations of chromatin that influence gene expression without changes in the sequence of DNA nucleotides.

In this review, based on available experimental evidence, we argue for the inclusion, at least in the cancer context, of yet another set of factors that may mimic epigenetic marks—DNA modifications induced by genotoxic agents.

DNA methylation: occurrence and enzymology

DNA methylation is a natural covalent DNA modification that is common in some eukaryotic species (vertebrates, plants) but not all (yeast and *Drosophila* have little methylation of their DNA). It involves the incorporation of a methyl group in the C-5 position of cytosine mainly in CpG dinucleotides, which leads to the formation of 5-methylcytosine (5-mC). In a normal cell, 5-mC constitutes approximately 0.75–1% of all nucleotides in DNA and affects 70–80% of all CpG dinucleotides in the genome (10,11). Cytosine methylation is also observed outside of CpG sequences (e.g. CpA or CpT); however, non-CpG methylation occurs relatively rarely in differentiated cells, appearing to represent a feature of stem cells (12,13). The latter conclusion is based on the first complete DNA methylation maps of the human genome at single-base pair resolution provided by Lister *et al.* in 2009, which compare patterns of this epigenetic modification between human embryonic stem cells, foetal fibroblasts and the latter reprogrammed towards a pluripotent state. In fibroblasts, 99.98% of all methylation occurred at CpG dimers, while in both embryonic and induced stem cells, as much as 25% of 5-mCs was found next to other nucleobases, adenosine in particular (13). Kriaucionis and Heinth (14) have changed the landscape of natural DNA modifications by the discovery of a new chemical mark in the mammalian genome—5-hydroxymethylcytosine (5-hmC). Previously considered to be a result of oxidative insult or rare modification found only in T-even bacteriophages, 5-hmC turns out to be relatively abundant in some (euchromatic) but not other (heterochromatic) nuclei of mammalian brain cells: 0.2 and 0.6% of the total number of nucleotides, respectively (14). Initially detected in brain cells, 5-hmC has been found in human cell lines beyond the ones previously recognised. The importance of these findings is further strengthened by the discovery of enzymatic activity responsible for converting 5-mC to 5-hmC in mammalian cells (15). The complete DNA methylation set, which currently must be understood as the pattern of 5-mC and 5-hmC distributions and their abundance in the genome, constitutes the methylome.

In eukaryotic cells, DNA methylation takes place most efficiently after DNA replication. It represents a heritable modification, which means that its pattern is preserved during cell divisions. The molecular model explaining how methylation can be passed from parent to daughter cells is depicted in Figure 1 and was originally put forward independently by Riggs (16) and Holliday and Pugh (17).

DNA methylation is catalysed by DNMTs, which recognise palindrome dinucleotides CpG (18). These enzymes catalyse the transfer of a methyl group from the donor *S*-adenosyl-L-methionine (SAM) to the C5 cytosine carbon atom (Figure 2). Four DNA cytosine-5-methyltransferase isoenzymes have been described to date: DNMT1, DNMT2, DNMT3A and DNMT3B (18). The role of DNMT1 is to maintain the original pattern of DNA methylation in hemimethylated DNA, in which after replication, 5-mC is present on only one parental DNA strand. This enzyme catalyses cytosine methylation in the newly synthesised strand that lies diagonally opposite 5-mC in the parent DNA strand. DNMT1 is a component of the multiprotein DNA replication complex that supports semi-conservative DNA replication, ensuring accurate propagation of DNA methylation with cell division (19). Hitherto, unknown mechanisms may be inducing subsequent oxidation of 5-mC to 5-hmC (Figure 2). The only mammalian enzymatic activity identified that is capable of catalysing this reaction involves TET1 protein, a 2-oxoglutarate- and Fe(II)-dependent oxygenase (15).

DNMT3A and 3B are responsible for *de novo* DNA methylation (18) and, therefore, for altering methylation pattern of the genome (Figure 1). New findings that shed some light on how histone and DNA methylation interact in gene silencing have demonstrated that symmetric methylation of histone H4 arginine 3 is required for subsequent DNA methylation. It serves as a direct binding site for DNMT3A (20). Furthermore, this isoenzyme has also been suggested to be responsible for cytosine methylation in non-CpG sequences (CpA, CpT) (12). *De novo* DNA methylation takes place mainly during embryonic development in mammals. It also plays an important role in parental genomic imprinting, i.e. in the epigenetic modification of one of the parental chromosomes in a gamete or zygote. This leads to a differentiated gene expression of two alleles of a gene in somatic cells (21), inactivation of the X chromosome (16,22), as well as carcinogenesis (22,23).

The function of DNMT2 is not clear, but it has been indicated that although this enzyme is characterised by a weak *in vitro* DNMT activity, it effectively catalyses tRNA methylation (24). It has been also suggested that methyltransferases can compensate each other's functions, e.g. DNMT3a is involved not only in *de novo* methylation but also in maintenance of DNA methylation.

In contrast to DNMTs, which have been fairly well characterised, the enzymes responsible for active DNA demethylation remain elusive. When methylation of newly synthesised DNA is inhibited during replication, it can proceed passively; however, the active demethylation reshaping the methylome still lacks a confirmed mechanism in mammals—this is the conclusion reached by Ooi and Bestor (25). The removal of 5-mC seems less controversial in plants, where a base excision repair mechanism (leaving behind an abasic

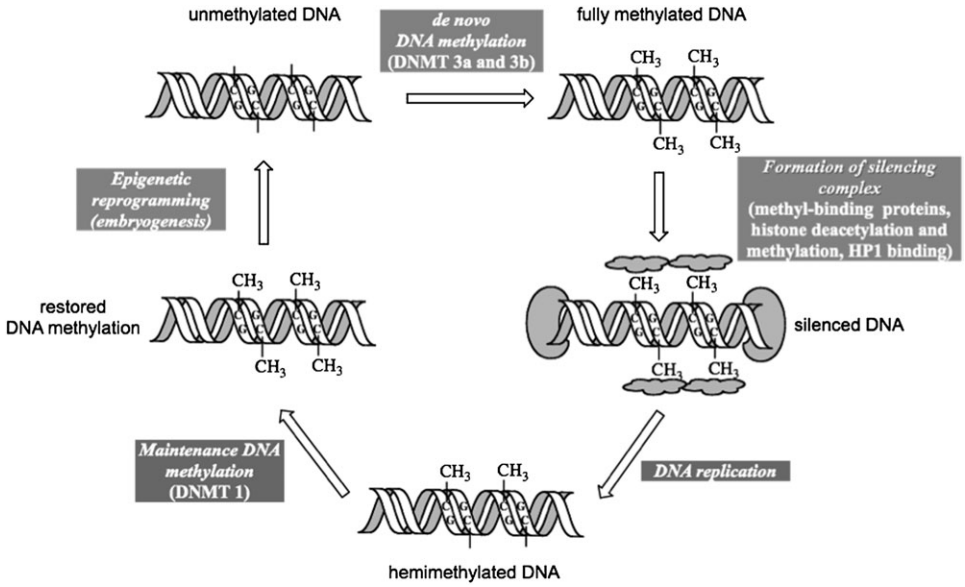

Fig. 1. A molecular model for the perpetuation of DNA methylation in eukaryotic cells (based on ref. 15). The DNA initially becomes methylated in both strands by *de novo* methylation. The methylation in a CpG island in the promoter region may be recognised by proteins capable of binding specifically to methylated DNA that provoke the recruitment of chromatin remodelling factors via a family of methyl-CpG-binding proteins. Conversion to the heterochromatin form inhibits gene expression. After replication, hemimethylated DNA is produced, which then becomes fully methylated by the action of DNMT 1 preferentially acting on hemimethylated CpG substrates. This process, called maintenance methylation, preserves the methylated condition in daughter cells. The reprogramming occurs only in a specific cell type or stage of development and is strictly regulated.

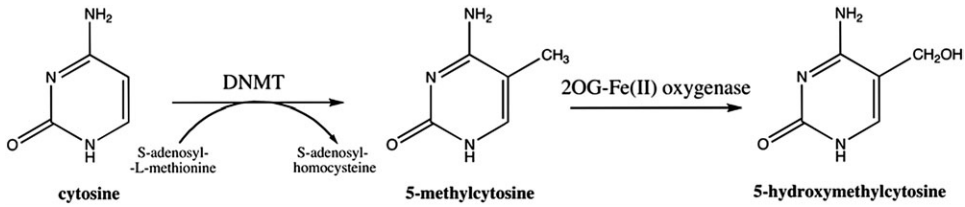

Fig. 2. The reactions of natural modifications of cytosine in the eukaryotic genome.

site) for active DNA demethylation has gained genetic support (26). The latest proposed mammalian mechanisms emphasise the role of cytosine deamination in active demethylation. Some reports suggest that demethylation in mammals is initiated by the same enzymes that are responsible for *de novo* methylation: DNMT3A and DNMT3B (27,28). According to Métivier *et al.* (28), the first step in the proposed mechanism involves oxidative deamination of 5-mC to thymine mediated in the absence of SAM by these two DNMTs. The T:G mismatch thus created is then converted by base excision repair to an unmethylated G:C pair. DNA deamination by activation-induced cytidine deaminases (AID) has been shown to be a key step in genome-wide erasure of DNA methylation in mouse primordial germ cells (29) as well as in reprogramming somatic cells towards pluripotency, whose initiation also requires active demethylation (30).

Apart from the enzymes mentioned above, turnover of DNA methylation involves a number of other proteins; those with established roles have been reviewed by Law and Jacobsen (3).

On the other hand, it may be worth noting that the methylome can be influenced by unspecific chemical processes such as spontaneous hydrolytic deamination of 5-mC to thymine. The latter remains incorporated and is duplicated during DNA replication (31), which results in DNA point mutation (31,32). This phenomenon explains the higher frequency of AT than GC pairs in the genomes of higher organisms. Precisely, the opposite effect, i.e. an elevated level of 5-mC, can be brought about by methyl radicals since the C-5 position of cytosine is an active site for free radical reactions. Methyl radicals are generated during the biotransformation of various endogenous and exogenous compounds, including some known environmental tumour promoters like cumene and *t*-butyl hydroperoxide (33).

The term 'DNA methylation' is sometimes used to describe the covalent modification of nucleic acids induced by certain alkylating agents like dietary *N*-nitrosamines, which react with nucleobases to form methylated derivatives, e.g. O^6-methylguanine. In this case, the reaction is non-enzymatic and represents DNA damage.

DNA methylation in gene silencing

It has been calculated that the human genome contains ~29 000 CpG-rich regions. CpG sequences in the genome are frequently grouped in clusters, so-called CpG islands. These islands are defined as regions rich in CpG dinucleotides of >500 base pairs, with a GC content >55%, located within the 5′-ends of promoter regions as well as exon 1 (11).

Roughly, 60% of human genes are associated with CpG islands, including those whose expression pattern is tissue-restricted. CpG islands in active genes are usually free from methylation and are the targets for proteins that initiate gene transcription. Cytosine methylation inhibits the transcription of eukaryotic genes and, being a very stable chemical modification, represents one of the main epigenetic mechanisms responsible for silencing gene expression. Two mechanisms are known for silencing gene expression as a result of CpG island methylation. The first takes place when the binding of transcriptional factors to methylated DNA is inhibited, as a consequence of which the level of CpG island methylation in promoter regions and regulatory genes may directly influence the efficacy of gene expression. The second mechanism involves the recognition of methylated DNA by methylated DNA-binding proteins (MBPs) and recruitment of co-repressors. The resultant protein complex causes gene silencing by inducing closed chromatin conformation within the area of the association of MBPs (34,35).

As mentioned earlier, cytosine methylation in DNA represents a modification of chromatin, which is reproduced during cell division with high fidelity (Figure 1). Since methylated CpG-rich sequences are distributed in a specific cell-type manner, the created patterns of methylation, the so-called differentially methylated regions (DMRs), are characteristic in regard to genes and tissues (11). However, DMRs are not unchangeable. Doi *et al*. (36) provided evidence for fluctuations in DMRs during differentiation, epigenetic reprogramming and cancer. The genome-wide analysis of nine human induced pluripotent stem cell lines revealed that their methylomes were significantly enriched in tissue- and cancer-specific DMRs compared to the fibroblasts these cell lines were derived from.

The dynamic changes of the methylome were also demonstrated by other researchers, who suggested that the methylation and demethylation of CpG sequences could be inducible transient cyclic phenomena (27,28) and that some genes were even de-repressed by DNA methylation, e.g. (37). With a periodicity of ~100 min, the methylation/demethylation cycle was observed in the case of sequences of promoters of five genes: *ER-α*, *TFF3*, *glutamate receptor*, *GRM4*, *J8-KCNJ8* in human breast cancer cells exposed to doxorubicin (27). The *survivin* gene, a member of the inhibitor of apoptosis gene family, whose expression is repressed by p53, may serve as an example of de-repression by DNA methylation. Methylation-specific polymerase chain reaction and pyrosequencing showed that the level of survivin in endometrial samples was correlated with the level of hypermethylation and progression from low- to high-grade tumours. This *survivin* overexpression in tumour samples probably resulted from the inhibition of p53 binding to methylated sequences and suggests that de-repression by methylation may be a general mechanism of p53 regulation (37).

DNA methylation in cancer-related diseases

Disturbances in the methylome were first proposed to underlie the aetiology of some complex congenital syndromes (22,38,39) and mental diseases (38). Subsequent research indicates that changed patterns of DNA methylation are related to such vital processes as aging and chronic inflammation and are observed following viral infections and cancer development. Aberrations from the DNA methylation pattern, leading to local hypermethylation in promoter regions of certain genes against a background of global hypomethylation, appear to be landmark features of carcinogenesis. The cancer cell genome is characterised by a decreased level of cytosine methylation relative to normal cells. Specific CpG-rich regions where hypomethylation occurs are in LINE-1 repetitive sequences, as well as in centromeres and microsatellite DNA, which in normal cells are highly methylated (7). It is thought that a lower level of DNA methylation in these regions increases genome instability, thereby promoting the development of cancer (39). Global hypomethylation is accompanied by region-specific DNA hypermethylation, particularly typical of the initiation stage, but also influencing further stages of tumour development (23). In normal cells, CpG islands are only sporadically methylated, so they may be used as binding sites for proteins responsible for the initiation of DNA transcription. In contrast, cancer cells display a high level of methylation of CpG islands, especially in promoters of genes preventing tumour growth, mainly tumour suppressor genes (40). It is estimated that in cancer cells, there are on average 600 regions rich in CpG islands with an altered DNA methylation pattern in comparison to normal cells (41).

DNMTs are responsible for DNA methylation in both normal and cancer cells; therefore, decreased or increased activity of these enzymes in cancer cells will lead to alterations in the level of 5-mC. In human prostate cancer cells PC-3, DUI145, LNCaP, DuPro, TsuPr1 and ND-1, an increased expression of *DNMT1* gene was found in comparison with benign prostatic hyperplasia tissue (42). Similarly, during the development of nodular invasive carcinomas of the bladder, aberrations in the DNA methylation pattern were correlated with the expression of this gene (43). Another proposed mechanism is the slower rate of 5-mC methyl group removal, which in effect could lead to DNA hypermethylation.

The epigenetic silencing of tumour suppressor genes induces such mechanisms as uncontrolled cell division, the ability to infiltrate other tissues, metastasis, avoiding apoptosis or maintaining angiogenesis all of which are responsible for promoting tumour development. As an example, tumour suppressor genes *CDKN2A*, *VHL* or *BRCA1* can be used, which, as a result of the methylation of their promoter regions, are inactive in cancer cells (43). Similarly, resistance to anticancer drugs targeting apoptosis may result from the methylation-silenced expression of *PTEN*, *caspase-9* or *Apat-1* (44). The elevated DNA methylation level in the CpG islands also influences the suppression of genes engaged in hormonal responses as well as cell adhesion (39). Another group of biomolecules whose inhibited expression via CpG island hypermethylation can contribute to cancer development and progression are microRNAs (miRNAs). It has been shown that DNA methylation-associated silencing of tumour suppressor miRNA (namely, miR-148a, miR-34b/c and miR-9) correlates with the appearance of lymph node metastasis (45).

In a growing tissue, the correct reproduction of the DNA methylation pattern after replication depends not only on the proper functioning of the DNA methylation machinery but also on the accessibility of donors of methyl groups and nutrients involved in the metabolism of methyl groups, i.e. folic acid, methionine, vitamin B_{12}, betaine and choline, derived mainly from the diet (46,47). Diets deficient in these nutrients tend to reduce SAM concentrations and in consequence can lead to global hypomethylation, a characteristic feature of cancer. It follows that an insufficiency of dietary methyl-group donors

may increase susceptibility to cancer. The increased risk of cancer in the elderly has been suggested to be associated with their reluctance to consume meat products (48). Epidemiological studies showing an inverse correlation between folate status and the risk of several malignances support this hypothesis (49). However, it has also been demonstrated that in animals, excessive folate supplementation (possibly increasing the probability of hypermethylation) may accelerate cancer progression (50). A similar effect of increased DNA methylation *in vivo* was reported following a high methionine intake, raising questions about the safety of this common dietary supplement (51). It remains to be established whether the above observations, as well as the impact of other dietary factors on cancer incidence, can be explained and to what extent in terms of nutrient–epigenome interactions.

The quantitative analysis of DNA methylation profiles carried out for panels of cancer-related genes also indicated a strong association between lifestyle indices. These include a high alcohol intake in the case of colorectal cancer associated with the hypermethylation of several genes (e.g. *APC-1A*, *CDKN2*, *RASSF1A*) and the connection between highly specific *MTHFR* gene hypermethylation in lung cancer and tobacco smoking (52,53). On the other hand, DNA hypomethylation in the leukocytes of healthy subjects and in patients with colon adenomas and carcinomas is positively correlated with cancer progression but is negatively correlated with the level of DNA oxidation (8-oxodG), which reflects human exposure to endo- and exogeneous reactive oxygen species (ROS) (54). All these data point to another issue awaiting experimental evidence, namely the precise role of epigenetic changes induced by diet, lifestyle and environment, a topic discussed in a recent review by Herceg (55).

The methylation of promoters, which silences the expression of tumour suppressor genes, has been accepted as one of the causes of tumour development. Another cause is the mutation of these genes, for example *p53*. A mutated *p53* suppressor gene is active in >50% of solid tumours, and its mutations in 25% of observed cases result from the already-mentioned spontaneous deamination of methylated cytosine, leading to the transition 5-mC → T in the area of the CpG-rich promoter sequence of this gene (56).

Variations from the pattern of 5-mC distribution in DNA are very often related to specific types of tumours even in the early phase of carcinogenesis; therefore, it is hoped that they could be used as a screening tool. The distribution of 5-mC in promoter regions of certain cancer-related genes has enabled characteristic gene maps to be defined, which can represent a marker for a given type of tumour (57–59). The best-established epigenetic markers are given in Table I. The most recently proposed biomarkers include the methylation suppressed expression of prolyl 3-hydroxylase P3H2 associated with oestrogen-receptor-positive breast cancers (73) and of the homeobox transcription factor EN1 (analysed in stool DNA), common in most colorectal cancers (74).

Compared to gene expression microarrays or proteomic approaches, the application of DNA methylation patterns in cancer diagnostics offers several advantages. DNA is a very stable molecule and the assays for individual markers are universal, i.e. independent of tumour type. Moreover, abnormal methylation patterns in cancer cells differ not only quantitatively but also qualitatively from those in normal cells, which ensure the high sensitivity and specificity of measurements. All this suggests the rapid development of convenient routine assays of tumour markers for cancer screening purposes and carcinogenic risk assessment. This is indeed the case: in 2009, the first such diagnostic tool was launched by Epigenomics AG under the trade mark Epi *pro*Colon, and it detects colorectal cancer based on the aberrantly methylated DNA of the *SEPT9* gene in blood plasma with >70% accuracy (75). In 2010, the same company released a similar Early Detection Assay for lung cancer—Epi *pro*Lung—based on the detection of the DNA methylation pattern of the human homeobox gene *SHOX2*, which allows ~60% of cancers to be predicted using bronchial lavage samples (www.epigenomics.com/en/diagnostic-products-1/epi-prolung).

Anticancer drugs targeting the methylome

Because of its key role for the appropriate functioning of the cells, the epigenome has become a promising target for anticancer therapy. The basic challenge in designing potential anticancer drugs functioning at the DNA methylation level is a specific recognition of molecular targets, which require epigenetic changes to prevent cancer growth. Currently, the greatest hopes are associated with the inhibitors of DNA methylation working as potential activators of tumour suppressor genes. DNMT inhibitors lead to the demethylation of the genome, thereby restoring expression of methylated genes, which is easily monitored by the estimation of mRNA levels (7,76).

Table I. Examples of genes silenced as a result of DNA methylation in relation to cancer type

Hypermethylated genes	Function	Tumour type	Reference
Rb	Cell-cycle regulation	Retinoblastomaoma	(60)
APC	Signal transduction	Colorectal carcinoma and other cancers	(61)
p14^{ARF}	Cell-cycle regulation	Colorectal carcinoma	(62)
p15/CDKN2B	Cell-cycle regulation	Leukaemias	(64)
p16/CDKN2A	Cell-cycle regulation	Various cancers	(63)
BRCA1	DNA repair	Breast carcinoma, ovarian carcinoma	(65)
VHL	Tumour suppressor	Kidney carcinoma	(66)
hMLH1	Mismatch repair	Breast carcinoma, gastric carcinoma, endometrial carcinoma	(67)
ER-α	Oestrogen receptor-α	Breast carcinoma and other cancers	(68)
Myf-3	Myoblast cell differentiation	Breast carcinoma	(69)
E-cadherin	Cell adhesion	Various cancers	(70)
Bcr-abl	Regulation of cell division and differentiation	Acute granulocytic leukaemia	(71)
GSTP1	Detoxification	Prostate carcinoma, kidney carcinoma, hepatocellular carcinoma, breast carcinoma	(76)
PTEN	Regulation of cell growth and apoptosis	Prostate carcinoma	(72)

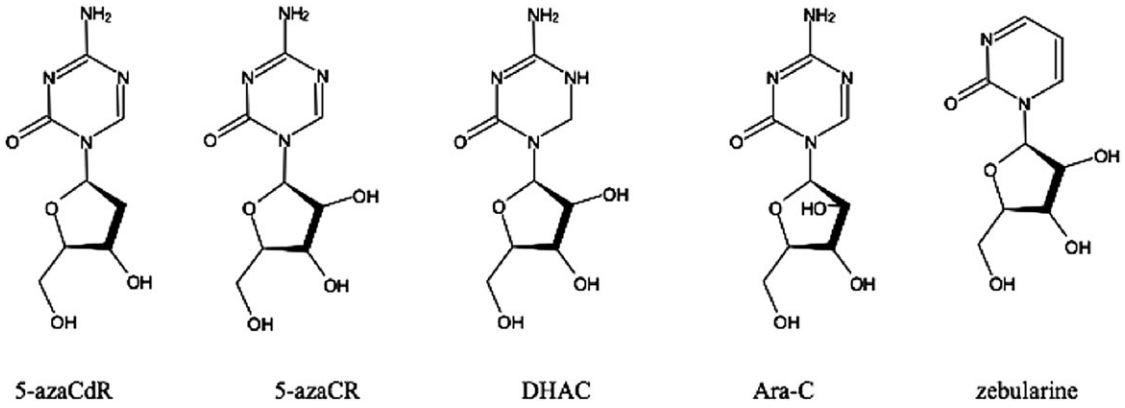

Fig. 3. Chemical structures of DNMT inhibitors belonging to the group of cytosine analogues.

Currently, five nucleoside DNMT inhibitors are undergoing different stages of preclinical and clinical trials as potential anticancer drugs (77). Chemically, they all are cytosine analogues (Figure 3): 5-azacytidine (5-azaCR, azacytidine, Vidaza™), 5-aza-2′-deoxycytidine (5-azaCdR, decitabine, Dacogen™), dihydro-5-azacytidine (DHAC), arabinosyl-5-aza-cytosine (Ara-C, fazarabine, kymarabine) and zebularine.

The two cytidine analogues of primary interest are 5-azaCR and 5-azaCdR. Both compounds have been extensively studied in clinical trials for treating solid tumours andhaemato-logical diseases as single agents or in combination therapy (78). 5-AzaCR was first described as a DNMT inhibitor >40 years ago (79). It is a ribose nucleoside analogue which, after metabolic modification by ribonucleotide reductase at the diphosphate stage and subsequent phosphorylation to a tri-phosphate, becomes incorporated into DNA in place of cytosine. It was shown that the reaction of cytosine methylation catalysed by DNMT is inhibited when this cytidine analogue is built into a polydeoxynucleotide chain. Inhibition results from the covalent trapping of DNMT via the sulfhydryl side chain of the catalytic cysteine by 5-azaCR and concomitant protein degradation (78). Subsequent passive DNA demethy-lation during replication occurs owing to the reduced levels of the enzyme catalysing the transfer of methyl groups, and a significant part of a newly synthesised DNA strand remains in a hemimethylated form, whereas the second round of DNA synthesis yields almost entirely demethylated DNA. After phosphorylation by uridine-citidine kinase, a part of 5-azaCR is also incorporated into RNA and affects protein synthesis with cytotoxic effects (80). 5-azaCR was approved in 2004 by the US Food and Drug Administration (FDA) for treating myelodysplastic syndrome (MDS) (81) and acute myeloid leukaemia, and it is also in phase II clinical trials for treating solid tumours and other types of leukaemias (10,58).

The cytosine analogue 5-azaCdR, like 5-azaCR, was synthesised in the 1960s. The proposed mechanism responsible for the antitumour activity of both drugs is generally the same; however, before being incorporated into cellular DNA, 5-azaCdR requires only phosphorylation, which makes its functioning more specific and is then less toxic than 5-azaCR (82). 5-azaCdR gained FDA approval in 2006 for the treatment of MDS and it is in phase II clinical trials for treating solid tumours (58); it is also in preparation for clinical trials for treating leukaemias (10). More detailed mechanistic studies have found that 5-azaCdR is capable of restoring apoptosis in cancer cells by the DNA demethylation of the *caspase-8* gene promoter, thereby overcoming resistance to cytostatic drugs, whose mechanism is based on inducing programmed cell death (83). It is also known that epigenetic suppression of the *hMLH1* gene, whose product is involved in the mismatch repair of cisplatin–DNA adducts, decreases apoptosis in response to platinum derivatives (58). Treatment of the resistant human ovarian cell line A2780/cp70 with 5-azaCdR results in partial reversal of DNA methylation, enhanced re-expression of the *hMLH1* gene and sensitisation to cisplatin both *in vitro* and *in vivo* (84).

Zebularine is another cytidine analogue which, after being incorporated into DNA, causes DNMT to be 'caught in a trap' while moving along DNA replication forks and in addition acts as a transition state analogue inhibitor of cytidine deaminase by binding covalently at the active site (7). Unlike 5-azaCR and 5-azaCdR, zebularine is a relatively stable chemical compound with a half-life of ∼21 days at pH 10, which means it can be administered orally (85). This compound has also been shown to be less toxic than 5-azaCR and to preferentially target tumour cells. Importantly, it reactivates genes often silenced in tumour cells (e.g. suppressor *p16* or *E-cadherin*) but does not induce the switching of the Epstein–Barr virus from the latent to the lytic form associated with the increased incidence of lymphomas (86). Zebularine is currently being prepared for preclinical trials for bladder cancers (10).

DHAC is a hydrolytically stable potential anticancer azacytidine analogue, which influences DNA methylation and inhibits RNA synthesis. During phase II clinical studies, the strongest therapeutic effect of this compound was observed in cases of mesothelioma; its activity towards solid tumours was limited. Ara-C, also a cytidine derivative, exhibited in preclinical studies the most promising activity against murine leukaemias as well as xenografts of human solid tumours, mainly in the colon and lungs. However, phases I and II of the clinical trials showed up the rather poor anticancer properties of this compound (87).

Other DNMT inhibitors include compounds that for many years have been used clinically for treating various diseases but were not regarded as potential anticancer drugs; phytochem-icals with proven anticarcinogenic properties and compounds created using modern technologies, e.g. computer-aided drug design. The most promising ones are hydralazine, procaine, procainamide, epigallocatechin 3-gallate (EGCG), MG98 and RG108. It is hoped that these non-nucleoside DNMT inhibitors

will be less toxic because they do not become incorporated into DNA.

Hydralazine is a drug used to treat high blood pressure and arrhythmia. Its inhibitory properties towards DNMT were first seen in patients in whom systemic lupus erythematosus developed owing to DNA hypomethylation in T cells (78). In the case of procaine used for anaesthesia, as well as procainamide, a drug used to treat cardiac arrhythmia, it has been observed that when bound within CpG sequences, they prevent DNMT from binding to DNA, which causes DNA hypomethylation and reduces cancer cell growth (77).

EGCG, a natural polyphenolic compound particularly abundant in green tea, exhibits a wide spectrum of properties preventing the development of cancer. EGCG has been reported as decreasing the level of DNA methylation and to reactivate methylation-silenced genes in human cancer cells as a result of its binding to the catalytic domain of DNMT (88). However, EGCG degradation liberates significant amounts of hydrogen peroxide, so DNMT oxidation may well account for the decreased activity of these enzymes and cytotoxic effects towards cancer cells. Many other polyphenolic compounds, for instance, catechol polyphenols or genistein, are also capable of DNMT inhibition, albeit to a lesser extent (89).

The compound RG108 is the result of rational drug design (80). It was developed on the basis of the molecular modelling of the human DNMT1 catalytic domain. RG108 blocks the active site of this enzyme, causing the demethylation and reactivation of tumour suppressor genes; interestingly, however, it does not affect the methylation of centromeric satellite sequences. These results establish RG108 as a DNMT inhibitor with fundamentally novel characteristics. Moreover, this compound exhibits a relatively low toxicity towards normal human cells, which makes it a promising lead structure for the further development of related anticancer drugs (78). Another potential epigenetic anticancer drug (MG98) developed by the rational design approach is an antisense deoxyoligonucleotide, whose sequence is complementary to human DNMT mRNA. Currently, MG98 is in phase II clinical trial (58), while EGCG, RG108 and procaine are in the process of preparation for clinical trials (78).

Epigenetic treatments of cancer have become a new and rapidly evolving branch of pharmacology, especially since the results obtained from clinical tests for potential anticancer drugs acting at the epigenome level are very promising. There are two main reasons why these compounds are regarded as the future of cancer therapy: widely understood epigenetic aberrations are the basis of many cancer-related diseases, and current anticancer chemotherapeutics, regardless of their mode of action, are highly toxic and often therapeutically ineffective.

Although epigenetic therapy is a promising approach for combating neoplastic diseases, the side effects associated with the use of potential anticancer drugs that influence DNA methylation and the long-term safety of these medications are problems that have not escaped notice. Some alterations in the methylome, including those that may be induced by DNMT inhibitors, have undesirable effects and can even be expected to promote the carcinogenic process. For example, 5-azaCR or procainamide cause a lupus-like autoimmune disease in animal models, an after-effect of the DNA hypomethylation of T lymphocytes (90). Another effect of diminished DNA methylation resulting from mutations in the *DNMT3b* gene is the immunodeficiency and instability of the centromere region underlying the immunodeficiency, centromere instability, facial anomalies syndrome (39). The role of DNA hypomethylation in the origin and pathogenesis of a number of human diseases, including cancer, has been discussed in detail by Pogribny and Beland (91). Non-selective remodelling of the chromatin structure may activate oncogenes or other genes, thereby subsequently initiate carcinogenesis (92). Moreover, the nucleotide analogues that become incorporated into DNA (like azacytosines) actually alter the DNA structure similarly to genotoxic compounds, so probably influence not only the epigenome but the genome function as well, a phenomenon well known in the case of other anticancer compounds acting as nucleotide analogues. Suffice it to mention 6-mercaptopurine, which has been shown to deplete SAM and subsequently decrease DNA methylation but also to initiate a number of genotoxic effects: single-strand breaks, SCE, DNA and DNA–protein cross-links among them (81). The unavoidability of overlaps between genotoxic and epigenetic effects induced by anticancer drugs and carcinogens will be discussed later.

Methods of DNA methylation analysis—challenges from DNA damage

Previous sections have described ways in which altered DNA methylation patterns, and therefore changes in the level of gene expression, may be the basis of many diseases and susceptibility to cancer. Therefore, the monitoring of fluctuations in the methylome, both at the global level as well as for single genes, can be very important in understanding the development and progression of these diseases, as is the identification of tumour markers, which can serve in early diagnostics for a particular type of tumour. The potential benefits of 'applied epigenomics', despite the controversy surrounding this concept, led to the launching of the multi-million-dollar epigenome project in 2008 by the US National Institutes of Health, one of the aims of which is to develop technologies that can precisely detect epigenetic markers in individual cells (93).

DNA methylation analysis for disease diagnostics holds the greatest promise. However, some researchers point to the usefulness of such determinations in basic initial toxicity assessments (94). Verification of the latter proposition for four model compounds showed that DNA methylation (global and GC-rich patterns) used in conjunction with traditional *in vitro* tests for genotoxicity and cytotoxicity gave a better indication of potentially problematic compounds, in particular non-genotoxins, thus enhancing the predictive value of basic assessments of their toxic potential and possible mechanisms of action in order to prioritise them for further evaluation.

The approaches to DNA methylation analysis can be divided into analytical chemistry-based methods and those employing the techniques of molecular biology. The former provide only information on the total level of 5-mC (and potentially 5-hmC) in the genome or the frequency of its occurrence within certain restriction sites but ensure quantitative accuracy of measurements and are capable of detecting non-natural modifications. For such determinations, the most convenient are chromatographic techniques such as reverse-phase high-performance liquid chromatography coupled with ultraviolet detection (254 and 280 nm) or mass spectrometry for the identification of separate nucleosides or bases in DNA digests. Although these methods are constantly being improved (e.g. (95)), one of their drawbacks is the relatively large amount of DNA needed for analysis (in the range of 10–100 μg). Other chromatographic methods involve the separation of ^{32}P-labelled

nucleotides by two-dimensional thin-layer chromatography and the determination of radioactivity associated with particular chromatographic spots. Though quantitatively less accurate (this can be overcome by the use of high-performance liquid chromatography with radiation specific detection), they are extremely sensitive, requiring only minute amounts of DNA (<1 μg). It comes as no surprise, therefore, that ^{32}P-labelling enabled the detection of 5-hmC in human neurons thereby changing the definition of the methylome (14).

Advanced molecular biology-based methods have the ability to determine both the frequency of 5-mC and their distribution within the DNA fragment or whole genome. Their application has enabled such undertakings as genome-scale DNA methylation mapping of clinical samples at single-nucleotide resolution (96). These throughput methods, involving specific antibodies, microarray technology and genome-wide next-generation sequencing, have been the subject of a number of recent reviews (9,97–99), or particularly informative comparisons (100,101), so there is no need to describe them. Therefore, here, merely the sources of errors that could jeopardise the correct interpretation of data during the analysis of DNA samples derived from organisms or cells exposed to genotoxic insult will be pointed out.

Regardless of further DNA processing, throughput methods in the initial step involve preparation of methyl-sensitive DNA, which is most frequently accomplished as a result of:

1. Chemical modification of cytosine by sodium bisulphite leading to the hydrolytic deamination of cytosine to uracil (Figure 4); this alters the susceptibility of DNA to hydrolysis by the specific restriction enzymes,
2. DNA digestion by restriction endonucleases capable of differentiating methylated and unmethylated DNA sequences, or
3. Immunoprecipitation of methylated DNA with specific antibodies recognising methylated DNA sequences.

All these methods exhibit a limited ability to differentiate 5-mC from other covalent DNA modifications. The dominant bisulphite sequencing techniques cannot distinguish between methylated and hydroxymethylated cytosines. Moreover, the change from GC to AT base pairing, the after-effect of the conversion of 5-mC to U, may also occur as a result of spontaneous or base-excision repair-induced removal of bulky adducts with the formation of an abasic site. During replication, the preferential incorporation of A opposite such a site takes place with the final effect of AT pairing. Other difficulties associated with the use of bisulphite in epigenome profiling have been summarised by Hayatsu (102).

When antibodies are employed in the detection of 5-mC, cross-reactivity with non-natural DNA modifications cannot be excluded, especially with regard to anti-5-hmC antibodies which may cross-react with other hydroxylated nucleosides found among the so-called oxidised DNA lesions. Formed by ROS, these lesions represent various chemical structures (~300 hundred are known to date) and can be very frequent, up to 1 modification per 10^5–10^6 base pairs, particularly in cells exposed to oxidative stress. The reliable profiling of 5-hmC seems less challenging since the elegant method of its two-step derivatisation has been proposed (103). The first stage involves the transfer of glucose, chemically modified to contain azide moiety, onto the hydroxyl group of 5-hmC in a reaction catalysed by T4 bacteriophage β-glucosyltransferase. The azide group is subsequently modified with e.g. biotin, which greatly enhances the sensitivity of detection.

The most vulnerable to artefacts resulting from covalent DNA modification by genotoxic compounds is the use of methyl-sensitive and non-sensitive restriction enzymes (HpaII/MspI tandem). We have developed a method of assessing the ability of genotoxins to bind to DNA that relies on the abolished ability of restriction endonucleases (MspI and Tru1I) to recognise their restriction sites once these sequences are chemically modified by low-molecular-weight compounds (104). This approach also allows the detection of the physicochemical binding of DNA-interacting compounds and oxygen adducts (B. Fedejko, A. Dopierała, A. Bartoszek, unpublished results). The soundness of our reservations expressed here is supported by the reported interference with enzymatic recognition of 5-mC in CpG arrays caused by benzo[*a*]pyrene (B[*a*]P)–DNA adducts (105).

The limitations of current techniques are recognised; consequently, new chemical methods that use derivatisation agents specifically reacting with methylated cytosine are being developed and may in the future be incorporated in the current high-throughput approaches (106). At the moment, it seems important, especially in so-called environmental epigenetics, where the probability of occurrence of non-natural DNA modifications is the greatest, to verify determinations obtained from bio-based assays by measuring the total methylation and hydroxymethylation of DNA in a given sample using precise chromatographic techniques.

The influence of DNA-binding compounds on epigenome function

The interactions between DNA and anticancer drugs, mutagens and carcinogens have been studied for many years. Therefore, the genotoxicity and alterations in the double helix structure resulting from such interactions are well understood and

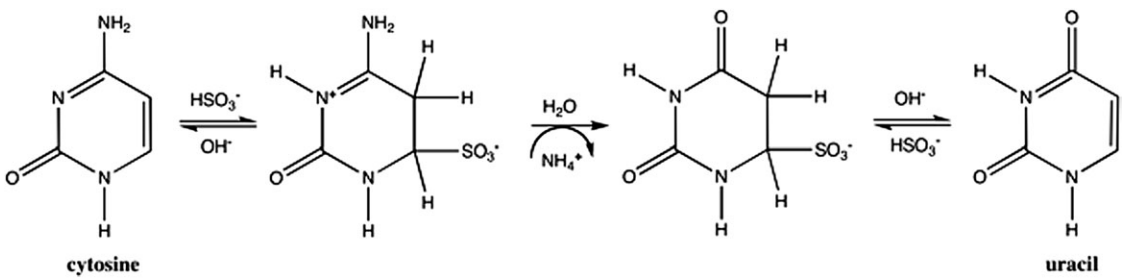

Fig. 4. Conversion of cytosine to uracil induced by bisulphite.

methods of their determination well established. As knowledge of chromatin chemistry and biology has grown, interest has shifted to the association of DNA binders with chromatin (92). As described earlier, chromatin is a very dynamic structure that can alternate between highly condensed and extended conformations strictly related to the transcriptional activity of DNA. The dynamics of chromatin remodelling is dependent on the subtle natural covalent modification of histones and DNA: for instance, 5-mC, which causes a local three-base pair long distortion in the polydeoxyribonucleotide chain, modulates the interactions between DNA and histone octamer (107), while non-canonical DNA structures (H- or Z-DNA) were shown to destabilise the mammalian genome *in vivo* (108). As local epigenetic modifications of DNA and/or histones can have a fundamental influence on all cellular processes in which DNA is a substrate, such as replication, transcription or DNA repair, it follows that non-natural DNA binders could impair epigenome function by both covalent modification and local disturbances in DNA structure, even at doses well below the thresholds necessary for causing detectable genotoxic damage or cytotoxicity. Most anticancer drugs, mutagens and carcinogens are known to form chemical bonds with both major chromatin components and to induce distortions in the double helix. The induction of chromatin condensation, abolishing its transcription by anticancer DNA-binding drugs, particularly intercalators, has been convincingly documented (109). Sporadic reports on the interference of DNA adducts with chromatin structure and function also suggest that the epigenetic consequences of these lesions deserve more systematic examination. There are a number of reasons that justify such undertakings.

Cytosine methylation occurs mainly within gene promoters or other sequences rich in CpG islands. For the majority of xenobiotics, guanine is a preferentially modified nucleobase (110), and CpG islands will therefore be their preferred binding region. Moreover, a local disturbance in the chromatin structure caused by 5-mC sometimes renders these sequences more accessible to DNA covalent modification by a foreign compound. Taking into account the fact that the average level of cytosine methylation is around one 5-mC per 400–500 nucleotides, the frequency of DNA adducts in cells treated with certain carcinogens estimated to be, on average, around one adduct per 10^5–10^{10} nucleotides, seems negligible. However, it can be expected that in exposed cells, non-natural covalent DNA modification, often constituting substantial steric hindrance, may nonetheless influence epigenetic mechanisms.

In much the same way as 5-mC or 5-hmC, a DNA adduct constitutes a distortion in chromatin, which in the case of bulky DNA binders may alter the structure of a sizable portion of genetic material. In general, a DNA adduct that gives rise to a greater steric hindrance than 5-mC can probably disrupt the chromatin structure, and thus its functioning, on a much larger DNA scale. A special case in this context is bifunctional DNA cross-linking compounds, including numerous important antitumour drugs, since they also form cross-links between DNA and protein; therefore, modification at the DNA-histone level enabling chromatin modelling is in a way warranted by their structure. Below we have summarised examples of epigenetic mechanisms induced by low-molecular-weight compounds, carcinogens and anticancer drugs, capable of covalent DNA modification.

The chemical compound whose ability to form DNA adducts has been best recognised is B[*a*]P, a carcinogen that undergoes metabolic transformation to a diol-epoxide, BDPE, in the human organism, and subsequently binds to guanine N^2 atom in DNA in the 5′-XGG-3′ or 5′-GGX-3′ sequence. The adducts occurring mainly in linker DNA are characterised by a variable but non-random distribution in the genome (111,112). It has been demonstrated that the presence of 5-mC at the CpG sequence creates a preferential binding site for B[*a*]P and explains the increased frequency of DNA damage and mutation at codon 14 of the *K-ras* gene often observed in human cancers (113), as well as G-to-T transversion mutations of *p53* gene commonly seen in smoking-associated lung cancers (114). Enhanced binding in the presence of 5-mC has also been reported for other carcinogens like AAF, aflatoxin B1, acrolein and acetaldehyde. Currently, there are hardly any investigations correlating genotoxic and epigenetic effects in humans exposed to chemical carcinogens. The study by Pavanello *et al.* (115) confirmed the interdependence between the percentage of 5-mC and markers of genotoxicity in a study involving Polish coke-oven workers. The increase in levels of B[*a*]P–DNA adducts (indicator of genetic damage) and micronuclei (indicator of chromosomal instability) in this group were parallelled by elevated global and gene-specific DNA methylation. Indirect support for the influence of B[*a*]P–DNA adducts on the epigenome has come from an already mentioned CpG microarray study designed to detect the epigenetic patterns susceptible to this carcinogen in MCF-7 cells. Perhaps not too surprisingly, during these experiments, the enzymes sensing 5-mC also recognised B[*a*]P–DNA adducts, suggesting that these two covalent modifications may be indistinguishable to actual DNA/chromatin processing machinery, also *in vivo* (105). Alterations in cellular epigenetic status (e.g. gene-specific hypermethylation) associated with liver and lung carcinogenesis have also been seen following exposure to other typical genotoxins, in liver and lung tissue, respectively, of rats exposed to 2-acetylaminofluorene (116) and 3-methylcholanthrene or diethylnitrosamine (117).

However, probably depending on the point in time and the extent of DNA damage, at least *in vitro*, the opposite effects of the higher affinity of B[*a*]P for CpG-rich sequences containing 5-mC may occur, i.e. DNMT inhibition and a decreased level of cytosine methylation in DNA (118). Furthermore, a B[*a*]P–DNA adduct causes a local disturbance in the chromatin structure, increasing accessibility of DNA to DNA repair systems (119), which can also take part in DNA demethylation (120). Accordingly, some studies comparing the relationship between DNA methylation and the level of DNA adducts formed by environmental carcinogens in human samples have demonstrated that the higher level of DNA adduction is associated with substantially depressed (by up to 50%) amounts of 5-mC (91,121). These data indicate that the epigenetic influence of DNA adducts formed by B[*a*]P and other carcinogens may affect the functions of the cells, both directly by inducing changes in the methylome, as well as indirectly by modifying the chromatin structure.

Anticancer drugs, such as cisplatin, doxorubicin, mitoxantrone, mitomycin C, cyclophosphamide or 1-nitroacridine derivatives, are compounds that covalently bind to DNA. Mitomycin C (MMC) covalently binds to N^2 of guanine in DNA, mainly in CpG, less often in GpC, sequences. It also forms cross-links between guanines in the complementary DNA strands. MMC is one of the few compounds whose epigenetic interactions have been described at the molecular

level. Cytosine methylation in the CpG sequence increases the number of cross-links formed by MMC. Their presence and interaction with MBPs deactivates the chromatin structure within the scope of the association of MBPs, resulting in suppressed gene expression (122).

Doxorubicin binds covalently to guanine N^2 in DNA, mainly in the 5′-GC sequence. It is suggested that the presence of the doxorubicin–DNA adduct in the promoter region can inhibit gene transcription and consequently gene expression. In contrast to other GC-binding compounds, the frequency of adduct formation by this drug was not influenced by DNA methylation (123).

Mitoxantrone also forms adducts with guanine, mainly in the CpG sequence, and inhibits transcription and influences gene expression (124). Furthermore, it forms more DNA adducts in CCGG sequences, which can be explained by a local change in DNA structure caused by cytosine methylation. The further opening of the chromatin structure by mitoxantrone-DNA adducts at these sites means, in turn, that such regions become more accessible to subsequent mitoxantrone molecules or other low-molecular-weight compounds (125).

Cisplatin, another anticancer guanine N^2 modifier, forms intrastrand cross-links between adjacent G-G and interstrand cross-links between guanines on opposite DNA strands at G-C sites (126), inducing distortion of the helix structure (127). It can therefore be expected that DNA–cisplatin adducts will also be frequent in CpG islands. It has been proven that as a result of covalent DNA modification by cisplatin, a disturbance in the DNA structure arises that encourages association with high mobility group proteins and further bonding of cisplatin molecules to DNA. This causes an increase in the level of DNA damage (128) and leads to a disturbance in the formation of the nucleosomal core (126).

1-Nitroacridines, including the registered anticancer drug Ledakrin and recently developed derivatives currently under clinical studies, also have a documented capability of forming DNA adducts with guanine, as well as cross-links between DNA and proteins (129,130). The ability of Ledakrin to inhibit transcription has also been demonstrated (131). It appears, then, that DNA adducts of 1-nitroacridines may influence chromatin function at both the genome and the epigenome level.

The most direct evidence of the impact of DNA-binding compounds on the epigenome comes from observations made for cyclophosphamide. It has been known that preconceptional paternal exposure to this anticancer drug leads to increased embryo loss, malformations and other deficiencies in offspring. The study in rats by Barton *et al.* (132) on the mechanisms underlying this male-mediated developmental toxicity revealed disruption of epigenetic programming of both parental genomes with male pronuclei dramatically hypomethylated and female pronuclei hypermethylated. These authors suggest that the DNA damage induced by cyclophosphamide in male germ cells interferes with chromatin remodelling after fertilisation and thereby affects subsequent generations.

In general, very few studies have been designed to investigate genotoxic effects in relation to DNA methylation and cancer risk, an exception being tamoxifen-induced rat hepatocarcinogenesis. Tamoxifen, an anti-oestrogen used in the treatment and long-term chemoprevention of breast cancer, is a potent genotoxic hepatocarcinogen in rats forming DNA adducts preferentially with guanine (133). In rats, the accumulation of tamoxifen–DNA adducts turned out to be parallelled by a number of epigenetic changes in target tissue, such as DNA hypomethylation, both globally and in regulatory sequences of *c-myc* proto-oncogene, resulting in its increased expression, as well as decreased expression of maintenance and *de novo* DNMTs and proteins guarding genomic stability (Rad51, Ku70, Polβ). Although the authors did not provide a molecular mechanism that could explain the translation of tamoxifen-induced DNA lesions into epigenetic effects, they demonstrated that genotoxic alterations could lead to the emergence of a specific cancer-related phenotype in liver but not in non-target tissues (134,135).

Another source of covalent DNA modification is ROS, effectively leading to the formation of oxidised DNA lesions, which can cause point mutation. The latest research indicates, however, that these DNA lesions can also have an influence on the methylome. One example is 8-oxodG, which inhibits the binding of MBPs to methylated CpG sequences and therefore disrupts gene expression (55). Since numerous xenobiotics including environmental pollutants, both mutagenic and non-mutagenic, are able to stimulate endogenous ROS production, they may disrupt the methylome in this indirect way. Such a mechanism was suggested to account for global DNA hypermethylation in sperm of mice exposed to particulate air pollution, in which an elevated level of DNA strand breaks, probably due to oxidative stress, was determined (136). Moreover, it may be expected that oxidised DNA lesions formed by the hydroxylation of pyrimidines (including 5-mC) may interfere particularly efficiently with epigenetic signals related to 5-hmC as a result of structural similarities.

Conclusions

The results of research into the relationship between epigenetic marks and cancer development gathered over the past decade show that most of the phenomena known to accompany carcinogenesis involve changes in DNA methylation patterns. Similar alterations in the methylome have been observed in DNA or the chromatin of cells challenged by genotoxic agents. First of all, CpG-rich sequences like CpG-islands are the major recipients of DNA methylation, but they are also hot spots for the covalent binding of genotoxins, as most of them preferentially modify guanine. It is conceivable that such non-natural chemical modifications may be sensed as DNA hypermethylation, preventing the association of transcription factors to gene promoter sequences and leading to reduced transcriptional activity. On the other hand, DNA adducts induce DNA repair which, while removing DNA damage, will erase existing DNA methylation and unblock silenced genes. Gene expression is also dependent on chromatin compaction, which is influenced by the methylation of cytosines and DNA adduction alike. Altogether, the altered pattern of gene expression seen in cancer as a result of epigenetic changes can also be brought about by the covalent DNA binding of genotoxins.

Another feature of the epigenome of cancer cells that can be modulated by epigenetic marks as well as genotoxic factors is global hypomethylation. There are two mechanisms by which genotoxins can induce such a chromatin state: either the above-mentioned removal of 5-mCs during the repair of DNA damage or as a result of DNMT inhibition.

Finally, according to very recent findings, the epigenome of pluripotent, and thus undifferentiated, cells is characterised by the increased occurrence of non-CpG methylations. It is not

known at the moment whether this shift in methylation pattern induces or is an effect of dedifferentiation. Should the former be the case, it would explain the dedifferentiation observed in the case of chemical carcinogenesis since covalent DNA modification by chemical carcinogens is a random process and may occur outside of CpG-islands mimicking non-CpG DNA methylation.

The multiple ways by which genotoxic agents can disrupt the normal pattern of DNA methylation, described in previous sections, encourage speculation on the relative importance of epigenetic effects induced by chemical carcinogens compared with DNA damage and mutations, especially in view of the fact that genotoxic lesions are under the strict control of DNA repair systems, as a result of which they can be rapidly eradicated. Although the repair systems sometimes introduce mistakes in the nucleotide sequence, the mutations must occur at specific sites to change the genetic code in a detrimental way. In contrast to the genome, at least according to the current state of knowledge, no such surveillance systems exist to ensure the stability of the epigenome. It follows that the epigenome may be regarded as much more vulnerable and its function more susceptible to disruptive factors than the genome. This reasoning leads to the conclusion that the carcinogenic properties of compounds (or factors) capable of covalent DNA modification result in the first place from their interference with the epigenome, while such deregulation can in turn accelerate genomic instability.

Some researchers have pointed to the relationship between DNA methylation and genotoxic effects, proposing different mechanisms to account for such interdependences (91,137). We suggest that a DNA adduct, apart from being a genotoxic lesion, also represents a misleading epigenetic mark—epimutation—that can directly and instantly impair chromatin function at the epigenome level and that epimutations are a preliminary step towards the malignant transformation induced by genotoxins.

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References

1. Kouzarides, T. (2007) Chromatin modifications and their function. *Cell*, **128**, 693–705.
2. Lippman, Z. and Martienssen, R. (2004) The role of RNA interference in heterochromatic silencing. *Nature*, **431**, 364–370.
3. Law, J. A. and Jacobsen, S. E. (2010) Establishing, maintaining and modyfying DNA methylation patterns in plants and animals. *Nat. Rev. Genet.*, **11**, 204–219.
4. Yu, W., Gius, D., Onyango, P., Muldoon-Jacobs, K., Karp, J., Feinberg, A. P. and Cui, H. (2008) Epigenetic silencing of tumour suppressor gene *p15* by its antisense RNA. *Nature*, **451**, 202–206.
5. Buhler, M. and Moazed, D. (2007) Transcription and RNAi in heterochromatic gene silencing. *Nat. Struct. Mol. Biol.*, **14**, 1041–1048.
6. Rosok, O. and Sioud, M. (2004) Systematic identification of sense-antisense transcripts in mammalian cells. *Nat. Biotechnol.*, **22**, 104–108.
7. Szyf, M. (2005) DNA methylation and demethylation as targets for anticancer therapy. *Biochemistry*, **70**, 533–549.
8. Kubicek, S., Schotta, G., Lachner, M. *et al.* (2006) The role of histone modifications in epigenetic transitions during normal and perturbed development. New Approaches to Cancer Therapy Series: Ernst Schering Foundation Symposium Proceedings, Vol. 57, Springer, Germany, pp. 1–27.
9. Baker, M. (2010) Epigenome: mapping in motion. *Nat. Methods.*, **7**, 181–185.
10. Peedicayil, J. (2006) Epigenetic therapy—a new developmnet in pharmacology. *Indian J. Med. Res.*, **123**, 17–24.
11. Espada, J. and Esteller, M. (2007) Epigenetic control of nuclear architecture. *Cell. Mol. Life Sci.*, **64**, 449–457.
12. Ramsahoye, B. H., Biniszkiewicz, D., Lyko, F., Clark, V., Bird, A. and Jaenisch, R. (2000) Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proc. Natl Acad. Sci. USA.*, **97**, 5237–5242.
13. Lister, R., Pelizzola, M., Dowen, R. H. *et al.* (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*, **462**, 315–322.
14. Kriaucionis, S. and Heintz, N. (2009) The nuclear base 5-hydroxymethylcytosine is present in Purkinje neurons and brain. *Science*, **324**, 929–930.
15. Tahiliani, M., Koh, K. P., Shen, Y. *et al.* (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science*, **324**, 930–935.
16. Riggs, A. D. (1975) X inactivation, differentiation, and DNA methylation. *Cytogenet. Cell Genet.*, **14**, 9–25.
17. Holliday, R. and Pugh, J. E. (1975) DNA modification mechanisms and gene activity during development. *Science*, **187**, 226–232.
18. Pradhan, S. and Estève, P.-O. (2003) Mammalian DNA (cytosine-5)methyltransferases and their expression. *Clin. Immunol.*, **109**, 6–16.
19. Vertino, P. M., Sekowski, J. A., Coll, J. M., Applegren, N., Han, S., Hickey, R. J. and Malkas, L. H. (2002) DNMT1 is a component of a multiprotein DNA replication complex. *Cell Cycle*, **1**, 416–423.
20. Zhao, Q., Rank, G., Tan, Y. T. *et al.* (2009) PRMT5-mediated methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing. *Nat. Struct. Mol. Biol.*, **16**, 304–311.
21. Wilkins, J. F. (2005) Genomic imprinting and methylation: epigenetic canalization and conflict. *Trends Genet*, **21**, 356–365.
22. Robertson, K. D. (2005) DNA methylation and human disease. *Nat. Rev. Genet.*, **6**, 597–610.
23. Jones, A. P. (2002) DNA methylation in cancer. *Oncogene*, **21**, 5358–5360.
24. Goll, G., Kirpekar, F., Maggert, K. A., Yoder, J. A., Hsieh, J. A., Zhang, X. and Golic, K. G. (2006) Methylation of tRNA Asp by the DNA methyltransferase homolog Dnmt2. *Science*, **311**, 395–398.
25. Ooi, S. K. T. and Bestor, T. H. (2008) The colorful history of active DNA demethylation. *Cell*, **133**, 1145–1148.
26. Kapoor, A., Agius, F. and Zhu, J.-K. (2005) Preventing transcriptional gene silencing by active DNA demethylation. *FEBS Lett.*, **579**, 5889–5898.
27. Kangaspeska, S., Stride, B., Metivier, R., Polycarpou-Schwarz, M., Ibberson, D., Carmouche, R. P., Benes, V., Gannon, F. and Reid, G. (2008) Transient cyclical methylation of promoter DNA. *Nature*, **452**, 112–116.
28. Métivier, R., Gallais, R., Tiffoche, C. *et al.* (2008) Cyclical DNA methylation of a transcriptionally active promoter. *Nature*, **452**, 45–50.
29. Popp, C., Dean, W., Feng, S., Cokus, S. H., Andrews, S., Pellegrini, M., Jacobsen, S. E. and Reik, W. (2010) Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. *Nature*, **463**, 1101–1105.
30. Bhutani, N., Brady, J. J., Damian, M., Sacco, A., Corbel, S. Y. and Blau, H. M. (2010) Reprogramming towards pluripotency requires AID-dependent DNA demethylation. *Nature*, **463**, 1042–1047.
31. Parry, T. E. (2006) Mutagenic mechanisms in leukemia and cancer: a new concept cytosine lack could be as mutagenic as cytosine deamination. *Leuk. Res.*, **30**, 1079–1083.
32. Zingg, J. M., Shen, J. C., Yang, A. S., Rapoport, H. and Jones, P. A. (1996) Methylation inhibitors can increase the rate of cytosine deamination by cytosine-5-DNA methyltransferase. *Nucleic Acid Res.*, **24**, 3267–3275.
33. Kasai, H. and Kawai, K. (2009) DNA methylation at the C-5 position of cytosine by methyl radicals: a possible role for epigenetic change during carcinogenesis by environmental agents. *Chem. Res. Toxicol.*, **22**, 984–989.
34. Zardo, G., Fazi, F., Travaglini, L. and Nervi, C. (2005) Dynamic and reversibility of heterochromatic gene silencing in human disease. *Cell Res.*, **15**, 679–690.
35. Klose, R. J. and Bird, A. P. (2006) Genomic DNA methylation: the mark and its mediators. *Trends Biochem. Sci.*, **31**, 89–187.
36. Doi, A., Park, I.-H., Wen, B. *et al.* (2009) Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat. Genet.*, **41**, 1350–1353.

37. Nabilsi, N. H., Broaddus, R. R. and Loose, D. S. (2009) DNA methylation inhibits p53-mediated survivin repression. *Oncogene*, **28**, 1–5.
38. Rodenhiser, D. and Mann, M. (2006) Epigenetics and human disease: translating basic biology into clinical applications. *CMAJ*, **3**, 341–348.
39. Erlich, M. (2002) DNA hypomethylation, cancer, the immunodeficiency, centromeric region instability, facial anomalies syndrome and chromosomal rearragements. *J. Nutr.*, **132**, 2424S–2429S.
40. Teodoridis, J. M., Strathdee, G. and Brown, R. (2004) Epigenetic silencing mediated by CpG island methylation: potential as a therapeutic target and as a biomarker. *Drug Resist. Updat.*, **7**, 267–278.
41. Szyf, M. (2003) Targeting DNA methylation in cancer. *Ageing Res. Rev.*, **2**, 299–328.
42. Zhang, Z., Karam, J., Frankel, E., Sagalowsky, A. and Hsieh, J.-T. (2006) The application of epigenetic modifiers on the treatment of prostate and bladder cancer. *Urol. Oncol.*, **24**, 152–160.
43. Ushijima, T. (2005) Detection and interpretation of altered methylation patterns in cancer cells. *Nat. Rev. Cancer*, **5**, 223–231.
44. Garcia, J. M., Silvia, J., Pena, C. *et al.* (2004) Promoter methylation of the PTEN gene is a common molecular change in breast cancer. *Genes Chromosomes Cancer*, **41**, 117–124.
45. Lujambio, A., Calin, G. A., Villanueva, A. *et al.* (2008) A microRNA DNA methylation signature for human cancer metastasis. *Proc. Natl Acad. Sci. USA.*, **105**, 13556–13561.
46. Davies, C. D. (2004) DNA methylation, cancer susceptibility, and nutrient interactions. *Exp. Biol. Med.*, **229**, 988–995.
47. Johanning, G. L., Heimburger, D. C. and Piyathilake, C. J. (2002) DNA methylation and diet in cancer. *J. Nutr.*, **132**, 3814S–3818S.
48. Huang, S. (2002) Histone methyltransferases, diet nutrients and tumor suppressors. *Nat. Rev. Cancer*, **2**, 469–472.
49. Prinz-Langenohl, R., Fohr, I. and Pietrzik, K. (2001) Beneficial role for folate in the prevention of colorectal and breast cancer. *Eur. J. Nutr.*, **40**, 98–105.
50. Kim, Y.-I. (2005) Nutritional epigenetics: impact of folate deficiency on DNA methylation and colon cancer susceptibility. *J. Nutr.*, **135**, 2703–2709.
51. Waterland, R. A. (2006) Assessing the effects of high methionine intake on DNA methylation. *J. Nutr.*, **136**, 1706S–1710S.
52. van Engeland, M., Weijenberg, M. P., Roemen, G. M. *et al.* (2003) Effects of dietary folate and alcohol intake on promoter methylation in sporadic colorectal cancer: the Netherlands cohort study on diet and cancer. *Cancer Res.*, **63**, 3133–3137.
53. Vaissiere, T., Hung, R. J., Zaridze, D. *et al.* (2009) Quantitative analysis of DNA methylation profiles in lung cancer identifies aberrant DNA methylation of specific genes and its association with gender and cancer risk factors. *Cancer Res.*, **69**, 243–252.
54. Guz, J., Foksinski, M., Siomek, A., Gackowski, D., Rozalski, R., Dziaman, T., Szpila, A. and Olinski, R. (2008) The relationship between 8-oxo-7,8-dihydro-2'-deoxyguanosine level and extent of cytosine methylation in leukocytes DNA of healthy subjects and in patients with colon adenomas and carcinomas. *Mutat. Res.*, **640**, 170–173.
55. Herceg, Z. (2007) Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. *Mutagenesis*, **22**, 91–103.
56. Singal, R. and Ginder, G. D. (1999) DNA methylation. *Blood*, **93**, 4059–4070.
57. Laird, P. W. (2003) The power and the promise of DNA methylation markers. *Nat. Rev. Cancer*, **3**, 253–266.
58. Balch, C., Montgomery, J. S., Paik, H.-I., Kim, S., Huang, T. H.-M. and Nephew, K. P. (2005) New anti-cancer strategies: epigenetic therapies and biomarkers. *Fron. Biosci.*, **10**, 1887–1931.
59. Zhu, J. and Yao, X. (2009) Use of DNA methylation for cancer detection: promises and challenges. *Int. J. Biochem. Cell Biol.*, **41**, 147–154.
60. Greger, V., Passarge, E., Hopping, W., Messmer, E. and Horsthemke, B. (1989) Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Hum. Gen.*, **83**, 155–158.
61. Jones, A. P. and Baylin, S. B. (2002) The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.*, **3**, 415–428.
62. Baylin, S. B. (2005) DNA methylation and gene silencing in cancer. *Nat. Clin. Pract. Oncol.*, **2**, S4–S11.
63. Herman, J. G., Merlo, A., Mao, L., Lapidus, R. G., Issa, J. P., Davidson, N. E., Sidransky, D. and Baylin, S. B. (1995) Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res.*, **55**, 4525–4530.
64. Herman, J. G. (1996) Hypermethylation-associated inactivation indicates a tumor suppressor role for p15 (INK4B). *Cancer Res.*, **56**, 722–727.
65. Esteller, M., Silva, J. M., Dominguez, G. *et al.* (2000) Promoter hypermethylation is a cause of BRCA1 inactivation in sporadic breast and ovarian tumors. *J. Natl Cancer Inst.*, **92**, 564–569.
66. Herman, J. G., Latif, F., Weng, Y. *et al.* (1994) Silencing of the VHL tumor suppressor gene by DNA methylation in renal carcinoma. *Proc. Natl Acad. Sci. USA.*, **91**, 9700–9704.
67. Herman, J. G., Umar, A., Polyak, K. *et al.* (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc. Natl Acad. Sci. USA*, **95**, 6870–6875.
68. Issa, J. P. (1994) Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nat. Genet.*, **7**, 536–540.
69. Hahnel, R., Harvey, J. and Kay, P. (1996) Hypermethylation of the myogenic gene Myf-3 in human breast carcinomas. *Anticancer Res.*, **16**, 2111–2115.
70. Yoshiura, K., Kanai, Y., Ochiai, A., Shimoyama, Y., Sugimura, T. and Hirohashi, S. (1995) Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc. Natl Acad. Sci. USA*, **92**, 7416–7419.
71. Zion, M., Yehuda, D. B., Avraham, A., Cohen, O., Wetzler, M., Melloul, D. and Neriah, Y. B. (1994) Progressive de novo methylation at the bcr-abl locus in the course of chronic myelogenous leukemia. *Proc. Natl Acad. Sci. USA.*, **91**, 10722–10726.
72. Dimitrijevic, B. (2005) Cancer epigenome: a review. *Arch. Oncol.*, **13**, 108–114.
73. Shah, R., Smith, P., Purdie, C., Quinlan, P., Baker, L., Aman, P., Thompson, A. M. and Crook, T. (2009) The prolyl 3-hydroxylases P3H2 and P3H3 are novel targets for epigenetic silencing in breast cancer. *Br. J. Cancer.*, **100**, 1687–1696.
74. Mayor, R., Casadome, L., Azuara, D., Moreno, V., Clark, S. J., Capella, G. and Peinado, M. A. (2009) Long-range epigenetic silencing at 2q14.2 affects most human colorectal cancers and may have application as a non-invasive biomarker of disease. *Br. J. Cancer.*, **100**, 1534–1539.
75. deVos, T., Tetzner, R., Model, F. *et al.* (2009) Circulating methylated *SEPT9* DNA in plasma is a biomarker for colorectal cancer. *Clin. Chem.*, **55**, 1337–1346.
76. Esteller, M. (2003) Relevance of DNA methylation in the management of cancer. *Lancet Oncol.*, **4**, 351–358.
77. Lyko, F. and Brown, R. (2005) DNA methyltransferase inhibitors and the development of epigenetic cancer therapies. *J. Natl Cancer Inst.*, **97**, 1498–1506.
78. Brueckner, B., Kuck, D. and Lyko, F. (2007) DNA methyltransferase inhibitors for cancer therapy. *Cancer J.*, **13**, 17–22.
79. Sorm, F., Piskala, A., Cihak, A. and Vesely, J. (1964) 5-Azacytidine, a new, highly effective cancerostatic. *Experientia*, **20**, 202–203.
80. Brueckner, B., Boy, R. G., Siedlecki, P., Musch, T., Kliem, H. C., Zielenkiewicz, P., Suhai, S., Wiessler, M. and Lyko, F. (2005) Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. *Cancer Res.*, **65**, 6305–6311.
81. De Abreu, R., Lambooy, L., Stet, E., Vogels-Mentink, T. and van den Heuvel, L. (1995) Thiopurine induced disturbance of DNA methylation in human malignant cells. *Advan. Enzyme Regul.*, **35**, 251–263.
82. Gore, S. D., Jones, C. and Kirkpatric, P. (2006) Decitabine. *Drug Discov. Today.*, **5**, 891–892.
83. Gopisetty, G., Ramachandran, K. and Singal, R. (2006) DNA methylation and apoptosis. *Mol. Immunol.*, **43**, 1729–1740.
84. Steele, N., Finn, P., Brown, R. and Plumb, J. A. (2009) Combined inhibition of DNA methylation and histone acetylation enhances gene re-expression and drug sensitivity *in vivo*. *Br. J. Cancer.*, **100**, 758–763.
85. Yoo, C. B., Cheng, J. C. and Jones, P. A. (2004) Zebularine: a new drug for epigenetic therapy. *Biochem. Soc. Trans.*, **32**, 910–912.
86. Rao, S. P., Rechsteiner, M. P., Berger, C., Sigrist, J. A., Nadal, D. and Bernasconi, M. (2007) Zebularine reactivates silenced *E-cadherin* but unlike 5-Azacytidine does not induce switching from latent to lytic Epstein-Barr virus infection in Burkitt's lymphoma Akata cells. *Mol. Cancer.*, **6**, 3–8.
87. Goffin, J. and Eisenhauer, E. (2002) DNA methyltransferase inhibitors: state of the art. *Annal. Oncol.*, **13**, 1699–1716.
88. Fang, M. Z., Wang, Y., Ai, N., Hou, Z., Sun, Y., Lu, H., Welsh, W. and Yang, C. S. (2003) Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res.*, **63**, 7563–7570.
89. Fang, M., Chen, D. and Yang, C. S. (2007) Dietary polyphenols may affect DNA methylation. *J. Nutr.*, **137**, 223S–228S.
90. Konstantinopoulos, P. A. and Papavassiliou, A. G. (2006) Chromatin-modulating agents as epigenetic anticancer drugs—''the die is cast''. *Drug Discov. Today*, **11**, 91–93.

91. Pogribny, I. P. and Beland, F. A. (2009) DNA hypomethylation in the origin and pathogenesis of human diseases. *Cell. Mol. Life Sci.*, **66**, 2249–2261.
92. Majumder, P., Pradhan, S. K., Devi, P. K., Pal, S. and Dasgupta, D. (2007) Chromatin as a target for the DNA-binding anticancer drugs. In Kundu, T. K. and Dasgupta, D. (eds.), *Chromatin and disease*, Springer-Verlag, Berlin, Germany, pp. 145–189.
93. Pearson, H. (2008) Outcry at scale of inheritance project. *Nature*, **455**, 845.
94. Watson, R. E., McKim, J. M., Cockerell, G. L. and Goodman, J. I. (2004) The value of DNA methylation analysis in basic, initial toxicity assessment. *Toxicol. Sci.*, **79**, 178–188.
95. Sandhu, J., Kaur, B., Armstrong, C., Talbot, C. J., Steward, W. P., Farmer, P. B. and Singh, R. (2009) Determination of 5-methyl-2'-deoxycitidine in genomic DNA using high performance liquid chromatography-ultraviolet detection. *J. Chromatogr. B*, **877**, 1957–1961.
96. Gu, H., Bock, C., Mikkelsen, T. S., Jäger, N., Smith, Z. D., Tomazou, E., Gnirke, A., Lander, E. S. and Meissner, A. (2010) Genome-scale DNA methylation mapping of clinical samples at single-nucleotide resolution. *Nat. Methods*, **7**, 133–136.
97. Schones, D. E. and Zhao, K. (2008) Genome-wide approaches to studying chromatin modifications. *Natl Rev. Genet.*, **9**, 179–191.
98. Ammerpohl, O., Martín-Subero, J. I., Richter, J., Vater, I. and Siebert, R. (2009) Hunting fir the 5th base: techniques for analyzing DNA methylation. *Biochim. Biophys. Acta*, **1790**, 847–862.
99. Hahn, M. A. and Pfeifer, G. P. (2010) Methods for genome-wide analysis of DNA methylation in intestinal tumors. *Mutat. Res.*, **693**, 77–83.
100. Laird, P. W. (2010) Principle and challenges of genome-wide DNA methylation analysis. *Nat. Rev. Genet.*, **11**, 191–203.
101. Bock, C., Tomazou, E. M., Brinkman, A. B. *et al.* (2010) Quantitative comparison of genome-wide DNA methylation mapping technologies. *Nat. Biotechnol*, **28**, 1106–1114.
102. Hayatsu, H. (2008) The bisulfite genomic sequencing used in the analysis of epigenetic states, a technique in the emerging environmental genotoxicology research. *Mutat. Res.*, **659**, 77–82.
103. Song, C.-X., Szulwach, K. E., Fu, Y. *et al.* (2010) Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. *Nat. Biotechnol*, **29**, 68–72.
104. Bartoszek, A., Sielenko, A. and Wesiora, M. (2003) A versatile method employing basic techniques of genetic engineering to study the ability of low molecular weight compounds to bind covalently with DNA in cell-free system. *Anal. Biochem.*, **313**, 53–59.
105. Sadikovic, B., Andrews, J. and Rodenhiser, D. I. (2007) DNA methylation analysis using CpG microarrays is impaired in benzopyrene exposed cells. *Toxicol. Appl. Pharamacol.*, **225**, 300–309.
106. Okamoto, A. (2009) Chemical approach toward efficient DNA methylation analysis. *Org. Biomol. Chem.*, **7**, 21–26.
107. Hodges-Garcia, Y. and Hagerman, P. J. (1992) Cytosine methylation can induce local distortions in the structure of duplex DNA. *Biochemistry*, **31**, 7595–7599.
108. Wang, G., Carbajal, S., Vijg, J., DiGiovanni, J. and Vasquez, K. M. (2008) DNA structure-induced genomic instability in vivo. *J. Natl Cancer Inst.*, **100**, 1815–1817.
109. Sen, D. and Crothers, D. M. (1986) Influence of DNA-binding drugs on chromatin condensation. *Biochemistry*, **25**, 1503–1509.
110. Swenberg, J. A., Richardson, F. C., Boucheron, J. A. and Dyroff, M. C. (1985) Relationship between DNA adduct formation and carcinogenesis. *Environ. Health Perspect.*, **62**, 17–24.
111. Beland, F. A. and Poireier, M. C. (1994) DNA adducts and their consequences. In Tardiff, R. G., Lohman, P. H. M., Wogan, G. N. (eds.), *Methods to assess DNA damage and repair: interspecies comparison*. pp. 24–54.
112. Millard, J. T. (1996) DNA modifying agents as a tools for studying chromatin structure. *Biochimie*, **78**, 803–816.
113. Hu, W., Feng, Z. and Tang, M. S. (2003) Preferential carcinogen-DNA adduct formation at codons 12 and 14 in the human K-ras gene and their possible mechanisms. *Biochemistry*, **42**, 10012–10023.
114. Yoon, J.-H., Smith, L. E., Feng, Z., Tang, M. S., Lee, C.-S. and Pfeifer, G. P. (2001) Methylated CpG dinucleotides are the preferential targets for G-to-T transversion mutations induced by benzo[*a*]pyrene diol epoxide in mammalian cells: similarities with the p53 mutation spectrum in smoking-related lung cancer. *Cancer Res.*, **61**, 7110–7117.
115. Pavanello, S., Bollati, V., Pesatori, A. C., Kapka, L., Bolognesi, C., Bertazzi, P. A. and Baccarelli, A. (2009) Global and gene-specific promoter methylation changes are related to*anti*-B[*a*]PDE-DNA adduct levels and influence micronuclei levels in polycyclic aromatic hydrocarbon-exposed individuals. *Int. J. Cancer.*, **125**, 1692–1697.
116. Bagnyukova, T. V., Tryndyak, V. P., Montgomery, B., Churchwell, M. I., Karpf, A. R., James, A. R., Muskhelishvili, L., Beland, F. A. and Pogribny, I. P. (2008) Genetic and epigenetic changes in rat preneoplastic liver tissue induced by 2-acetylaminofluorene. *Carcinogenesis*, **29**, 638–646.
117. Liu, W.-b., Liu, J.-y., Ao, L., Zhou, Z.-y., Zhou, Z.-h., Cui, Z.-h., Yang, H. and Cao, J. (2009) Dynamic changes in DNA methylation during multistep rat lung carcinogenesis induced by 3-methylchloranthrene and diethylnitrosamine. *Toxicol. Lett.*, **189**, 5–13.
118. Sadikovic, B. and Rodenhiser, D. (2006) Benzo[*a*]pyren exposure disrupts DNA methylation and growth dynamics in breast cancer. *Toxicol. Appl. Pharamacol.*, **216**, 456–468.
119. Zou, Y., Shell, S. M., Utzat, D. C., Luo, C., Yang, Z., Geacintov, N. E. and Basu, A. K. (2003) Effects of DNA adduct structure and sequence context on strand opening of repair intermediates and incision by UvrABC nuclease. *Biochemistry*, **42**, 12654–12661.
120. Jiricny, J. (2002) DNA repair: bioinformatics helps reverse methylation damage. *Current Biol.*, **12**, R846–R848.
121. Pfohl-Leszkowicz, A., Grosse, Y., Carriere, V., Cugnenc, P.-H., Berger, A., Carnot, F., Huc, A., Beaune, P. and de Waziers, I. (1995) Impaired DNA methylation and DNA adduct concentrations in human peritumoral and tumoral colorectal samples and normal colon. *Clin. Chem.*, **41**, 1924–1926.
122. Johnson, W. S., He, Q.-Y. and Tomasz, M. (1995) Selective recognition of the m5CpG dinucleotide sequence in DNA by mitomycin C for alkylation and cross-linking. *Bioorg. Med. Chem.*, **3**, 851–860.
123. Cutts, S. M., Swift, L. P., Raphaeli, A., Nudelman, A. and Phillips, D. R. (2003) Sequence specifity of adriamycin-DNA adducts in human tumor cells. *Mol. Cancer Ther.*, **2**, 661–670.
124. Parker, B. S., Cutts, S. M. and Phillips, D. R. (2001) Cytosine methylation enhances mitoxantrone-DNA adduct formation at CpG nucleotides. *J. Biol. Chem.*, **276**, 15953–15960.
125. Parker, B. S., Buley, T., Evison, B. J., Cutts, S. M., Neumann, G. M., Iskander, M. N. and Phillips, D. R. (2004) A molecular understanding of mitoxantrone-DNA adduct formation. *J. Biol. Chem*, **279**, 18814–18823.
126. Galea, A. M. and Murray, V. (2002) The interaction of cisplatin and analogues with DNA in reconstituted chromatin. *Biochim. Biophys. Acta*, **1579**, 142–152.
127. Fichtinger-Schepman, A. M., van der Veer, J. L., den Hartog, J. H., Lohman, P. H. and Reedijk, J. (1985) Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation. *Biochemistry*, **29**, 707–713.
128. Davies, N. P., Hardman, L. C. and Murray, V. (2000) The effect of chromatin structure on cisplatin damage in intact human cells. *Nucleic Acids Res.*, **28**, 2954–2958.
129. Bartoszek, A., Dackiewicz, P. and Konopa, J. (1997) ^{32}P-postlabelling analysis of nucleobases involved in the formation DNA adducts by antitumor 1-nitroacridines. *Chem. Biol. Interact.*, **103**, 131–139.
130. Woynarowski, J. M., McNamee, H., Szmigiero, L., Beerman, T. A. and Konopa, J. (1989) Induction of DNA-protein crosslinks by antitumor 1-nitro-9-aminoacridines in L1210 leukemia cells. *Biochem. Pharmacol*, **38**, 4095–4101.
131. Gniazdowski, M., Ciesielska, E. and Szmigiero, L. (1981) Some properties of the irreversible complexes of nitracrine (Ledakrin, C-283) with polynucleotides. *Chem. Biol. Interact*, **355**, 355–366.
132. Barton, T. S., Robaire, B. and Hales, F. H. (2005) Epigenetic programming in the preimplantation rat embryo is disrupted by chronic parental cyclophosphamide exposure. *Proc. Natl Acad. Sci. USA*, **102**, 7865–7870.
133. Phillips, D. H. (2001) Understanding the genotoxicity of tamoxifen? *Carcinogenesis*, **22**, 839–849.
134. Tryndyak, V. P., Muskhelishvili, L., Kovalchuk, O., Rodriguez-Juarez, R., Montgomery, B., Churchwell, M. I., Ross, S. A., Beland, F. A. and Pogribny, I. P. (2006) Effect of long-term tamoxifen exposure on genotoxic and epigenetic changes in rat liver: implications for tamoxifen-induced hepatocarcinogenesis. *Carcinogenesis*, **27**, 1713–1720.
135. Tryndyak, V. P., Kovalchuk, O., Muskhelishvili, L., Montgomery, B., Rodriguez-Juarez, R., Melnyk, S., Ross, S. A., Beland, F. A. and Pogribny, I. P. (2007) Epigenetic reprogramming of liver cells in tamoxifen-induced rat hepatocarcinogenesis. *Mol. Carcinogenesis*, **46**, 187–197.
136. Yauk, C., Polyzos, A., Rowan-Caroll, A. *et al.* (2008) Germ-line mutations, DNA damage, and global hypermethylation in mice exposed to particulate air pollution in an urban/industrial location. *Proc. Natl Acad. Sci. USA*, **105**, 605–610.
137. Jaffe, L. A. (2003) Epigenetic theories of cancer initiation. *Adv. Cancer Res.*, **30**, 209–230.