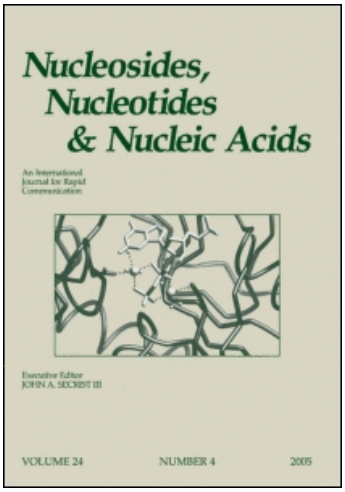

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Zebularine: A Unique Molecule for an Epigenetically Based Strategy in Cancer Chemotherapy. The Magic of its Chemistry and Biology

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ZEBULARINE: A UNIQUE MOLECULE FOR AN EPIGENETICALLY BASED STRATEGY IN CANCER CHEMOTHERAPY. THE MAGIC OF ITS CHEMISTRY AND BIOLOGY

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▫ *1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one (zebularine) is structurally 4-deamino cytidine. The increased electrophilic character of this simple aglycon endows the molecule with unique chemical and biological properties, making zebularine a versatile starting material for the synthesis of complex nucleosides and an effective inhibitor of cytidine deaminase and DNA cytosine methyltransferase. Zebularine is a stable, antitumor agent that preferentially targets cancer cells and shows activity both in vitro and in experimental animals, even after oral administration.*

Keywords Zebularine, Ring-Expanded Nucleoside Analogues, Isouridine, Cytidine Deaminase Inhibition, DNA Methyltrasferase Inhibition, Antitumor Activity

INTRODUCTION

Zebularine [1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one, **1b**] is the pyrimidine counterpart of the purine natural product nebularine (**2b**).[1] The first synthesis of zebularine was described in 1961,[2] and improved methods of preparation appeared later.[3–9] Preliminary biochemical investigations identified zebularine as a bacteriostat,[10] and several years later our laboratory and others established it as a potent, mechanism-based inhibitor of cytidine (**1a**) deaminase

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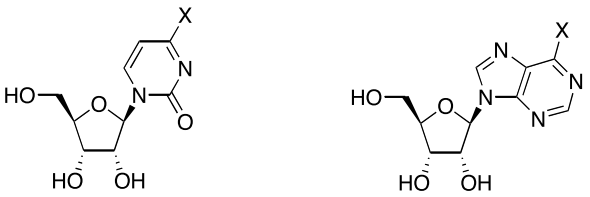


1a, X = NH_2
1b, X = H

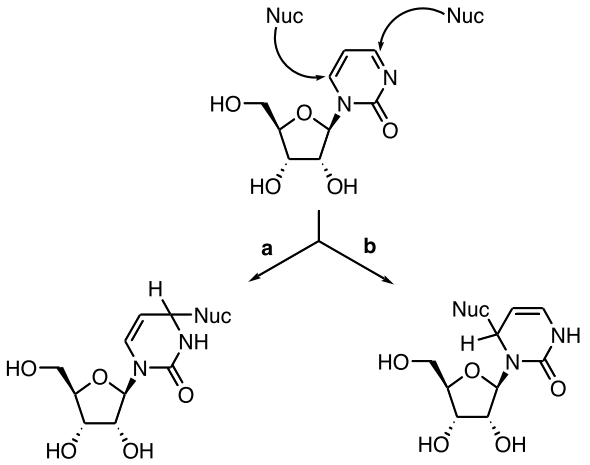
2a, X = NH_2
2b, X = H

(CDA).[8,11] Because of the similar mechanisms of CDA and adenosine (**2a**) deaminase (ADA),[12] it is not surprising that zebularine (**1b**) and nebularine (**2b**) behave as potent inhibitors of CDA and ADA, respectively.

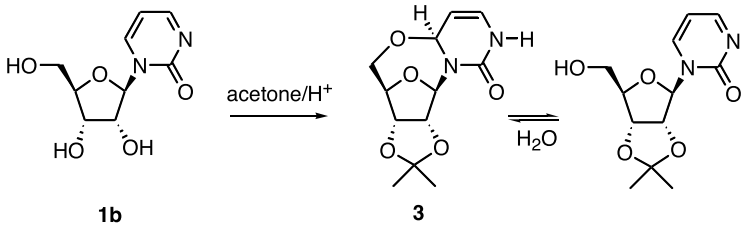
During the course of our investigations, it became clear that the simple structural change of removing the 4-amino group from cytidine (**1a**) caused a profound increase in the electrophilic character of the aglycon. This increase in electrophilicity explains the ease with which nucleophilic attack takes place at C4 and C6 on the 2-oxopyrimidine ring, and clarifies zebularine's activity as a potent CDA and DNA methyltransferase inhibitor (Scheme 1). For the same reason, the chemistry of zebularine is similarly rich and it has served as a versatile starting material for a number of syntheses.

ZEBULARINE AS A STARTING MATERIAL: THE MAGIC OF ITS CHEMISTRY

The smooth formation of the O^6,5′-cyclonucleoside **3**, which occurs during the synthesis of the acetonide of zebularine, results from an acid-catalyzed intramolecular "hydration" with participation of the 5′-hydroxyl group (Scheme 2).[13] Formation of **3** appears to be facilitated by the rigidity and skewing imposed on the



SCHEME 1



SCHEME 2

furanose ring by the acetonide group since a similar intramolecular reaction could not be observed with zebularine. The acetonide **3** exists in equilibrium with its open form in aqueous solution as revealed by NMR spectroscopy in D_2O. However, after lyophilization the compound cyclizes back to **3** and remains cyclized when dissolved in aprotic organic solvents.[13] The absolute configuration at C6 was later established by X-ray crystallography (vide infra).[14]

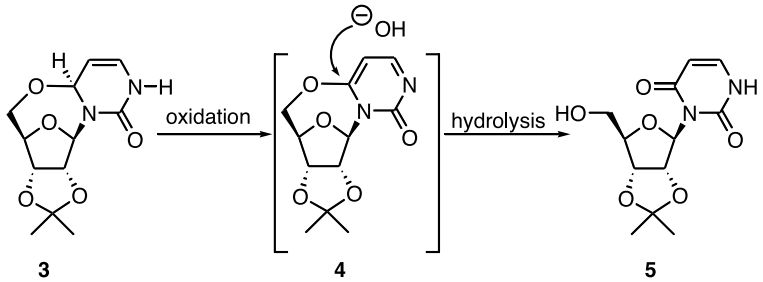
Synthesis of Isouridine

Previous reported examples on the chemical transformations of anhydronucleosides indicated that oxidation of **3** to stage **4** (Scheme 3) might provide a simple, one-step synthesis of isouridine after ring opening of the anhydride linkage.

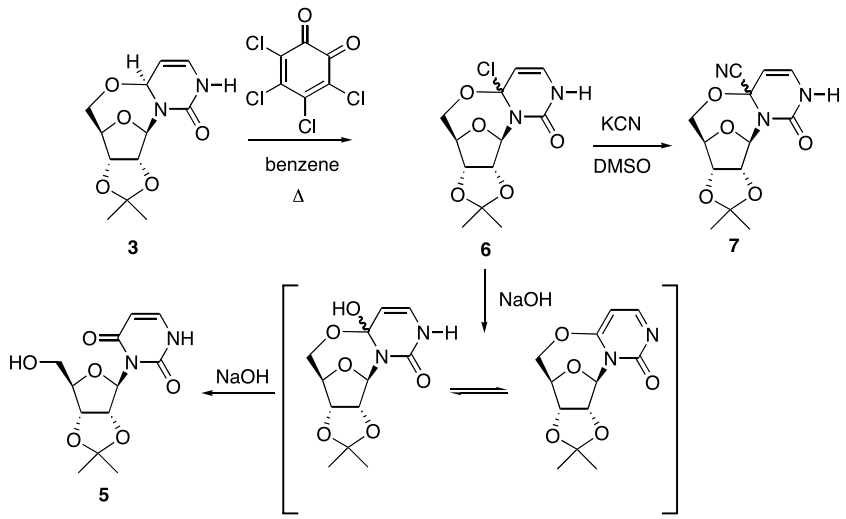
In practice, however, treatment of **3** with dichlorodicyanobenzoquinone (DDQ) provided only a low yield (10%) of the acetonide of isouridine (**5**). When *o*-chloranil was used, a totally different compound from **4** and **5** was obtained (Scheme 4). The new compound (**6**), which incorporated a chlorine atom from chloranil, had a reactive C-Cl bond which reacted readily with nucleophiles, such as KCN, to give **7**. When a similar exchange reaction was performed with NaOH, the intermediate spontaneously rearranged to **5**, which afforded the target isouridine (**8**) in excellent yield after removal of the acetonide group.[15]

2,4-Diazabicyclo[4.1.0]-Heptan-3-One Nucleosides and 5-Carbon-Substituted Zebularine Analogues

After protection of the NH group with benzoyl (**9**) or trimethylsilyl groups, the double bond in **3** can undergo smooth dihalocarbene insertion with :CX_2 (X = Cl

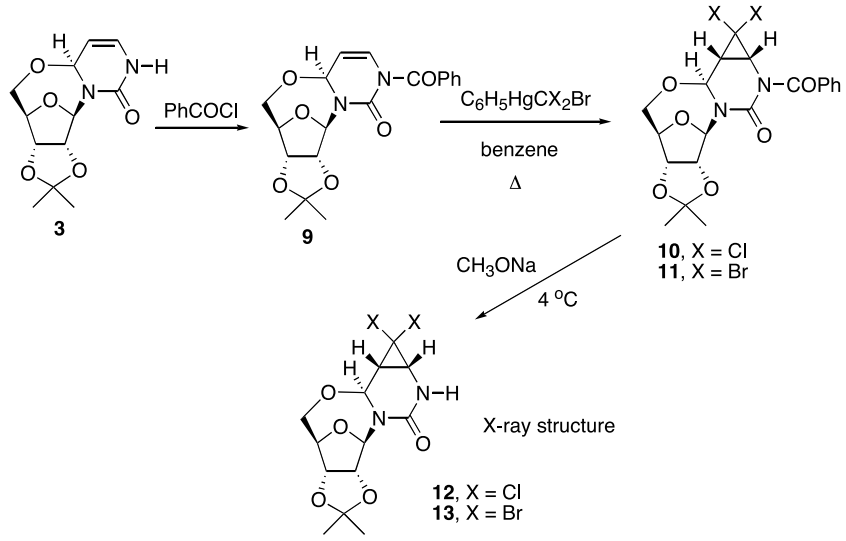


SCHEME 3

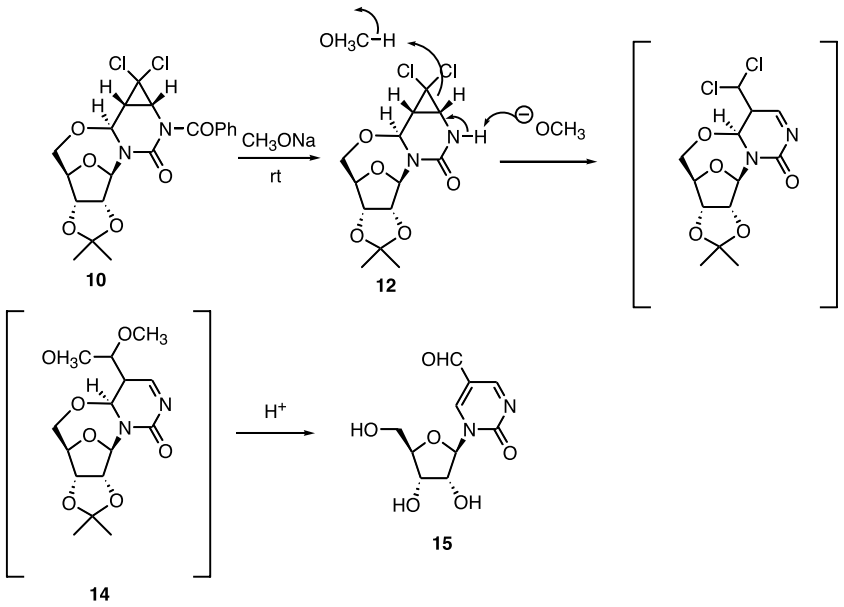


SCHEME 4

or Br) to give the corresponding adducts in excellent yields.[14] The reaction is completely sterospecific and only one isomer was isolated in each case. The use of the trimethylsilyl group permitted the synthesis of **12** directly from **3** in a single operation when X = Cl, but failed in the case of X = Br. A more general method consisted of using the *N*-benzoylated intermediate **9**, which after conversion to **10** or **11** reacted with one equivalent of NaOMe at low temperature to afford the desired targets **12** and **13** (Scheme 5).[14] In all these compounds, the C6(H)



SCHEME 5



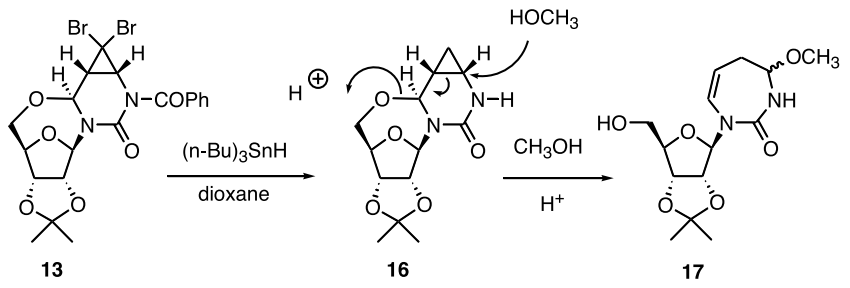
SCHEME 6

proton resonates at very low field (δ 4.81–5.53) indicating that the hydrogen has the down stereochemistry pointing to the furanose oxygen. This stereochemistry was later unequivocally confirmed by the X-ray structure of **12**.[14]

When the NaOMe treatment was performed at room temperature or higher, aldehyde **15** was obtained in excellent yield.[14] Under these conditions, bond cleavage of the external C–Cl bond in **12** is likely to proceed through an intermediate, which in the presence of NaOMe forms the dimethyl acetal derivative **14**. This dimethyl acetal converted readily to the conjugated aldehyde (**15**) during acidic workup (Scheme 6). Aldehyde **15** is an excellent starting substrate for the synthesis of 5-modified zebularine analogues.[16]

Ring Expansion Approach to 1,3,4,5-Tetrahydro-2*H*-1,3-Diazepin-2-One Nucleosides

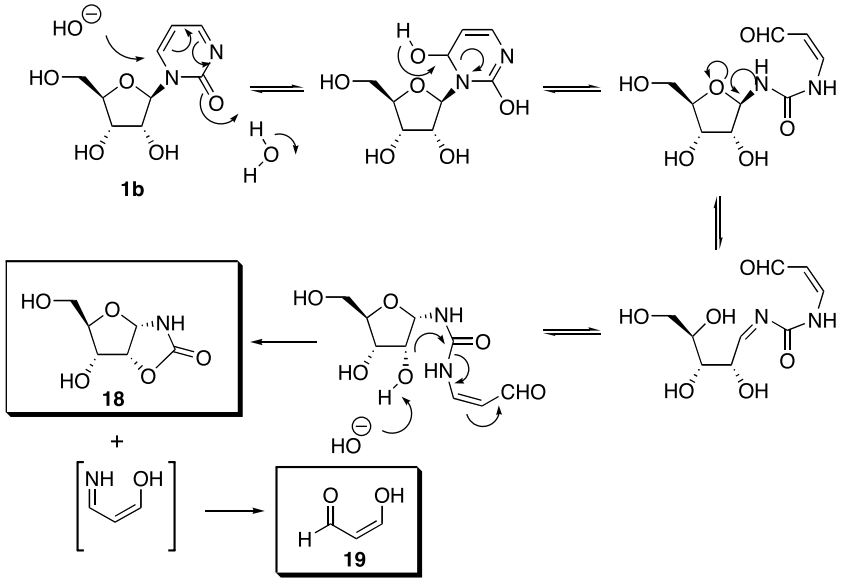
As seen above, the 2,4-diazabicyclo[4.1.0]heptan-3-one ring in **12** and **13** underwent facile ring opening with NaOMe. Conversely, the compounds were quite resistant to acid-catalyzed methanolysis at room temperature, probably due to the steric hindrance afforded by the halogen atoms. Indeed, after taking away the halogen atoms from **13** with *n*-Bu_3SnH, and following the removal of the *N*-benzoyl group with one equivalent of NaOMe, the deprotected compound (**16**) solvolyzed rapidly and quantitatively to the diazepinone nucleoside **17** in methanol at 0°C after the pH of the mixture was lowered to 4 (Scheme 7). As expected this material was obtained as a mixture of C4 epimers.



SCHEME 7

Base-Catalyzed Decomposition of Zebularine

The acid resistance of zebularine is in contraposition to its behavior in alkali. In 1969, Oyen had shown that a rapid and irreversible reaction occurred when zebularine was dissolved in aqueous 0.1 N NaOH.[3] This reaction was characterized by a red shift in the parent UV maximum from 303 to 315 nm, followed by a blue shift back to 277 nm after neutralization. Since the parent base and its *N*1-methyl derivative are resistant to alkali, the ribose moiety must play an important role in the sensitivity to alkali, probably acting as a strong electron-withdrawing group. Participation of the 5′-hydroxyl group to form a O^6,5′-cyclonucleside of the type shown in Scheme 1 was ruled out since the 5′-*O*-methylzebularine analogue underwent a similar decomposition. We were able to demonstrate that hydroxide attack at C6 initiates a cascade of reactions that are consistent with both UV and NMR spectral observations during decomposition and



SCHEME 8

after neutralization (Scheme 8).[17] The complete characterization of the α-*cis*-fused carbamate (**18**) and malonaldehyde (**19**) as final degradation products is consistent with the proposed mechanism, which requires an anomerization step. That the stereochemistry of the carbamate appears to be controlled by the stereochemistry of the 2′-hydroxyl group was demonstrated by the isolation of the β-*cis*-fused carbamate when ara-zebularine was decomposed in alkali.[17]

ZEBULARINE AS A CYTIDINE DEAMINASE INHIBITOR

Preliminary studies indicated that zebularine functioned as a competitive inhibitor of both yeast and mammalian cytidine deaminase (CDA) with a K_i value *ca*. two orders of magnitude lower than the K_m value of cytidine.[8] Since covalent hydration is extremely unfavorable in free solution (Scheme 1, path **a**, Nuc = OH), the 3,4-hydrate of zebularine, with an sp^3 carbon at C4 was expected to possess extremely high affinity for the enzyme to account for the inhibition observed. Taking into account the unfavorable equilibrium for hydration, Wolfenden estimated the equilibrium constant for dissociation of a single isomer of hydrated zebularine with bacterial CDA to be 1.2 pM, more than 8 orders of magnitude lower than the K_m for the substrate cytidine.[18] This enzyme-generated transition-state analogue has been detected by X-ray analysis with the inhibitory hydroxyl oxygen interacting with the zinc atom at the active site of the enzyme, similar to the case of adenosine deaminase with nebularine (**2b**).[19]

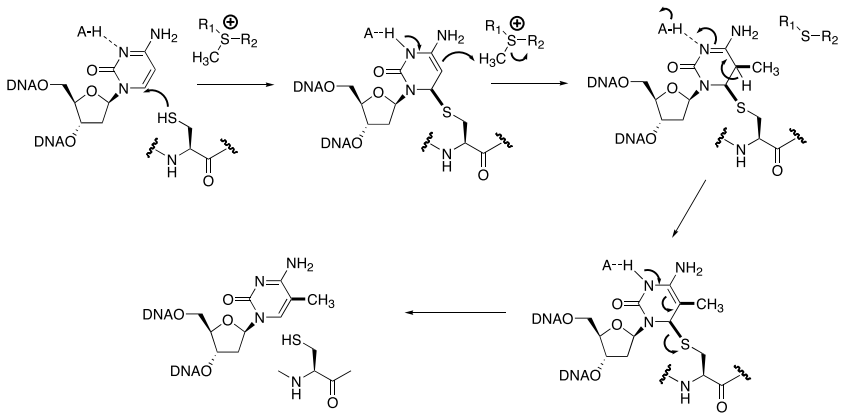
As a CDA inhibitor, zebularine in solution is very stable (Table 1), more so than the prototypic inhibitor, tetrahydrouridine, thus making it a useful adjuvant for oral administration with drugs such as arabinofuranosyl cytosine (ara-C)[9] and 5-azacytidine-2′-deoxyriboside,[20] both of which are readily deaminated by CDA. Coadministration of zebularine with either one of these drugs produced significant increases in life span of tumor bearing mice with L1210 or P388 leukemia.[9,20]

ZEBULARINE AS A DNA METHYLASE INHIBITOR

In contrast to the ability of zebularine to undergo nucleophilic attack at C4 (Scheme 1, path **a**), which is responsible for its CDA inhibitory properties, the capacity of zebularine to function as an inhibitor of DNA methylation is associated with its complementary ability to undergo covalent hydration at C6 (Scheme 1, path **b**). Two other drugs known to inhibit DNA methylation, 5-azacytidineriboside (5-aza-CR) and 5-azacytidine-2′-deoxyriboside (5-aza-CdR), undergo nucleophilic attack at C6 in a similar fashion.[21] Unfortunately, both 5-aza-CR and 5-aza-CdR are

TABLE 1 Stability of Zebularine (50 μM) in Aqueous Solution at 37°C

pH	1.0	2.0	5.0	7.4	>12
$t_{1/2}$ (h)	44	68	Stable	510	dec.



SCHEME 9

very unstable and the triazine ring undergoes rapid decomposition, even at neutral pH.[22]

The mechanism for DNA methyl transfer is known to involve nucleophilic attack at C6 of a cytosine ring by a thiol group of an invariant cysteine residue at the active site of the enzyme (Scheme 9).[23] The target cytosine residue is embedded in the DNA duplex with a recognition sequence of G*C*GC (substrate italicized) in the case of bacterial M.*H*haI DNA methyltransferase. Initially, it was difficult to understand how the DNA methyltransferase acted on its target cytosine entrenched in a double helix, away from a seemingly inaccessible concave active pocket of the enzyme. This puzzle was solved by the crystal structure of the bacterial enzyme showing the cytosine base completely rotated out of the DNA in a process now recognized as "base flipping."[24] Recently a 13-mer oligodeoxynucleotide (ODN) containing a 5′-CXGC-3′/5′-GCGC-3′ recognition sequence with the 2-(1*H*)-pyrimidinone ring of zebularine (X) at the target site was shown by X-ray crystallography with the base rotated out of the helix and into the catalytic pocket of the enzyme, forming the expected covalent adduct of the type illustrated in Scheme 1 (path b, Nuc = SH).[25]

When two double-stranded ODNs incorporating either 5-azacytosine or 2-(1*H*)-pyrimidinone were tested as inhibitors of methyl transfer to a 24 bp long ODN containing a hemimethylated 5′-G*C*GC-3′ DNA substrate (5-methylcytosine [M] opposite to the target *C*), the level of inhibition observed was similar for both ODNs when X was replaced by either nucleobase (Figure 1). This result showed that the two heterocyclic moieties of 5-aza-CR (or 5-aza-CdR) and of zebularine have comparable reactivity towards nucleophilic attack by the cysteine's SH group.[26]

Induction of *p16* Gene Expression and Inhibition of DNA Methylation

Gene silencing by the abnormal methylation of promoter regions of regulatory genes is commonly associated with cancer.[27] Therefore, silenced tumor suppressor

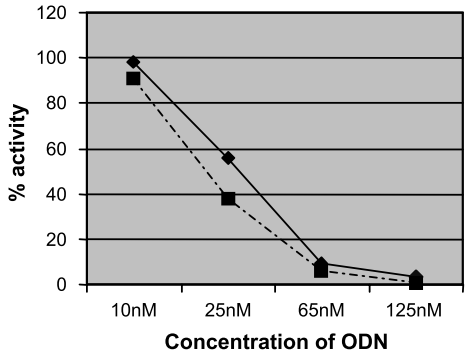


FIGURE 1 Relative inhibition of M.*H*haI methyl transfer by 5′-TGTCA*GXGC*ATGG-3′/3′-ACAGTCGMG-TACC-5′. X = 5-azacytosine (◆) or X = 2-(1*H*)-pyrimidinone (■).

genes present themselves as obvious targets for reactivation by methylation inhibitors such as 5-azacytosine nucleosides (5-aza-CR and 5-aza-CdR) and zebularine. One of the regulatory genes that commonly appear silenced by hypermethylation in several human cancer cell lines is *p16*.[28] When T24 human bladder carcinoma-derived cells, which contain a transcriptionally silent hypermethylated *p16* gene promoter, were treated with 5-azacytosine nucleosides, or with zebularine, induction of *p16* expression was successfully achieved.[29] In the case of zebularine, strong induction of *p16* expression was observed at 100 μM (Figure 2). Although zebularine was not as potent as 5-aza-CR or 5-aza-CdR, which induced comparable *p16* expression at doses *ca.* 10-and 100-fold lower (data not shown), zebularine's stability allows it to be administered continuously to cells resulting in robust *p16* expression.[29]

Zebularine was less toxic than 5-aza-CR or 5-aza-CdR as assessed by measuring average plating efficiency. Similarly, doses of zebularine that induced equivalent levels of *p16* expression as 5-aza-CR or 5-aza-CdR were minimally cytotoxic.[29] It is also of interest to note that dormant *p16* was effectively reactivated in tumors by zebularine in vivo after intraperitoneal and oral administration of the drug at doses of 500–1000 mg/kg.[29]

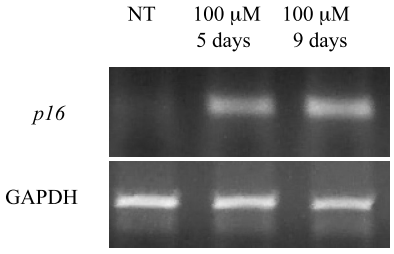


FIGURE 2 Reverse transcription polymerase-chain reaction (RT-PCR) analysis performed on total cellular RNA isolated from T24 cells treated continuosly with zebularine. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression served as control for input DNA. NT = no treatment.

Remethylation and resilencing of tumor suppressor genes is a common problem for drugs, such as 5-aza-CdR, and zebularine is no exception. This represents a potential complication in the clinical application of these drugs, which requires continuous administration. The remethylation problem was successfully circumvented by continuous exposure to zebularine owing to its low toxicity. Indeed, continuous exposure to zebularine from 100 μM to 400 μM for up to 40 days led to an induction of *p16* expression from day 5, which increased over time with no signs of cellular toxicity.[30] A sustained high level of *p16* expression was achieved by sequentially treating the cells with a single dose of 5-aza-CdR (1 μM) followed by continuous treatment with 500 μM of zebularine, auguring well for the use of both drugs in combination.[30]

The lower toxicity of zebularine following continuous administration can be appreciated by looking at colony formation in both normal LD419 bladder fibroblasts and T24 tumor cells (Figure 3). The effect of zebularine on normal fibroblast is practically absent, whereas the effect on T24 cells is dramatic. In these experiments, the average doubling time for normal LD419 fibroblasts changed from 26.8 h (untreated) to 27.0 h (100 μM continuous zebularine for 14 days), whereas for T24 cells it increased from 19.7 h (untreated) to 26.6 h (100 μM continuous zebularine for 14 days).

Selective Depletion of DNMT1 Methyltransferase

The mammalian DNA methyltransferases (DNA methyltransferase 1 [DNMT1], DNMT3a, and DNMT3b) seem to work cooperatively to help establish

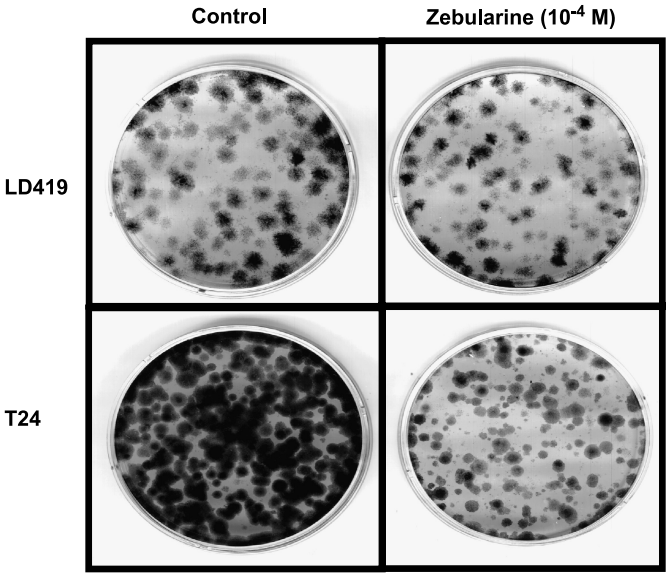


FIGURE 3 Effects of continuous zebularine on colony formation in normal LD419 fibroblasts and T24 bladder cancer cells.

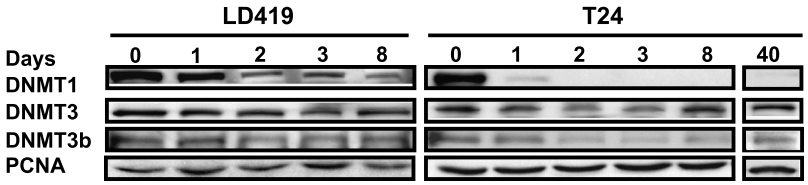


FIGURE 4 Levels of DNMT-1, -3a, and -3b proteins after continuous zebularine (100 μM) administration for the indicated days in T24 cells and normal LD419 fibroblasts. PCNA serves as internal control for cellular division.

and maintain genomic methylation patterns, which are of critical importance in biological processes. DNMT1 is mostly associated with maintenance methylation, whereas DNMT3a and DNMT3b appear to be implicated in de novo methylation. Western blot analysis showed a drastic depletion of DNMT1 by day 1 of treatment with zebularine in T24 cells, with virtually no extractable DNMT1 protein present in cells growing in the presence of drug (100 μM) for 40 days (Figure 4).[30] DNMT3b was affected next, followed by DNMT3a, both of which were affected to lesser extents. The levels of these enzymes were mostly unaffected in normal LD419 fibroblasts with partial depletion observed for DNMT1 (Figure 4). Because the levels of DNMT RNA transcripts were unaffected by zebularine (data not shown), the depletion of DNMT protein levels is most likely due to trapping of the enzymes to the zebularine-substituted DNA via the mechanism shown in Scheme 9.

Why are 5-aza-CR and 5-aza-CdR More Potent than Zebularine? A Metabolic Bottleneck

The inhibition of DNA methyltransferase is envisioned to result from the formation of a covalent complex between the enzyme and zebularine-substituted DNA.[26] As described in Figure 1, inhibition of DNA methyltransferase was similar for ODNs containing either 5-azacytosine or 2-(1*H*)-pyrimidinone nucleobases. However, higher doses of zebularine are required to achieve comparable levels of *p16* reactivation in T24 cells. Metabolic activation of zebularine requires that it be phosphorylated and incorporated into DNA. Incorporation of zebularine into DNA necessitates critical levels of 2′-deoxyzebularine-5′-triphosphate (dZTP) which can be formed by a complex metabolic route that could explain its weaker potency (Figure 5). A quantitative assessment of the phosphorylation and DNA incorporation of zebularine in T24 cells using 2-[^{14}C]-zebularine revealed that the drug is readily phosphorylated to form the corresponding 5′-mono-(ZMP), di-(ZDP) and triphosphates (ZTP) in a dose-and time-dependent manner.[31] Two additional zebularine-containing metabolites were also observed and identified as diphosphocholine (ZDP-Chol) and diphosphoethanolamine adducts.[31] Intracellular concentrations of ZTP and ZDP-Chol were comparable and greatly exceeded those of the other metabolites. When DNA and RNA levels of incorporation were compared, RNA incorporation surpassed DNA incorporation by at least 7-fold.[31] These results were confirmed by reverse-phase HPLC analysis of the free

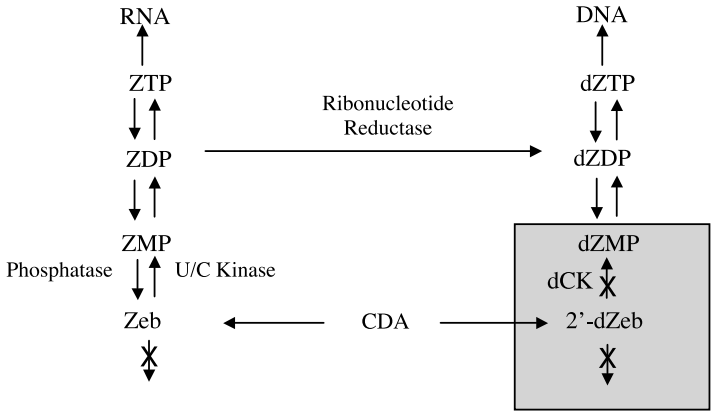


FIGURE 5 Proposed metabolic pathway of zebularine (U/C kinase = uridine/cytidine kinase; CDA = cytidine deaminase; dCK = deoxycytidine kinase).

nucleosides generated from the complete enzymatic digestion of the isolated DNA and RNA, which revealed that the radiolabel co-eluted with zebularine itself in RNA and with authentic 2′-deoxyzebularine (2′-dZeb) in DNA. Because formation of zebularine riboside metabolites appears to be quite robust, conversion of zebularine-5′-diphosphate (ZDP) to 2′-deoxyzebularine-5′-diphosphate (dZDP) by ribonucleotidediphosphate reductase (Figure 5) appears to be the rate-limiting step and may explain zebularine's weaker potency. Unfortunately, the use of 2′-deoxyzebularine (2′-dZeb) did not overcome this deficiency and was completely ineffective, perhaps due to lack of recognition by the activating enzyme deoxycytidine kinase (dCK).[29]

The monophosphorylation of zebularine is most likely mediated by uridine-cytidine kinase (U/C Kinase) (Figure 5). To test this hypothesis, the metabolic activation of zebularine with and without cyclopentenyl uracil (CPEU), a potent non-cytotoxic inhibitor of uridine-cytidine kinase, was investigated.[32] The phosphorylation of zebularine was substantially reduced by 10 μM CPEU and almost completely abrogated by 50 μM CPEU, suggesting that uridine-cytidine kinase indeed catalyzes the initial phosphorylation step.[31] Interestingly, of the endogenous uridine-cytidine kinase substrates uridine and cytidine, only cytidine was effective in competing with zebularine and inhibiting its phosphorylation. In this regard, 50 μM cytidine was almost as effective as 50 μM CPEU.[31]

It can be surmised from the foregoing discussion that in biochemical terms zebularine behaves like a cytidine analogue, albeit one with unique properties. Its metabolic activation to form a 2′-deoxy-5′-triphosphate metabolite required for incorporation into DNA (Figure 5) is complex and inefficient. The results reported here point to a prodrug strategy for 2′-deoxyzebularine-5′-monophosphate that could be investigated as a means of increasing DNA incorporation and thus increasing its potency. Of course, increased incorporation into DNA carries with it the risk of additional toxicity and the abrogation of zebularine's relatively benign

toxicity during extended treatment. It is this minimal toxicity of zebularine that, when coupled with the favorable chemical stability which allows facile oral administration, makes it such a promising clinical candidate for reversing DNA methylation and use as a drug for both cancer chemotherapy and possible chemoprevention.

CONCLUSIONS

The seemingly small change brought about by the removal of the exocyclic amino group of cytidine to engender zebularine is accompanied by a tremendous transformation in its chemistry that manifests itself in a plethora of unique chemical and biological properties that can be exploited synthetically, as well as pharmacologically, for two important areas of cancer therapy: cytidine deaminase and inhibition DNA methyl transferase.

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