DNA Methyltransferase and its Clinical Applications

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Abstract. This review mainly discusses the DNA methyltransferases (Abbreviation for mouse: Dnmt; for human: DNMT) with a concentration on its importance in the mechanism of DNA methylation. Besides, the clinical application, especially the pharmaceutical application of cytosine agents as Dnmt inhibitors is also the main point of discussion. The significant effect of several Dnmt inhibitors for the therapy of oncological diseases is demonstrated. The paper will elaborate on three most representative Dnmt inhibitors: namely azacytidine, decitabine, and the developing drugs zebularine. The advantages of zebularine among the other agents will be analyzed. As a conclusion, zebularine is emphasized as the future research direction.

1. Introduction
The family of DNA methyltransferases including Dnmt 1 and Dnmt 3a/b is one of the enzymes for DNA methylation. The deregulation of the DNMT family induces aberrant DNA methylation on numerous tumor suppressors and cancer-related genes. It could induce many types of cancer. But the frequent epigenetic modification represents a reversible mechanism for clinical intervention during the tumorigenesis. Thus, an inhibitor to repress the function of DNMT could be practical for oncological therapy. This review consists of two parts and elucidates the DNMT inhibition. The first part concentrates on the mechanism of the catalyzation of Dnmt. Besides, the relation between DNA methylation and relevant diseases will be mentioned. The second part discusses the ongoing development of relevant pharmaceutical derivatives for the regulation of DNA methylation. Nowadays, two of these DNMT inhibitors have been approved by the Food and Drug Administration of the United States (FDA). They are azacytidine (Vidaza; Celgene (NJ 07901, US)) and decitabine (Dacogen; SuperGen (MK14 5BU, UK)) [1]. Another inhibitor, zebularine, is also appreciated by the academic community. This paper will evaluate these three demethylating agents both positively and negatively. A conclusion will be proposed toward this issue at the end.

2. Analysis

2.1 Mechanism of DNA methylation
DNA methylation is a major epigenetic mechanism. It is identified as the methylation on cytosine. The most common pattern of methylation is 5-methylcytosine (5mC) [1]. The following three parts will respectively elaborate on the correlation between DNA methylation and CpG island, the deregulation of DNA methylation, and the models of catalyzation.

2.1.1 The correlation between DNA methylation and CpG island
DNA methylation is highly related to the CpG island. The research demonstrated that the nucleotides are hypermethylated if the CpG density is under 30%. If the density is over 30%, it is in comparison
more likely to be a hypomethylation on the DNA sequence [2]. In other words, CpG islands enhance the accessibility of DNA and promote transcription factor binding [3]. Since roughly 70% of the promoters reside within the CpG island [4], DNA methylation is significant for the expression of the whole gene sequence. Generally, low density or a lack of DNA methylation in the promoter region is correlated with an "on" configuration of chromatin and activates the gene expression. By contrast, hypermethylation of CpG islands in gene promoters indicates an "off" configuration of chromatin that leads to gene silencing [5]. The methylation pattern is read by the family of methyl-CpG-binding domain protein (MBD). It binds to the transcriptional repression domain (TRD) such as histone deacetylase (HDAC) to silence the gene [4].

2.1.2 DNA methylation and diseases
The DNA methylation can be deregulated due to the environmental changes. It has been measured that air pollution alters DNA methylation and impairs health. Long-term exposure to the NO2 air pollution during the pregnancy is correlated to the differential DNA methylation in offspring [3].

The disorder of DNA methylation is associated with various diseases. It has been demonstrated that differential methylation is strongly associated with attention-deficit/hyperactivity disorder (ADHD) [3]. Once several methylations occur in the apoptotic gene sequences, a resistance against cell death can be developed. The cancer cells utilize this methylation pattern to escape the program cell death and to establish a chemoresistance to the drugs[6]. Hence, the disorder of DNA methylation is one of the reasons that initiate different types of solid and hematological cancers.

2.1.3 The catalyzation of DNA methylation
The present researches for the regulation concentrate on the catalyzation of DNA methylation. The enzyme for the methylation as well as for the transcription of the methylation pattern in the family of DNA methyltransferase (Dnmt). This family consists of Dnmt1, Dnmt3a/b. Dnmt2 and Dnmt3L are also in this family, however, they will not be discussed in this review because they don't have catalytic region. To attract and to regulate the catalyzation, CpG islands bind with the transcription factors for Dnmt [4].

Up to date, two different models of the catalyzation are suggested. The old one emphasizes the two-steps of functions. Dnmt3a and Dnmt3b are de novo methyltransferase and attach the methyl group from S-adenosyl methionine (SAM) on the previously unmethylated CpG dinucleotides. Specifically, Dnmt3a is necessary for maternal imprinting at differentially methylated regions, whereas Dnmt3b is required for methylation of pericentromeric repeats and CpG islands on inactive X-chromosomes [7]. In comparison, Dnmt1 is responsible for the maintenance of the methylation pattern. During the replication, Dnmt1 binds on the newly synthesized strand, mimics the methylation pattern of the original DNA and restores this pattern on the new strand [4, 8]. The problem of this model lies in the bias with the observation. In the in vitro experiments, if the Dnmt3a/b are knocked out and the function of Dnmt1 has remained, there is still 30% of the replicated DNA which is hemimethylated. Moreover, it is observed that the Dnmt1 can also complete the de novo methylation.

Fig. 1. An illustration for the respective tasks of Dnmt1 and Dnmt3a/b [4]
Because of the inefficiency of Dnmt1 in the maintenance of the methylation pattern, the new model suggests that Dnmt1 and Dnmt3a/b catalyze cooperatively and simultaneously [2]. The cooperation can compensate for their disadvantages. This implication fixes the old model. It also implies that the DNMT inhibitor should be able to suppress both DNMT1 and DNMT3a/b.

Fig. 2. An illustration for the coordinative work of the whole DNMT family UHRF1 (Ubiquitin-like, containing PHD and RING finger domains, 1): a protein that binds on the strand to regulate the chromatin structure and gene expression [2].

2.2 DNMT inhibitors in its application
Among all developed strategies to inhibit the DNA methylation, the DNMT inhibitor is the most advanced since its imperative role for DNA methylation. Another strategy is i.e. to utilize the HDAC inhibitors to repress the reading of the methylation, but that will not be elucidated in this review.

The DNMT inhibitors are identified as cytosine analog, which means that they are modified from cytosine. Therefore, they can be competitors with 5mC. These nucleotide analogs can incorporate into the DNA and link the enzymes covalently. The connection between DNA and DNMT will be disrupted. The productivity of DNMT will be degraded [9].

The following section of the review will elucidate the pathway of metabolization of the DNMT inhibitors. Their effects and the existing drawbacks will be evaluated. This section divides into two parts. The first part will analyze the two approved cytidine analogs, namely azacytidine and decitabine. The second part will explicate the still developing DNMT inhibitor zebularine.

2.2.1 Present application of DNMT inhibitors
Two major inhibitors, 5-azacytidine (Vidaza; Celgene (NJ 07901, US)) and decitabine (Dacogen; SuperGen(MK14 5BU, UK) have been approved by the US Food and Drug Administration (FDA) for the treatment of patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). Specifically, azacytidine has also been approved by the European Medicines Agency (EMA) against chronic myelomonocytic leukemia (CMML). Besides, regardless of their success in treating the hematological malignancies, researches show that they can also provide the treatment for the solid tumor types [1, 10].
Fig. 3. The chemical structure of cytidine and the three DNMT inhibitors which are discussed [1].

With a molecular perspective, the azacytidine and decitabine are modified from cytidine that a
nitrogen atom replaces the 5th carbon of their pyrimidine ring.
Azacytidine has two hydroxyl groups on the ribose ring just like cytidine, by contrast, that decitabine
has only one on the third carbon of the ribose.

Fig. 4. An illustration of the metabolization pathway of the three DNMT inhibitors [1]

Both inhibitors enter cells by transportation by human concentrative nucleoside transporter 1. Afterward, they react to the triphosphate form. First, 5-azacytidine converts to the 5-azacytidine-5-
diphosphate (5-Aza-CDP) by mean of uridine cytidine kinase (UCK) and cytidine monophosphate
kinase (CMP kinase), whereas decitabine is catalyzed by the deoxycytidine kinase and deoxy-CMP
kinase, and becomes 5-aza-2-deoxycytidine-5-diphosphate (5-Aza-dCDP). Then, one more phosphate
attaches to both diphosphates and they respectively become the 5-azacytidine-5-triphosphate (5-Aza-
CTP) and 5-aza-2-deoxycytidine-5-triphosphate (5-Aza-dCTP). Finally, the triphosphates are
incorporated into the gene. [1]Azacytidine is predominately incorporated into RNA, in comparison,
decitabine is only incorporated into DNA. Therefore, decitabine is much more potent than azacytidine.
The research confirmed that the potency of decitabine is ten times higher than azacytidine [1].

In humans, both azacytidine and decitabine are metabolized by cytidine deaminase (CDA) to 5-aza-
uracil (5-aza-U). The in vitro experiment shows that at 37°C and in the neutral aqueous solution, the
half-lives for azacytidine were 7 hours and for decitabine were 21 hours. However, in the in vivo
environment, the half-lives for azacytidine reduce to mere 15-25 minutes and the half-lives for
decitabine remain only 40 minutes. The reason is that the optimal temperature for both enzymes is 4°C, which is dramatically distinguished from the body temperature. Besides, the high concentration of CDA in a human's body accelerates the metabolization. These factors contribute to the chemical instability of both drugs [1].

The direct causality of the repression of the DNA methylation is the reactivation of the silenced gene. Since the DNMT inhibitors don't have drug targets, with other words, the agents are non-specified drugs, there might be deregulation of a methylation pattern. A few silenced gene such as tissue inhibitor of metalloproteinase 3 (TIMP3), p15, p16, cyclin-dependent kinase inhibitor 1C (CDKN1C), RAS-association domain family 1 (RASSF1), which are responsible for the apoptosis, normal cell cycle and the DNA repair, will be varied by these agents. The treatment thus induces the cytotoxicity, interferes with the DNA synthesis and damages the DNA [1]. Yet the doses-dependence should not be ignored. Because azacytidine is at least 10-fold more cytotoxic than decitabine, a long dosing schedule with low intensity is suggested [11,12]. By contrast, the dosing schedule of decitabine doesn't affect the early death rate of patients [13]. Nevertheless, the pharmaceutical experiment concludes that such long exposure with a low intensity to the malignant cell may bring an improved response and prolong the therapeutic effect [12]. Moreover, the age of patients and their plausible gene mutation should also be considered during the exposure, since the potential risks differ from groups [13].

2.2.2 The future DNMT inhibitor zebularine
In spite of the present pharmaceutical compounds of DNMT inhibitors, another cytosine agent, zebularine, is appreciated by the researchers as well. Zebularine does not have the amino group on the nucleic acid ring. Besides, a hydroxyl group on the second position of the ribose ring is missing. The potency of zebularine is proven with a good result in the in vitro experiments. The clinical trial in the future is hence encouraged. Zebularine is characterized by chemical stability, apparent bioavailability and low level of cytotoxicity. Similar to azacytidine, zebularine, which is incorporated in DNA, can also trap the DNMT with the covalent bond. Zebularine is phosphorylated in the body through UCK. It can be incorporated into RNA through the catalyzation of nucleotide kinase. Besides, it can also be deoxidated by ribonucleotide reductase, be phosphorylated to its triphosphate form and substitute for cytosine in DNA. The pharmacokinetic research indicates the better chemical stability of zebularine. Its half-lives are >500 hours in the neutral aqueous solution. The lower toxicity is demonstrated in both in vitro and in vivo experiments [1,14].

Zebularine can completely deplete DNMT1 and inhibit DNMT3a and DNMT3b partially [15]. Some cytidine analogs, such as 4'-Thio-2'-deoxycytidine (TdCyd), are less effective as zebularine because it can only inhibit DNMT1 [9]. To compare the effect between azacytidine and zebularine, the research estimates that they might have the same magnitude of inhibition, although zebularine is chemically more stable than azacytidine. The reason might be due to the low frequency of incorporation into DNA and vulnerable binds with DNMT [16].

The combination of the epigenetic demethylating agents with the classical chemotherapeutics such as 5-fluorouracil (5-FU), cisplatin and doxorubicin can enhance the effect. The in vitro experiments demonstrate the potentiating of chemotherapy by mean of epigenetic drugs. Recent researches also suggest combining epigenetic therapy with immunotherapy [5]. The DNMT inhibitors can variate the DNA methylation pattern. The researchers hypothesize that it can reverse the elements of tumor immune evasion and enhance immune checkpoint therapy [17].

3. Future Perspectives
For future perspectives, the author suggests approving zebularine as a new epigenetic drug. Attempting to find more novel compounds of DNMT inhibitors to create diversity for selection is encouraged. Better half-lives and low toxicity are primary for the novel compounds. Besides, it is necessary to enhance the knowledge of the treatment with demethylating agents for more solid tumors. However, even though there are more and more cytidine analogs have been developed, their effects are not enough satisfactory to replace the azacytidine and decitabine. Hence, it is recommended to maintain its application in
epigenetic therapy. The academic community should concentrate on how to target the aberrant methylated gene so that to minimize the influence on the normal function of DNA methylation.

4. Conclusion
DNA methylation is an epigenetic variation that occurs predominantly on CpG-island. The aberrant DNA methylation due to the environmental changes induces various diseases. The input of the DNA methylation pattern is catalyzed by the Dnmt family. Because the new model hypothesizes the complementary and simultaneous work of the whole Dnmt family, the DNMT inhibitors must deplete all three members together.

Azacytidine and decitabine are the two present applied DNMT inhibitors. They have similar magnitudes of demethylation. However, they are characterized by short half-lives in the \textit{in vivo} experiments and cytotoxicity. Thus, the presently approved drugs can only apply with low intensity and a long schedule of treatment. However, zebularine shows better chemical stability and bioavailability and lower level of cytotoxicity than azacytidine and decitabine. Furthermore, the combination of epigenetic demethylating agents with chemotherapy and immunotherapy is discovered with an enhanced effect.

References
[1] A. Gnyyszka, Z. Jastrzębski, S. Flis, DNA Methyltransferase Inhibitors and Their Emerging Role in Epigenetic Therapy of Cancer. \textit{Anticancer Research}, \textbf{33}, 8 (2013)
[2] Z. Li, H. Dai and S. Martos, Distinct roles of DNMT1-dependent and DNMT1-independent methylation patterns in the genome of mouse embryonic stem cells. \textit{Genome Biology}, \textbf{16}, 115 (2015)
[3] A. J. Titus, R. M. Gallimore, L. A. Salas and B. C. Christensen, Cell-type deconvolution from DNA methylation: a review of recent applications. \textit{Human Molecular Genetics}, \textbf{26}(R2), R216–R224 (2017)
[4] L. D. Moore, T. Le and G. Fan, DNA Methylation and Its Basic Function. \textit{Neuropsychopharmacology}, \textbf{38}, 23 (2012)
[5] D. Brocks, C. R. Schmidt and M. Daskalakis, DNMT and HDAC inhibitors induce cryptic transcription start sites encoded in long terminal repeats. \textit{Nature Genetics}, \textbf{49}(7) (2017)
[6] S. J. Choi and S. W. Jung. Alteration of DNA Methylation in Gastric Cancer with Chemotherapy. \textit{J. Microbiol. Biotechnol}, \textbf{27}(8), 1367–1378 (2017)
[7] H. Meng, Y. Cao and J. Qin, DNA Methylation, Its Mediators and Genome Integrity. \textit{Int J Biol Sci}, \textbf{1}(5) (2015)
[8] W. Zhang and J. Xu, DNA methyltransferases and their roles in tumorigenesis. \textit{Biomarker Research}, \textbf{5}(1) (2017)
[9] C. Omar, P. Depreux and L. Halby, DNA Methylation Targeting: The DNMT/HMT Crosstalk Challenge. \textit{Biomolecules}, \textbf{7}(1), 3 (2017)
[10] E. L. Walton. On the road to epigenetic therapy. \textit{Biomedical Journal}, \textbf{39}, 161–165(2016)
[11] J. K. Christman, 5-Azacytidine and 5-aza-2’-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. \textit{Oncogene}, \textbf{21}(35), 5483–5495 (2002)
[12] C. R. Cogle, B. L. Scott, T. Boyd, G. Garcia-Manero, Oral Azacitidine (CC-486) for the Treatment of Myelodysplastic Syndromes and Acute Myeloid Leukemia. \textit{Oncologist}, \textbf{20}(12), 1404–1412 (2015)
[13] P. He, J. Zhou, D. Yao, et al. Efficacy and safety of decitabine in treatment of elderly patients with acute myeloid leukemia: A systematic review and meta-analysis. \textit{Oncotarget}, \textbf{8}(25), 41498–41507 (2017)
[14] K. Nakamura, K. Nakabayashi, K. Htet Aung, et al. DNA methyltransferase inhibitor zebularine induces human cholangiocarcinoma cell death through alteration of DNA methylation status. \textit{PLoS One}, \textbf{10}(3), e0120545 (2015)
[15] J. Cheng, D. Weisenberger, F. Gonzales, et al. Continuous zebularine treatment effectively sustains demethylation in human bladder cancer cells. *Mol Cell Biol*, 24(3), 1270–1278 (2004)

[16] P. Griffin, C. Niederhuth, R. Schmitz, A Comparative Analysis of 5-Azacytidine- and Zebularine-Induced DNA Demethylation. *G3 (Bethesda)*, 6(9), 2773–2780 (2016)

[17] K. B. Chiappinelli, C. A. Zahnow, N. Ahuja, S. B. Baylin, Combining Epigenetic and Immunotherapy to Combat Cancer. *Cancer Res*, 76(7), 1683–1689 (2016)