International Journal of Life science & Pharma Research

EPIGENETIC CHANGES AND THEIR REVERSAL IN CANCER

ABHIMANYU KUMAR JHA[1*], MEENAKSHI JHA[2], VINAY DWIVEDI[1], RASHMI CHANDRA[1], VIVEK KUMAR[1] AND JAGDEEP KAUR[2]

1-Department of Biotechnology, IMS Engineering College, Ghaziabad (Uttar Pradesh), INDIA
2-Department of Biotechnology, Panjab University, Chandigarh, INDIA

ABSTRACT

Cancer is caused by genetic as well as epigenetic changes. Chemotherapy is considered the mainstay of cancer therapy. But multiple side effects of chemotherapy have created a demand for developing other novel and specific targets for cancer therapy. The potential reversibility of epigenetic changes has resulted in the reactivation of epigenetically silenced tumor suppressor genes being an emerging strategy for the treatment of cancer. Epigenetic modifiers like DNA methyltransferase (DNMT) inhibitors and histone deacteylase (HDAC) inhibitors induce the re-expression of epigenetically silenced genes *in vitro* and *in vivo*. Moreover, they demonstrate safety and efficacy against neoplastic diseases in clinical trials. DNMT inhibitors like 5-azacytidine and 5-aza-2'-deoxycytidine have recently been approved by FDA for the treatment of myelodysplastic syndrome. Still the mechanism of action behind their clinical efficacy remains unclear. In this review, the different epigenetic changes taking place during tumor progression and their reversal has been discussed.

Key words: chemotherapy, DNMT, HDAC, epigenetic modifiers,

INTRODUCTION

The genetic basis of cancer as a disease is well established. However, the involvement of factors other than changes in nuclear DNA sequence in cancer development and progression gained much attention in the last thirty years. Epigenetics refers to heritable reversible changes in gene expression that occur without any changes in the DNA sequence. Epigenetic modifications affect the nuclear DNA and/or the nucleosomes-incorporated histones and consequently modify gene expression. Ongoing clinical trials intend to identify tumor suppressor genes that upon re-expression can induce remission and cure in patients. The DNA methyltransferase (DNMT) inhibitors azacitidine and decitabine are among the new agents approved by the FDA for the treatment of myelodysplastic syndrome (MDS). In this review, various epigenetic changes, like histone modifications the development of different DNMT inhibitors as epigenetic modifiers for the treatment of cancer are discussed.

HISTONE MODIFICATIONS

The posttranslational modifications of the amino terminal tail of histones like methylation, acetylation, phosphorylation, ubiquitination and SUMOylation can directly affect the packing of nucleosomes and chromatin architecture (Shilatifard A, 2008; Jones PA and Baylin SB, 2002; Weake VM and Workman JL, 2008). In accordance with this, the histone code hypothesis (Paro R, 2000; Turner BM, 2000) proposes a combinatorial code of histone modifications that complement the information stored in the DNA sequence and mediate downstream events. Despite the diversity of

histone modifications, histone acetylation is the most recognized as a drug target for modulation of gene expression. The process of histone acetylation is carried out by; histone acetyltransferase (HAT) and histone deacetylase (HDAC). HAT is associated with a transcriptionally active state chromatin (euchromatin), while HDAC is associated with a transcriptionally inactive state chromatin (heterochromatin).

Consequently, inhibition of HDAC enzymes to activate the transcription of silenced tumor suppressor genes (TSG) in cancer is a rational approach. However, global genomic approaches demonstrate that HDAC inhibitors induce and repress a small (~2%) set of genes (Van Lint C. et al., 1996); emphasizing the coordinated role of other histone modifications and/or DNA cytosine methylation in remodeling the chromatin architecture. Additionally, HDAC inhibitors induce hyperacetylation of non-histone proteins like NF-kB (Dai Y et al., 2005), p53 (Zhao Y et al., 2006) and Hsp90 (Kovacs J J et al., 2005). Hyperacetylation of both histones and non-histone proteins by HDAC inhibitors indicate that these compounds are in fact "lysine deacetylase"inhibitors and not just HDAC inhibitors. Interestingly, hyperacetylation of non-histone proteins like Hsp90 by HDAC inhibitors induces DNMT1 protein downregulation by promoting the ubiquitin-dependent proteasomal degradation of DNMT1 (Zhou Q et al., 2008) highlighting an indirect effect of HDAC inhibitors on the DNA methylation machinery and adding another layer of complexity to the interactive effects of combined HDAC inhibitors and DNMT inhibitors therapy. The contribution of the acetylation of histones and non-histone proteins to the clinical efficacy of these compounds is not clear (Bolden JE et al, 2006).

Paradoxically, HAT inhibitors are also known as anticancer agents because aberrant lysine acetylation mediates oncogenesis (Yang X, 2004). The natural product anacardic acid has been used as a lead compound to develop HAT inhibitors (Eliseeva, ED et al., 2007). HAT inhibitors demonstrate selective antitumor effect in cancer cell lines and prevent cardiac failure in rodent models. In contrast to HDAC inhibitors, none of these compounds has advanced to clinical trials as anticancer agents.

DNA METHYLATION

Inactivation of tumor suppressor genes is central to the development of all common forms of human cancer. This inactivation often results from epigenetic silencing associated with hypermethylation rather than intragenic mutations (Vogelstein B. & Kinzler KW,1998).

DNA methylation in mammalian cells occurs at the 5-position of cytosine within the CpG dinucleotide. CpG islands are the sites present in the promoter of most of the tumor suppressor genes, and hypermethylation at CpG island leads to silencing of the expression of these genes. The CpG islands have the following important characteristics: (i) G+C content of 0.50 or greater (ii) observed to expected CpG dinucleotide ratio of 0.60 or greater and (iii) both occurring within a sequence window of 200 bp or greater.

CpG dinucleotides methylation in mammals represent the target for the covalent modification of DNA (Bao Y *et al.,* 2007). Although CpG islands account for only about 1% of the genome and for 15% of the total genomic CpG sites, these regions contain over 50% of the unmethylated CpG dinucleotides. There are about 45,000 CpG islands, most of which reside within or near the promoters or first exons of genes and are unmethylated in normal cells, with the exception of CpG islands on the inactive X chromosome in females. Cellular DNA methylation patterns is established by a complex interplay of at least three independent DNA methyltransferases: DNMT1, DNMT3A and DNMT3B. The first methyltransferase to be discovered was DNMT1. Pioneering work has established that DNMT1 has a 10–40-fold preference for hemimethylated DNA (Pradhan S et al., 1999; Pradhan S et al., 1997). By providing both enhanced transcriptional control and protection against mutation, the methyl-CpG binding proteins could have facilitated the expansion of the methylated DNA compartment within the evolving vertebrate genome. MBD2 and MBD3 are the only vertebrate methyl-CpG binding proteins and in mammals, MBD2 and MBD3 genes have an identical genomic structure, differing only in the

sizes of their introns, and they encode proteins that are 70% identical (Baylin S et al., 2002).

CpG islands are associated with at least half of all cellular genes and are normally methylation-free. Dense methylation of cytosine residues within islands results in strong and heritable transcriptional silencing. Such silencing normally occurs almost solely at genes subject to genomic imprinting or to X chromosome inactivation. Aberrant methylation of CpG islands associated with tumor suppressor genes has been proposed to contribute to carcinogenesis (Antequera F and Bird A*,* 1993). In addition to carcinogensis and genomic imprinting, DNA methylation has also been found to regulate memory formation and synaptic plasticity in the adult rat hippocampus (Miller CA et al*.,* 2008). The understanding of chromatin with respect to the components that specify for states of gene expression is growing rapidly, and this knowledge is establishing a base from which abnormal as well as normal gene expression events can be understood. In this regard, an especially active field in cancer research is concerned with patterns of aberrant gene promoter hypermethylation that have been associated with loss of transcription of a growing list of genes in virtually every type of human cancer (Greenblatt MS et al*.,* 1994).

Several mechanisms have been proposed to account for transcriptional repression by DNA methylation. The first mechanism involves direct interference with the binding of specific transcription factors to their recognition sites in their respective promoters. Several transcription factors, including AP-2, c-Myc/Myn, the cyclic AMP-dependent activator CREB, E2F and NFkB, recognize sequences that contain CpG residues, and binding of each has been shown to be inhibited by methylation (Baylin S et al., 1998). The second mode of repression is the direct binding of specific transcriptional repressors to methylated DNA. Hypomethylation is the second kind of methylation defect that is observed in a wide variety of malignancies (Jones PA et al*.,* 1999). It is common in solid tumors such as metastatic hepatocellular cancer, cervical cancer, prostate tumors, and also in hematologic malignancies such as B-cell chronic lymphocytic leukemia. The global hypomethylation seen in a number of cancers, such as breast, cervical, and brain, show a progressive increase with the grade of malignancy (Kim Y et al*.,* 1994). A mutation of *DNMT3b* has been found in patients with immunodeficiency, centromeric instability, and facial abnormalities, which causes the instability of the chromatin (Okano M et. al., 1999). Hypomethylation has been hypothesized to contribute to oncogenesis by activation of oncogenes such as *cMYC* and *H-RAS* or by activation of latent retrotransposons (Alves G et al*.,* 1996) or by chromosome instability (Tuck-Muller CM *et al.,* 2000). More attention in the methylation field has focussed on CpG islands, primarily because of the propensity of such sequences to become aberrantly hypermethylated in tumours, resulting in the transcriptional silencing of the associated gene (Kochanek S et al*.,* 1995; Jones PL et al., 1998).

Two important additional links between DNA methylation and chromatin structure have also been observed. First, DNMT1 forms a complex with Rb, E2F1, and HDAC1 and represses transcription from E2F responsive promoters (Robertson KD et al*.,* 2000). The second link between chromatin structure and methylation comes from patients with mutations in a putative ATP-dependent chromatin-remodelling factor of the SNF2 family, termed *ATR*-X (Gibbons RJ et al*.,* 2000).

Interplay between DNA methylation and histone modifications

It is important to note that there is a direct link between DNA methylation and histone modifications. A number of proteins involved in DNA methylation (e.g. DNMTs and MBDs) directly interact with histone modifying enzymes such as histone methyltransferases (HMTs) and histone deacetylases (HDACs). The growing evidence for dynamic inter/intra-regulation of these modifications, position and modification-specific protein interactions, and biochemical/biophysical interaction between modifications has strengthened the 'histone code' hypothesis, in which histone modifications are integral to regulating the expression of the genome (Strahl BD et al*.,* 2000).

There are now several examples of modification patterns and sequences that relate to gene activation, some of which occur on the same histone tail or on the same amino acid. In fact, it is now believed that DNA methylation and histone methylation are tied

together in a loop where one modification is dependent on the other. Altering this relationship will almost certainly have severe consequences on the epigenome and chromatin organization. Thus most, if not all, factors that affect DNA methylation levels also affect histone modifications. For instance, it appears that H3K9 methylation and DNA methylation are linked (Fuks F, 2005). In mammals, DNA methyltransferases interact with Suv39h H3K9 methyltransferases and loss of H3K9 methylation inSuv39h-knockout embryonic stem cells decreases Dnmt3b-dependent CpG methylation at major centromeric satellites (Lehnertz B et al., 2003).

Methyl-CpG-binding proteins may recruit histone deacetylase complexes to deacetylate histone tails so that the tails become suitable for serving as substrates for methylation. In contrast with this sequential process, MBD-containing HMTs may bind directly to methylated DNA to methylate histone tails. Alternatively, it is also possible that chromodomain-containing proteins bind to methylated histone tails and recruit DNA methyltransferase (DNMT) to methylate adjacent CpG sequences. Irrespective of the sequence of events, it is likely that a concerted action of HMT and HDAC complexes may play an important role in methylated DNA silencing (Zhang S et al., 2001).

In contrast to the above predictons it has been also observed that transcription of mouse DNA methyltransferase 1 (DNMT 1) is regulated by both E2F-Rb-HDAC dependent and -independent pathways. It has been identified that the promoter region and major transcription start sites of mouse Dnmt1 and found two important cis-elements within the core promoter region. One is an E2F binding site, and the other is a binding site for an as yet unidentifed factor

As DNA methylation is found to be linked to histone deacetylation in the same manner, methylation of histone H4 by arginine methyltransferase PRMT1 is essential in vivo for many subsequent histone modifications knocking out of PRMT1 gene leads to a domain-wide loss of histone acetylation on both histones H3 and H4, as well as an increase in H3 Lys9 and Lys27 methylation, both marks associated with inactive chromatin (Huang Y et al., 2005).

EPIGENETIC THERAPY: AN EMERGING AREA OF PHARMACOLOGY

Epigenetic therapy, the use of drugs to correct epigenetic defects, is a new and rapidly developing area of pharmacology. Because so many diseases, such as cancer, involve epigenetic changes, it seems reasonable to try to counteract these modifications with epigenetic treatments. These changes seem an ideal target because they are by nature reversible, unlike DNA sequence mutations. The most popular of these treatments aim to alter either DNA methylation or histone acetylation. The emerging use of drugs that modulate epigenetic alterations, including the hypomethylating agents and histone deacetylase inhibitors, is an exciting advance for cancer treatment. These agents have shown great promise in the treatment of several hematologic malignancies, especially myelodysplastic syndromes, acute myeloid leukemia, and cutaneous T-cell lymphoma. The potential reversibility of epigenetic states offers an exciting opportunity for novel cancer drugs that can reactivate epigenetically silenced tumor-suppressor genes (Esteller *et al.,* 2005; Jha *et al.,* 2010).

DNA methylation inhibitors:

Reactivation of hypermethylated genes has been carried out *in vitro*; however, translating this capability in humans has proved difficult. One obstacle is that the drugs used to demethylate DNA cannot be used to target particular genes as they are non-specific (Villar-Garea A and Esteller M, 2003). Demethylating agents such as 5-aza-cytidine or 5-aza-2 -deoxycytidine inhibit DNA methyltransferases and cause global hypomethylation (Christman JK, 2002). Furthermore, the 5-aza-2-deoxycytidine affects all human cancer cell lines universally (Paz MF et al, 2003). New inhibitors of DNA methylation are available, eg, zebularine, procainamide, but the issue of non-specificity is still there. DNMT inhibitors can be categorized into three types, which are being discussed separately.

Nucleoside analogue DNMT inhibitors

These drugs are analogues of cytosine, the nucleotide base that is methylated by DNMTs. The examples of these drugs are 5-azacytidine (5-aza-

CR) and decitabine (5-aza- 2' - deoxycytidine or 5-aza-CdR) which were initially developed as cytotoxic drugs(Egger G et al., 2004). Both these drugs are phosphorylated to the deoxynucleotide triphosphate and then incorporated instead of cytosine into replicating DNA. Hence, these are S-phase specific drugs, acting as potent inhibitors of DNMTs. Disadvantage of 5-aza -CR and decitabine is that these must be administered parenterally and are myelotoxic, resulting in cytopenia (Herman JG and Baylin SB, 2003). The myelotoxicity of these drugs is thought to be due to their incorporation into DNA and not related to their DNA hypomethylation effects. Zebularine is a newer cytosine analogue which is less toxic and can be administered orally. However, it has the disadvantage of being much less potent than 5-azacytidine and decitabine and needs to be administered in high doses. Another problem is that at high doses, these agents seem to have toxic effects on normal cells. But despite their drawbacks, these compounds and their derivatives have achieved some therapeutic success in the clinic, especially in haemopoietic disorders such as myelodysplastic syndrome and acute myeloid leukaemia (Wijermans PW et al., 1997) Lower doses of 5-aza-cytidine, which are associated with inhibitors of histone deacetylases— eg, trichostatin, depsipeptide, suberoylanilide hydroxamic acid, sodium butyrate—may also reactivate tumor suppressor genes.

Non-nucleoside analogue DNMT inhibitors

The myelotoxic effects of the nucleoside analogue inhibitors has encouraged the search for inhibitors of DNA methylation that are not incorporated into DNA because of structural differences from cytosine. These non-nucleoside analogue inhibitors are undergoing preclinical trials. Some of these drugs such as procainamide and procaine have the potential advantage as these have already been extensively used in clinical practice.

Due to the cytotoxic effects to normal cells of these demethylating compounds, the focus now has shifted to the discovery of orally administered and non-toxic natural and/or dietary compounds. Certain dietary polyphenols, such as (–)-epigallocatechin 3-gallate (EGCG) from green tea and genistein from soybean, have recently been demonstrated to inhibit DNA methyltransferases (DNMT) *in vitro*. This inhibitory activity is associated with the demethylation of the CpG islands in the promoters and the reactivation of methylation-silenced genes such as $p16^{INK4a}$, retinoic acid receptor *β*, O^6-methylguanine methyltransferase, human mutL homolog 1, and glutathione *S*-transferase-π. These activities have been observed in human esophageal, colon, prostate, and mammary cancer cell lines, and the activity can be enhanced by the presence of histone deacetylase inhibitors or by a longer-term treatment (Fang M et al, 2007**)**. Curcumin and one of its major metabolites, tetrahydrocurcumin can inhibit M. SssI, an DNMT1 analog, activity (Liu Z et al, 2009). Several phytochemicals have been found to inhibit the DNA methyltransferase activity with betanin being the weakest while rosmarinic and ellagic acids were found to be the most potent modulators (Paluszczak J et al., 2010). Curcumin and genistein cause reversal of hypermethylation and reactivation of RARβ2 gene in SiHa cell line (a squamous cervical cancer cell line) (Jha AK et al, 2010**)**.

Tea polyphenols are strong antioxidants and tea preparations demonstrate inhibitory activity against carcinogenesis (Lu, G et al, 2008). (-)-Epigallocatechin-3-gallate (EGCG), the major polyphenol obtained from green tea, is a potent inhibitor of catechol-O-methyltransferase activity (COMT) (Chen D et al, 2005). The structural similarity between DNMTs and COMT suggests possible inhibition of DNMTs by EGCG. EGCG inhibits DNMTs activity in KYSE 510 esophageal cells in a dose-dependent manner and induces re-expression of hypermethylated genes like CDKN2A, RARβ2 and MGMT. EGCG demethylating activity does not involve competitive binding to CG rich regions of DNA; instead, it is mediated by direct binding to DNMT1 through hydrogen bonding (Fang M Z et al., 2003; Lee W J et al., 2005). The binding of EGCG to other DNMTs has not been tested but is likely to occur because DNMTs share a highly conserved catalytic pocket. Genistein, a polyphenolic compound, demethylates DNA and increases histones acetylation at the transcription start sites of CDKN2A and p21WAF1 with consequent induction of gene expression (Majid S et al., 2008). Additionally, genistein enhances gene re-

expression when combined with trichostatin A or DAC (Fang MZ et al., 2005). A major concern associated with the use of natural products is product standardization. Multiple sources can provide extracts with different activities and therefore create discrepancies in their reported demethylating activity.

Antisense oligonucleotides

Antisense oligonucleotides are short, defined sequences of nucleotides that are complementary to mRNAs and hybridize with them and make them inactive, thereby blocking translation. Antisense oligonucleotides that are complementary to mRNA for human DNMT1 are undergoing preclinical (Yan L et al., 2003) as well as clinical (Davis AJ et al., 2003) trials.

STRATEGIES FOR COMBINING EPIGENETIC MODIFIERS

Different strategies of epigenetic therapy can be formulated based on the pleiotropic effects of these agents. The successful outcome of using epigenetic modifiers as single agents in the treatment of cancer encourages their use in sequential or simultaneous combinations to harness their additive or synergistic effect on gene re-expression and cytotoxicity.Tumor resistance to chemotherapy is a major obstacle in cancer therapy. Epigenetic silencing of TSG disrupts the apoptotic machinery in cancer cells with consequent resistance development to chemotherapy. Pretreatment with DNMT or HDAC inhibitors can restore the expression of TSG and sensitizes tumor cells to chemotherapeutic agents.

Pretreatment of solid tumors in vitro with DAC sensitizes tumor cells to cisplatin treatment (Frost, P et al., 1990). Also concomitant treatment with 5AC and doxorubicin induces synergistic cytotoxicity in multiple myeloma cells (Kiziltepe T et al., 2007) cytotoxicity. Surprisingly, cytarabine inhibits DAC induced global hypomethylation in leukemia cells, probably due to the selective killing of hypomethylated cells by cytarabine (Qin T et al., 2007). Conversely, sequential treatment with chemotherapy to debulk the tumor followed by DNMT or HDAC inhibitors to restore the differentiation program of chemotherapy resistant tumor-initiating cells is also conceivable. However, this approach is still not validated.

The combination of DNMT inhibitors and HDAC inhibitors is currently under investigation in several clinical trials. Sequential administration of DAC followed by the HDAC inhibitor TSA induces optimal re-expression of densely promoter-methylated genes, which cannot be re-expressed by TSA alone (Cameron, E. E. et al, 1999). This observation suggests a hierarchical organization of the different epigenetic modifications and incites the sequential use of DNMT inhibitors followed by HDAC inhibitors but not the reverse sequence. Interestingly, the sequential administration of DAC followed by different HDAC inhibitors in leukemia cells induces synergistic reexpression of p21, which lacks promoter CpG methylation. Apoptotic synergy and DNA damage induction are also observed by the same sequential treatment with consequent p21 upregulation in a p53-dependent fashion. This effect highlights the importance of DNA damage as an off-target effect of epigenetic modifiers in regulating gene expression.

CONCLUSIONS

Several epigenetic modifiers are currently undergoing clinical trials. A few of them are already FDA approved. The mechanism behind the clinical efficacy of 5AC and DAC in hematological malignancies is still not known completely. Although epigenetic reversal of DNA methylation is postulated as the mechanism of action, recent reports do not show an association of clinical response with methylation reversal of CDKN2B (p15) and other TSG. The diverse range of biological activities of these compounds may suggest the involvement of mechanisms other than methylation reversal like cytotoxicity, activation of immune response or induction of cellular

senescence in the neoplastic clone in mediating their clinical activity. Development of small molecule inhibitors that specifically inhibit different DNMT isotypes is a possible strategy; however, the in vivo antitumor activity of these compounds needs to be demonstrated. Testing lead compounds like RG108 in the recently developed mouse models and

other leukemia mouse models can help in showing their efficacy as anticancer agents and this can result in initiating the design of new molecular entities that specifically inhibit DNMTs based on structure activity relationship.

ACKNOWLEDGEMENTS

The authors kindly acknowledge the financial assistance provided by CSIR (Council of Scientific and Industrial Research), INDIA to AKJ.

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