Recent Developments of Two NCI Epigenetic Drugs: Zebularine and 3-Deazaneplanocin-A

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Background

DNA methylation and chromatin remodeling are at the heart of gene regulation, as they provide additional layers of control that are independent of the underlying DNA sequence of the gene. These self-regulating epigenetic mechanisms control the accessibility of DNA for transcription factors and play an essential role in the regulation of gene expression. Although different sets of genes may be targeted by these epigenetic events, the combined outcome of DNA demethylation/methylation at CpG residues in promoter regions of genes, coupled with additional post-translational “on/off” modifications of histone lysine tails, such as acetylation/deacetylation and demethylation/methylation, results in a chromatin structure that is either accessible or inaccessible to the transcription machinery. For many years, evidence has accumulated suggesting that CpG methylation in mammalian DNA is involved in gene silencing (1). In addition, multi-protein complexes that mediate transcriptional repression via deacetylation of the core histones H3 and H4 (2), as well as high levels of histone H3 trimethylation at lysines 9 and 27 (3), result in a silent chromatin.

Epigenetic events play a critical role in the development and progression of cancer (4). This process is often deregulated in tumor cells and frequently leads to the aberrant silencing of normal tumor-suppressor genes. The reversal of such a silenced status by pharmacological means requires the development of effective compounds that inhibit the processes of DNA methylation, histone deacetylation, and histone methylation at specific sites on the nucleosome. In this review, we will discuss two NCI epigenetic drugs, zebularine (Zeb) and 3-deazaneplanocin (DZNep), which inhibit, respectively, the methylation of cytosines in DNA (5) and the trimethylation of specific lysine residues (6) (Figure 1). These pharmacological events are often synergistic with the third class of histone modifiers known as histone deacetylase (HDAC) inhibitors (7) (Figure 2). Indeed, methyl CpG binding proteins are associated with histone deacetylation and EZH2 requires HDAC activity for gene silencing; EZH2 can also recruit DNA methylase to certain gene promoters to control DNA methylation.

Discussion

Zeb (1-(β-d-ribofuranosyl)-1,2-dihydropyrimidin-2-one) is the older of the two drugs. It was originally identified as a bacteriostat and several years later our laboratory and others described it as a potent, mechanism-based inhibitor of cytidine deaminase (CDA) (8). CDA
is responsible for the rapid inactivation of important antitumor agents such as arabinosylcytosine (cytarabine) and 5-aza-2'-deoxycytidine (decitabine). As a result, Zeb is able to significantly enhance the antitumor activity of decitabine, mainly through its inhibition of CDA (9). Structurally, Zeb is basically the 4-desamino analogue of cytidine. This simple structural change increases the electrophilicity of the ring and is the basis for the formation of a stable,
covalent hydrate at the active site of CDA that results in the potent inhibition of the enzyme. In a similar fashion, the removal of the 4-amino group facilitates nucleophilic attack at position 6 of the pyrimidine ring, which is the basis for the most recently discovered activity of Zeb as a DNA methyl transferase inhibitor (10). Zeb requires phosphorylation and conversion to its deoxynucleotide before it is incorporated into DNA. Once incorporated, its base is paired with guanine, and, after flipping out of the DNA helix, it forms a tight complex with the DNA methyltransferase through an invariant cysteine residue at the active site that leads to inhibition of DNA methylation (11). The two other drugs known to inhibit DNA methylation, azacytidine and decitabine, have a similar mechanism of action once they become incorporated into DNA. At the molecular level, modified oligodeoxynucleotides carrying either the 2’-deoxynucleotide moieties of Zeb or decitabine, are equipotent in their ability to inhibit DNA methylation (12). On the practical side, decitabine is more effectively metabolized and incorporated into DNA, resulting in a more potent agent, albeit somewhat toxic. Decitabine, as well as azacytidine, are very unstable and undergo rapid decomposition even at neutral pH. Furthermore, they are rapidly inactivated by CDA.

Zeb, on the other hand, is a very stable compound, which explains the following results:

- Continuous administration of Zeb to T-24 bladder cells results in promoter demethylation of the CDKN2A gene and induction of its m-RNA (13).
- Continuous Zeb treatment produces complete depletion of the Dnmt1 enzyme required for DNA methylation (14).
- Oral or IP administration of Zeb to nude mice with EJ6 xenograft tumors inhibits tumor growth without significant animal toxicity; and it preferentially targets tumor cells (5).
- Long-term administration of Zeb was effective against the development of murine T-cell lymphoma with minimal toxicity (15).
- Chronic, oral administration of Zeb in drinking water decreased DNA methylation in the Min mouse model; activity was specific for the target tissue, inducing gene expression and dramatically reducing polyp formation (16).

DZNep [(1S,2R,5R)-5-(4-amino-1H-imidazo[4,5-c]pyridin-1-yl)-3-(hydroxymethyl)-cyclopent-3-ene-1,2-diol] is structurally analogous to the natural product, neplanocin A, except for the missing nitrogen at position 3 on the purine ring (17). DZNep and neplanocin A share many similar pharmacological properties, including their ability to inhibit the target enzyme, S-adenosylhomocysteine hydrolase (AdoHcyase). This enzyme controls intracellular levels of S-adenosylhomocysteine (AdoHcy), which is a potent, product inhibitor of some S-adenosylmethionine (Ado-Met)-dependent methyltransferases. Pharmacological inhibition of AdoHcyase leads to indirect inhibition of methyltransferases by increasing the AdoHcy pool. Initially, both neplanocin A and DZNep were found to have potent antiviral activity. However, neplanocin A is readily phosphorylated with the ensuing formation of the AdoMet triphosphate analogue as the major metabolite. High levels of this metabolite were associated with cytotoxicity. For that reason DZNep, which lacks a nitrogen atom on the purine ring, was designed to bypass phosphorylation by adenosine kinase (17). Because DZNep is not phosphorylated it is much less toxic than neplanocin A.
Recently there has been an avalanche of interest in DZNep as a novel epigenetic drug. Over-expression of the polycomb group (PcG) protein enhancer zeste homologue 2 (EZH2) is known to occur in several malignancies, including prostate cancer, breast cancer, and glioblastoma multiforme. EZH2 is a histone-lysine N-methyltransferase with a conserved SET domain that contains the active site for the methylation of histone H3 on Lys27 (H3K27m3 methylation) (18). This methylation mark is a central feature of PcG-silenced chromatin. Expectedly, pharmacologic disruption of EZH2 by DZNep selectively induces beneficial changes in various cancer cells:

- DZNep is able to induce down-regulation of EZH2 as early as 2 h after treatment resulting in inhibition of H3K27m3 methylation (6).
- DZNep is 20-fold more effective in killing BRCA1-deficient mammary tumor cells which depend on elevated EZH2 levels for growth than BRCA1-proficient tumor cells (19).
- Pharmacologic disruption of EZH2 by DZNep strongly impairs glioblastoma cancer stem cells self-renewal in vitro and tumor-initiating capacity in vivo (20).
- DZNep produced marked upregulation of \( ADRB2 \) transcripts at 5 \( \mu \)M after 48 h. H3K27m3 methylation silencing of the \( ADRB2 \) gene (adrenergic receptor \( \beta-2 \)), a critical mediator of \( \beta \)-adrenergic signaling, enhances cell invasion and transformation in benign prostate cells (21).

**Future Directions**

The exploration of these two drugs is a constantly evolving field. Beginning with Zeb, several important aspects need to be defined:

- Determining the minimum concentrations required for optimal activity in vitro. Initial studies with T24 cells used rather high concentrations (100 \( \mu \)M, 500 \( \mu \)M, and even 1000 \( \mu \)M) to achieve p16 expression levels equivalent to those obtained with just 3 \( \mu \)M of decitabine (5). However, a recently completed study determined that a concentration of just 30 \( \mu \)M was able to induce measurable levels of the cyclin-dependent kinase inhibitor 2A (CDKN2A) m-RNA and p-16 protein (13).
- Establishing an effective dose in animals for antitumor activity. Because plasma levels of 30 \( \mu \)M can be safely achieved in mice following a daily dose of 5 mg/ml of Zeb in drinking water (13), doses of 500 and 1,000 mg/kg (ip and po) and 400 mg/kg ip used in previous studies may have been too high.
- Combining the appropriate minimally effective dose and time of administration is critical. In a cancer prevention study, significant activity was achieved using an even lower concentration of 0.2 mg/ml in drinking water for a period of 113 days (16). The detection of stable metabolites of Zeb, such phosphodiester conjugates of ethanolamine and choline, are capable serving as active reservoirs of the drug (22).
- Determining the effective dose for treatment is very important, particularly since Zeb showed severe toxicity in primates after continuous i.v. administration at a rate of 33/ mg/kg/h for 120 h (23).
• Understanding the role of liver metabolism by liver aldehyde oxidase (AO). Zeb is rapidly converted to uridine by AO, but there are significant differences in AO activity amongst species; some animals like dogs totally lack AO, while in mice only males have the enzyme (24). When AO is involved, some studies have questioned the validity of toxicity studies in monkeys as the sole predictor of human toxicity, and, surprisingly, there is a higher degree of homology between the human AO and the mouse enzyme (25). Toxicity studies in animals lacking AO must be completed to establish the role of this enzyme in Zeb's toxicity.

• Devising useful protocols for Zeb in combination with other agents. There is already evidence that Zeb in combination with decitabine is useful (9). The combination of Zeb with an AO inhibitor, such as the clinical agent raloxifene (24), needs to be tested, particularly to determine if the toxicity in primates could be ameliorated.

For DZNep there are a number of in vitro studies that have already established an effective dose of ca. 5 μM. However, the emerging picture is that DZNep is more potent when used in combination with other epigenetic drugs to remodel the chromatin to a transcriptional competent state. Given the complexity of epigenetic regulation, it is expected that combination of drugs targeting multiple components will provide an effective clinical approach for a combined gene reactivation therapy.

References