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Review

DNA Methyltransferase Inhibitors and Their Emerging Role in Epigenetic Therapy of Cancer

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Abstract. *The DNA methyltransferase (DNMT) inhibitors azacytidine and decitabine are the most successful epigenetic drugs to date and are still the most widely used as epigenetic modulators, even though their application for oncological diseases is restricted by their relative toxicity and poor chemical stability. Zebularine (1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one), a more stable and less toxic cytidine analog, is another inhibitor of DNMT with concomitant inhibitory activity towards cytidine deaminase. Unfortunately, there is no new information related to the possible clinical applications of zebularine. Although many new inhibitors of DNMT have been identified, none of them can so far replace azacytidine, decitabine and, to a lesser degree, zebularine. This review summarizes the current data and knowledge about azacytidine, decitabine and zebularine, and their role in present and possible future epigenetic cancer therapy. We also discuss the molecular modes of action of these agents with consideration of their different toxicities and demethylation profiles, reflecting their complex and partially overlapping biological effects.*

The term 'epigenetic' was coined by C. H. Waddington in 1942 and from that time, the definition of 'epigenetic' has evolved (1). Finally, in 2008 a consensus definition of 'epigenetics' was established as "stably-heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence", which may lead to disease *e.g.* Beckwith-Wiedemann and Prader Willi/Angelman syndromes, as well as cellular aging and cancer (2). Although for many years scientists have been reporting that epigenetic changes may influence cancer development, the methodology to prove this had some limitations. Nowadays, it is known that epigenetic alterations are as equally responsible for carcinogenesis as are genetic mutations. In cancer, gene silencing through methylation occurs at least as frequently as mutations or deletions, and leads to aberrant silencing of normal tumor-suppressor function (3). It has also been proven that epigenetic changes can be detected in carcinogenesis earlier than the well-known genetic origins of cancer (4).

Epigenetic modifications rely on re-building of chromatin structure resulting in an open or closed configuration and thereby expressing or repressing genes which control such basic cellular processes such as differentiation, proliferation and apoptosis, and as a consequence, cell functions. Two covalent modifications are responsible for these processes: DNA methylation and nucleosomal histone tail acetylation, which influence the epigenetic regulation of the gene expression pattern.

DNA methylation is the most characterized epigenetic phenomenon described as a stable epigenetic marker (5). This process involves enzymes belonging to the DNA methyltransferase family (DNMTs). In humans, DNMTs bind a methyl group ($-CH_3$) at the carbon 5-position of the pyrimidine ring of cytosine in CpG dinucleotides (Figure 1A). In somatic cells, most CpG dinucleotides are methylated, except those located in CpG islands (6-8). These islands are mainly located near or in the promoter regions (nearly 60% of mammalian gene promoters) in repeated sequences as long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) (9), as well as in CpG island shores, where the methylation status depends on the tissue origin (10).

The regulation of histone acetylation is controlled by two enzyme families: histone acetyltransferases (HATs) and histone deacetylases (HDACs). The latter promotes a higher-order chromatin structure, which is equated with transcriptional gene repression.

Histone proteins are not the only target of HATs and HDACs. It is important to note that HDACs might also

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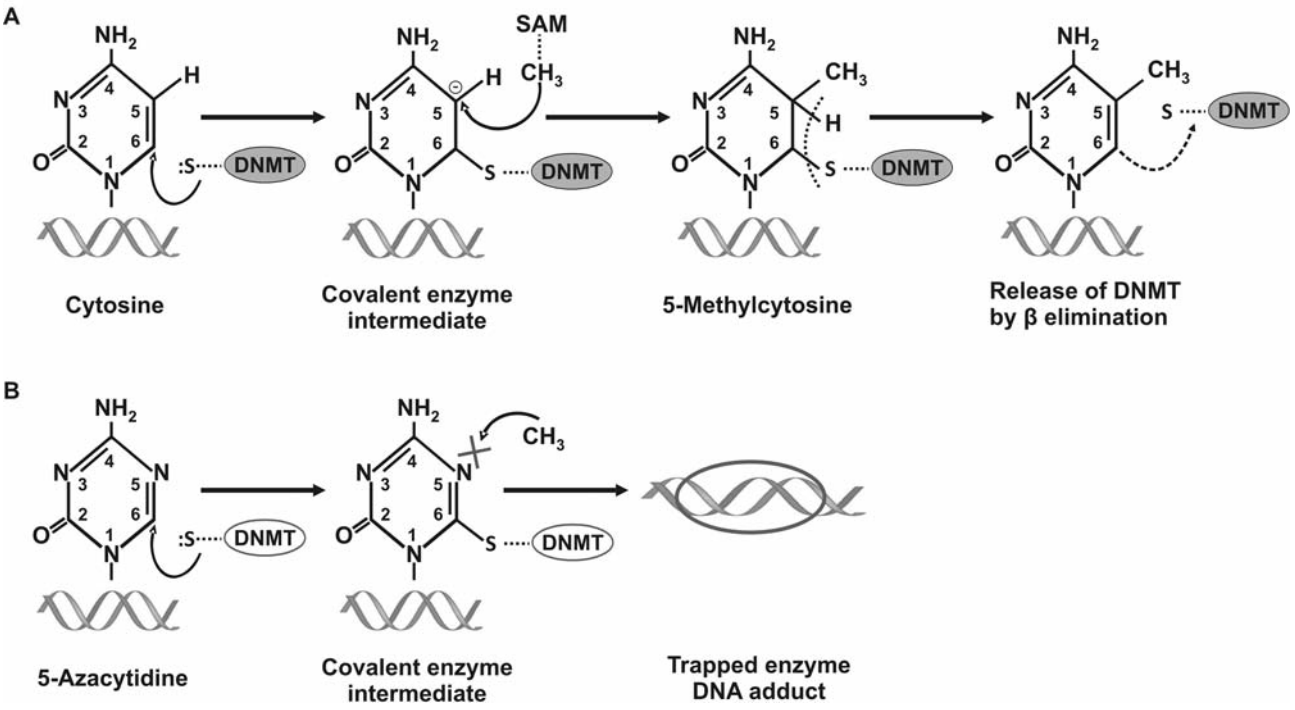

Figure 1. *Scheme of DNA methylation (A) and DNA methyltansferase inhibition (B). A: DNA methylation at the 5-position of the pyrimidine ring of cytosine is catalyzed by DNA methyltransferase. The methyl group (-CH_3) is transferred from the cofactor S-adenosyl-L methionine (SAM), resulting in creation of 5-methylcytosine, then the enzyme is released by β elimination. B: Trapping reaction relies on prevention of β elimination due to the presence of the nitrogen atom at the 5-position of azacytidine, resulting in a covalent irreversible complex (6).*

directly modulate acetylation of the non-histone proteins such as p53, signal transducer and activator of transcription (STAT), transcription factor E2F and others. Hence, HDACs do not only act in an epigenetic manner. Nevertheless, DNA methylation and histone modifications are closely related. Methylated CpG sites in gene promoter regions are easily recognized by specific methyl CpG binding proteins (MBPs) which act as adapters between methylated DNA and chromatin modifying factors. MBPs can recruit co-repressors such as HDAC, methyltransferase and chromatin remodeling factors, creating the protein complex which regulates gene expression (11). If the promoter region is methylated, the corresponding gene is repressed due to its poor recognition by transcription factors (12). Indeed, DNMTs affect protein DNA interactions by chromatin remodeling, determine the accessibility of DNA to transcription factors, and are associated with under- or overexpression of certain proteins, ultimately leading to diverse pathologies, among which cancer (13).

Distinct changes of DNA methylation are termed 'epimutations' and appear to play an important role in carcinogenesis. Consequences of epimutation are similar to those of classic genetic mutations because the affected genes are silenced and functional gene products cannot be generated (14). In many types of tumors, the genomic DNA methylation pattern is changed either through hypermethylation (increased methylation) or hypomethylation (decreased methylation). In cancer cells, hypomethylation of CpG dinucleotides, especially in the pericentromeric regions of the chromosome, may lead to genomic instability. On the other hand, dense methylation of CpG islands, particularly in the promoter region of tumor suppressor genes, is associated with aberrant silencing of transcription (15). An elevated level of methylation in cancer cells probably results from increased activity of DNMTs, often as a consequence of overexpression. De-regulation of the DNMTs has been shown in many types of cancer including of the lung, breast, stomach and colon, and as well as in leukemia (16). Fortunately, epigenetic alterations are potentially reversible, unlike genetic mutations. Therefore, such alterations have become an attractive target for cancer therapy. Since hypermethylation of tumor suppressor genes and overexpression of DNMTs have been established as the major key players in carcinogenesis, demethylating agents seem to be especially promising as anticancer drugs. Re-expression of aberrantly silenced genes and restoration of their normal function can be achieved through the use of DNMT inhibitors which are incorporated into the growing DNA strand and covalently bind DNMTs.

Currently, two DNMT inhibitors (azanucleosides) have been approved by the US Food and Drug Administration (FDA): azacytidine (Vidaza; Celgene) and decitabine (5 aza 2' deoxycytidine) (Dacogen; SuperGen). These two types of drugs are the first molecules that have been characterized as the archetypal DNMT inhibitors and the only epidrugs that have been approved for the treatment of patients with acute myeloid leukemia (AML) and myelodyplastic syndrome (MDS). Azacytidine has also been approved by the FDA and the European Medicines Agency (EMA) for use against chronic myelomonocytic leukemia (CMML) (10). Therapeutic use of DNMT inhibitors can provide new and effective solutions for patients not only with hematological malignancies but also with other tumor types (especially since azacytidine and decitabine are currently in phase I clinical trials in patients with solid tumors) (17). The third novel member of the nucleoside DNMT inhibitor family is zebularine, a cytidine analog, which has been characterized as a potent and promising agent because of good results achieved in *in vitro* experiments, encouraging zebularine use for future clinical trials.

In the present article, we discuss the action mechanism of DNMT inhibitors (azacytidine, decitabine and zebularine) with consideration of their different activities towards cancer cells which may impact future clinical trials.

Azacytidine and decitabine are cytidine analogs modified in position 5 of the pyrimidine ring (Figure 2). Both compounds were synthesized by Sorm and co-workers in 1964 (18). The compounds were initially used as antimetabolite agents in leukemia chemotherapy until their hypomethylating properties were discovered. These cytidine analogs are transported into cells by human concentrative nucleoside transporter-1 and converted to active triphosphate forms, *i.e.* azacytidine by uridine cytidine kinase to 5-azacytidine 5'-triphosphate and decitabine by deoxycytidine kinase to 5-aza-2'-deoxycytidine-5'-triphosphate and then degraded by cytidine deaminase (CDA). Azacytidine, being a ribonucleoside, is incorporated into RNA and, to a lesser extent, into DNA, whereas decitabine, as a deoxyribose analog, is incorporated only into DNA strands (Figure 3A). To be active, these compounds need to be integrated into the genome of rapidly proliferating cells during the S phase of the cell cycle (19). The incorporated 5-azanucleoside disrupts the interaction between DNA and DNMTs through nitrogen instead of carbon, in the 5-position of the modified pyrimidine, which precludes the resolution of the complex and finally promotes its proteosomal degradation (Figure 1B). Thus, the enzyme remains covalently bound to DNA and its DNMT and the function is blocked. In addition, the covalent protein adduction also compromises the functionality of DNA and triggers DNA damage signaling, resulting in the degradation of the trapped DNMTs (20, 21). Therefore, further methylation of cytosine residues is

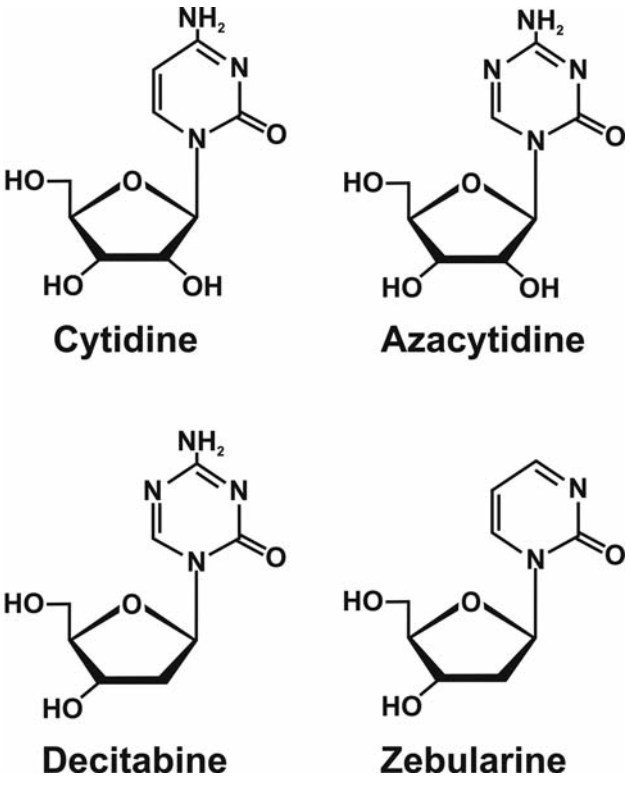

Figure 2. *Nucleoside analog inhibitors of DNA methyltranferase.*

inhibited, causing the passive loss of cytosine methylation in the daughter cells after replication.

Another demethylating agent in the family of nucleoside analogs is zebularine, which is characterized by chemical stability, apparent bioavailability and low cytotoxicity (22). Such properties distinguish zebularine among nucleoside inhibitors (Figure 2). Zebularine was originally synthesized and evaluated as an inhibitor of CDA to prevent de-amination of nucleoside analogs. It acts, however, primarily as a DNMT inhibitor by trapping the DNMT protein and forming tight covalent complexes between the DNMT protein and zebularine-substituted DNA (23). Zebularine is also activated after incorporation into DNA and metabolized presumably in a similar way to azacytidine. The initial phosphorylation is most likely mediated by uridine-cytidine kinase, followed by conversion to the 2'-deoxyzebularine-5'-diphosphate by ribonucleotide reductase. Furthermore, 2' deoxyzebularine-5'-diphosphate is converted to 2'-deoxyzebularine-5' triphosphate, which appears to be crucial for its incorporation into DNA, but this step of metabolic activation still needs to be clarified. In brief, inactivation of the DNMT by zebularine may be related to the absence of the 4-amino group in the 6-position of its pyrimidine ring (Figure 3B). In this way, zebularine does not allow the activation of cytosine C5

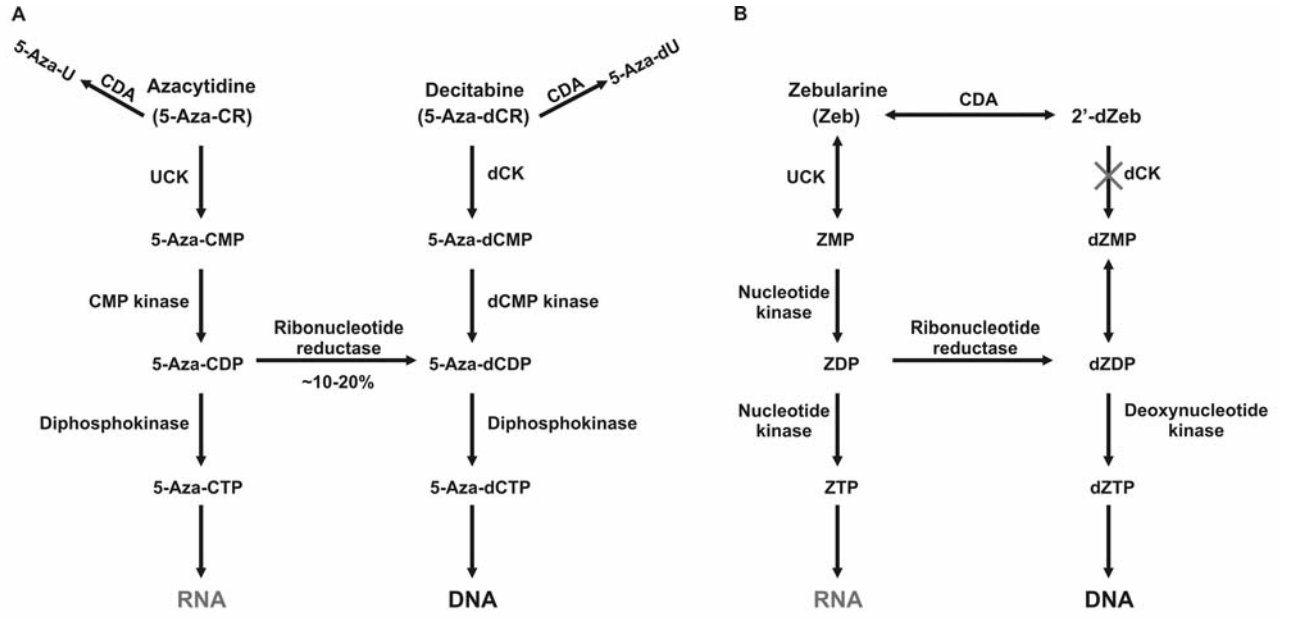

Figure 3. *Metabolic activation of 5 azanucleosides (A) and zabularine (B). After cellular uptake 5-azanucleosides and zebularine are modified by different metabolic pathways as described in the text. CDA: Cytidine deaminase; UCK: uridine cytidine kinase; dCK: deoxycytidine kinase; CMP kinase: cytidylate kinases; dCMP: deoxycytidylate kinase; 5-Aza-U: 5-aza-uridine; 5-Aza-CTP: 5 azacytidine 5'-triphosphate; 5-Aza-dCTP: 5-aza 2'-deoxycytidine-5'-triphosphate; ZTP: zebularine-5'-triphosphate; dZTP: 2'-deoxyzebularine-5'-triphosphate (19, 22).*

position and methyl group transfer. The lack of methylation, even if it is reversible, can clarify the stabilization of the zebularine containing DNA binding complex and slowing its dissociation (24).

Even though azacytidine and decitabine are used in the clinic, they are characterized by poor chemical stability, which depends on temperature and pH. In alkaline solutions both agents undergo irreversible decomposition. *In vitro* studies have shown that at 37˚C, in neutral aqueous solutions, the half-lives were 7 hours for azacytidine and 21 hours for decitabine, whereas the corresponding times *in vivo* are 41 minutes for azacytidine and from 15 to 25 minutes for decitabine. At 4˚C, both agents have considerable chemical stability, but with elevation of the temperature, they undergo more rapid degradation (21, 25, 26).

In humans, azacytidine and decitabine are metabolized by CDA, the enzyme which renders these drugs inactive by converting them into 5-azauridine compounds. The high level of CDA in the human liver and spleen is largely responsible for shorter half-lives of both agents *in vivo* than *in vitro*. Therefore, an increase in CDA activity may reduce efficacy by lowering drug levels and shortening the half-life times.

Unlike azacytidine and decitabine, zebularine is highly stable at acid and neutral pH. Zebularine has a half-life of ~44 hours at 37˚C in phosphate buffered saline (PBS) at pH 1.0 and ~508 hours at pH 7.0; such properties allow for its oral administration (27). Moreover, taking into consideration the half-life times of zebularine after intravenous and oral dosing in mice, rats and rhesus monkeys, it is likely that relatively frequent dosing or continuous intravenous infusion of zebularine will be necessary to maintain prolonged DNMT inhibition, which seems to be possible because of the low toxicity of zebularine (28).

Azanucleosides in Cancer Therapy – Mechanism of Action

The importance of DNA hypermethylation of CpG islands as a key player during cancer development and progress is not questioned. Especially since this phenomenon occurs infrequently in normal cells, the abnormal transcriptional silencing of tumor suppressor genes by hypermethylation of CpG islands has become an attractive and selective tumor specific therapeutic cancer target (3, 27). During gene methylation, the DNA sequence, as well as protein product, remains unaltered. Therefore, pharmacological intervention in the form of chemical inhibitors seems to be one of the possible ways for de-repression of inappropriately silenced genes and restoration of their normal functions.

The ability of azacytidine and decitabine to deplete the DNA methylating activity of DNMT can be achieved at low doses of both agents. Stresemann *et al.* have shown that azacytidine and decitabine are able to induce demethylation of epigenetically silenced genes at least at concentrations that

exceed 20% inhibition of cell growth (IC_{20}). *In vitro* experiments on whole-genome methylation, as well as local methylation at a defined genomic locus revealed that azacytidine and decitabine at 5-fold the IC_{20} concentration strongly reduces the genomic DNA methylation level in lymphoid cancer cells as compared to controls, *i.e.* by 60% and 40%, respectively. A similarly strong concentration-dependent demethylation was observed after incubation of colon carcinoma cells with both agents at concentrations centered on the respective drug specific IC_{20} concentration (29). Independent studies have shown that these two epidrugs may significantly reactivate silenced genes [tissue inhibitor of metalloproteinase 3 (*TIMP3*), *p15*, *p16*, cyclin-dependent kinase inhibitor 1C (CDKN1C), RAS-association domain family 1 (*RASSF1*)] responsible for basic cellular mechanisms such as apoptosis, cell cycle, and DNA repair (29), and that the concentrations required of both agents to achieve this effect are not high. Azacytidine and decitabine should be used at low inhibitory concentrations because at higher concentrations these agents exert strong cytotoxicity, interfere with DNA synthesis and cause DNA damage (30, 31). Apart from the use of low concentrations, for their demethylation function, the S phase of the cell cycle is needed. The DNA replication phase of the cell cycle enables selective and effective incorporation of these substances into the DNA of rapidly dividing cancer cells, reducing, thereby, hypomethylation in normal cycling cells (32). However, the hypomethylation activity of these two agents is not equal. It has been shown that azacytidine has only about 10% the potency of decitabine at inhibiting DNA methylation (33). Decitabine as a deoxyribonucleotide is directly incorporated into the DNA after phosphorylation to inhibit DNA methylation, whereas azacytidine is additionally incorporated into RNA. The overall incorporation of azacitydine into RNA can account for about 80-90% (34). Decrease of tRNA acceptor activity, polyribosome breakdown and incorporation into mRNA causing the subsequent inhibition of protein synthesis and enzyme induction are the functional consequences of RNA synthesis inhibition by azacytidine (35). All these in effect may influence both cancer and normal cells, resulting in greater *in vitro* and *in vivo* side-effects.

It has been proven that the mechanism of action of both these agents is dose-dependent. Therefore, the concept of a dual mechanism of action of these two agents has arisen. For decitabine, the 'dual mechanism' refers to the inhibition of cell proliferation at high doses and to the DNA hypomethylation-mediated effect on gene re-expression at low doses affecting the processes of cell differentiation and tumor suppression, whereas for azacytidine the 'dual mechanism' refers to the cytotoxicity at high doses, *via* incorporation into RNA and DNA, and to the DNA hypomethylation effect at lower doses (34). Their mechanism of action at the highest doses is related to the formation of covalent DNMT-DNA adducts in aza-containing DNA, leading to DNA damage and cytotoxicity. Experiments conducted on human tumor cell lines have shown that treatment with these agents causes growth inhibition by cell cycle arrest (specific to G_2/M phase for decitabine and cell cycle non-specific for azacytidine) and reduction in clonogenic survival. Moreover, decitabine and azacytidine can induce apoptosis in p53-dependent or p53-independent manners, respectively (36-38).

Unfortunately, these agents also have some limitations. In spite of their clinical efficacy, azacytidine and decitabine are characterized by poor bioavailability, instability in physiological media and high toxicity, restricting their use. For this reason, one of the most promising molecules appears to be zebularine. It has been characterized as a potential antitumor agent, based on its stability (half-life of >500 hours at pH 7.4) and minimal toxicity both *in vitro* and *in vivo* (39-41). Once incorporated into DNA, zebularine is involved in the reactivation of the silenced genes, such as cyclin-dependent kinase inhibitors (CKI) *p15* in AML cells, *p16* in bladder, colon and pancreatic cells, and *p57* in myeloid leukemia cells (42, 43). Zebularine also leads to the re-expression of the cell cycle and apoptosis modulator Ras association domain family 1 isoform A (*RASSF1A*) as a result of induced demethylation of its promoter region in ovarian cancer cells. Moreover, microarray analysis demonstrated that decitabine and zebularine may demethylate 78 genes, with 32 exclusive to decitabine and only eight specific for zebularine (44). This might suggest that both agents exert their demethylating effects by different mechanisms. Cheng *et al*. observed that zebularine caused complete depletion of DNMT1 and partial depletion of DNMT3A and DNMT3B2/3 in cancer cells (45). This suggests zebularine preference for DNMT1 over DNMT3A and DNMT3B. Moreover, DNMT1 may be an important indicator of the demethylating ability of zebularine. It has also been reported that DNMT3A/DNMT3B double-null embryonic stem cells are more resistant to decitabine than are DNMT1 null cells, suggesting that decitabine may be more effective for selected types of cancer cells, in which DNMT3 expression is up-regulated (46). Furthermore, DNMTs have a higher affinity for zebularine-containing DNA than for the unmodified DNA (24). Such results indicate that the differential specificity and affinity of these drugs for DNMT isoforms could lead to divergent cellular responses. It has been demonstrated that higher doses of zebularine are required to obtain demethylation and gene re-expression levels comparable to those that are induced by azacytidine and decitabine (29). This might be a result of lower binding affinity of uridine-cytidine kinase for zebularine and its slow conversion to 2'-deoxyzebularine-5'-diphosphate (40). However, owing to its minimal toxicity profile, zebularine, unlike azacytidine and decitabine, can be

used at high micromolar concentrations in prolonged treatment periods (41, 47). The lower toxicity of zebularine, as a ribonucleoside, may result from its different incorporation into RNA and DNA in normal and neoplastically-transformed cells, due to the overexpression of uridine-cytidine kinase in cancer cells, facilitating zebularine insertion into nucleic acids (44). Moreover, Champion *et al*. have shown that the absence of the 4-amino group in zebularine does not allow for the activation of the cytosine C5 position after covalent intermediate formation and methyl group transfer (24). Such lack of zebularine methylation seems to prevent dissociation of the DNA-enzyme complex, indicating the stabilization of the zebularine-containing DNA-binding complex.

Future medical use of zebularine could involve combinations of this drug with other therapeutic modalities, such as chemotherapy, immunotherapy or radiotherapy. Clinical trials with azacytidine or decitabine administered as single agents or in co-administration with other chemotherapeutics resulted in significant toxicity (48, 49). Therefore, zebularine and other demethylation agents with similar properties could be less detrimental and more promising drug candidates.

Conclusion and Future Perspectives

Azacytidine, decitabine and zebularine have differential activity, complex and partially overlapping mechanisms of action. Studies have indicated that the methylation patterns of tumor suppressor genes might differ depending on the drug being used. Azacytidine and decitabine induce a strong demethylating effect, leading at lower doses to re expression of aberrantly silenced genes associated with reduced proliferation, cell differentiation, apoptosis, and senescence, while at higher doses, their main effect consists of DNA damage after incorporation into genomic DNA. The impact of zebularine on the DNA methylation level is more moderate. However, this cytidine analog is less toxic and can, therefore, be given continuously at high doses. Additionally, zebularine seems to target tumor cells preferentially.

The use of demethylating agents azacytidine and decitabine in the treatment of myelodysplastic syndromes is well-documented, in spite of their high toxicity. But the status of knowledge for using demethylating agents for solid tumors is still insufficient and needs to be further evaluated. It appears to be reasonable to use demethylating agents in combination with chemotherapeutic agents. *In vitro* experiments indicate that decitabine can potentiate the cytotoxic effect of classical chemotherapeutics, such as doxorubicin, 5-fluorouracil and oxaliplatin, and induce highly synergistic effects (50, 51). Interestingly, encouraging results were obtained with combination of decitabine and carboplatin in patients with solid tumors (52). The authors concluded that decitabine combines safely with carboplatin and that the specific regimen causes epigenetic changes. In another phase I study, a combination of cisplatin with decitabine resulted in one partial response in a patient with cervical cancer, and two minor responses: one in a patient with non-small cell lung cancer and another in a patient with cervical cancer (17).

Generally, combinations of azacytidine or decitabine with standard chemotherapy clearly have clinical activity, but it is difficult to distinguish the effect of the epigenetic therapy from the cytotoxic therapy (17). On the other hand, the present demethylating agents used in clinics are cytotoxic, mutagenic and exhibit lack of specificity towards genes, which may limit their clinical application. Thus, the next generation of DNMT inhibitors with lower toxicity might be more fruitful for future research. Zebularine is an alternative derivative of cytidine and promises to be a better drug than azacytidine and decitabine for epigenetic cancer therapy. Based on its stability, it was the first DNMT inhibitor showing *in vivo* antitumor activity against T-cell lymphoma after oral administration (53). Attention has also been attributed on the identification of small non-nucleoside DNMT inhibitors, such as epigallocatechin-3-gallate, hydralazine, procainamide, procaine and RG108, which bind directly to the catalytic region of DNMTs without incorporation into DNA. However, the results of *in vitro* studies showed that non-nucleoside compounds induce limited epigenetic changes in living cells (54). Among the novel agents of demethylation, the most intensively studied are *DNMT1* antisense and siRNA. Down-regulation of DNMT1 by antisense or siRNA is sufficient to restore the expression of aberrantly hypermethylated genes (55, 56), but such therapy is still controversial mainly because of the problems associated with the administration to humans, which include instability, toxicity, risk of non-specific effects, and complexity in developing a suitable delivery system.

Even though many DNMT inhibitors other then cytidine analogues have been developed, their effects are not satisfactory and they do not seem to be able to replace azacytidine and decitabine, as yet. Therefore, azacytidine and decitabine, in spite of their limitations, are still used for combination epigenetic therapy. However, attempts to discover and to develop novel compounds targeting DNMTs should be continued. It is important to find more selective and less toxic DNMT inhibitors which will be effective especially in patients with solid tumors. It would be a challenge to design inhibitors whose mechanism of action will rely only on reactivation of abnormally silenced suppressor genes.

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