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Metabolic activation of zebularine, a novel DNA methylation inhibitor, in human bladder carcinoma cells

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Abstract

Zebularine (2(1H)-pyrimidinone riboside, Zeb), a synthetic analogue of cytidine that is a potent inhibitor of cytidine deaminase, has been recently identified as a general inhibitor of DNA methylation. This inhibition of DNA methyltransferase (DNMT) is hypothesized to be mechanism-based and result from formation of a covalent complex between the enzyme and zebularine-substituted DNA. Metabolic activation of Zeb thus requires that it be phosphorylated and incorporated into DNA. We have quantitatively assessed the phosphorylation and DNA incorporation of Zeb in T24 cells using 2-[^{14}C]-Zeb in conjunction with gradient anion-exchange HPLC and selected enzymatic and spectroscopic analyses. The corresponding 5′-mono-, di- and triphosphates of Zeb were readily formed in a dose- and time-dependent manner. Two additional Zeb-containing metabolites were tentatively identified as diphosphocholine (Zeb-DP-Chol) and diphosphoethanolamine adducts. Intracellular concentrations of Zeb-TP and Zeb-DP-Chol were similar and greatly exceeded those of other metabolites. DNA incorporation occurred but was surpassed by that of RNA by at least seven-fold. Equivalent levels and similar intracellular metabolic patterns were also observed in the Molt-4 (human T-lymphoblasts) and MC38 (murine colon carcinoma) cell lines. For male BALB/c *nu/nu* mice implanted s.c. with the EJ6 variant of T24 bladder carcinoma and treated i.p. with 500 mg/kg 2-[^{14}C]-Zeb, the in vivo phosphorylation pattern of Zeb in tumor tissue examined 24 h after drug administration was similar to that observed in vitro. The complex metabolism of Zeb and its limited DNA incorporation suggest that these are the reasons why it is less potent than either 5-azacytidine or 5-aza-2′-deoxycytidine and requires higher doses for equivalent inhibition of DNMT.

Keywords: Zebularine; 2(1H)-Pyrimidinone riboside; T24 bladder carcinoma; Phosphorylation; DNA and RNA incorporation; DNA methyltransferase

1. Introduction

Gene silencing by abnormal methylation of the promoter regions of regulatory genes is frequently associated with cancer [1]. Among the regulatory genes that are commonly methylated in cancer cells are *RB1* in retinoblastomas, *VHL* in sporadic renal cell carcinomas, *H19* in Wilms' tumors, *p15* in leukemias [2] and the *p16* tumor suppressor gene in several human cancer lines [3]. Consequently, therapeutic use of DNA methylation inhibitors has been proposed as a strategy to reactivate antiproliferative, apoptotic and differentiation-inducing genes in cancer cells [4–6]. The most well characterized and widely used agents to inhibit DNA cytosine methylation and reactivate silenced genes are the U.S. Food and Drug Administration-approved nucleoside analogues 5-azacytidine (azaciditine) and 5-aza-2′-deoxycytidine (decitabine) [7]. Although 5-aza-C and 5-aza-dC are currently undergoing extensive clinical evaluation, especially for the treatment of acute myeloid leukemia and myelodysplastic syndrome [8], their toxicity and chemical instability in solution has complicated their clinical use. Zebularine (2(1H)-pyrimidinone riboside) (Fig. 1) is a recently identified inhibitor of DNA methylation, that has demonstrated activity both in vitro and in vivo in mammalian cells by effecting the

Abbreviations: 5-Aza-C, 5-azacytidine; 5-Aza-dC, 5-aza-2′-deoxycytidine; Zeb, zebularine; Zeb-MP, zebularine-5′-monophosphate; Zeb-DP, zebularine-5′-diphosphate; Zeb-TP, zebularine-5′-triphosphate; Zeb-DP-EA, zebularine-5′-diphosphoethanolamine; Zeb-DP-Chol, zebularine-5′-diphosphocholine; 2′-dZeb-TP; 2′-deoxyzebularine-5′-triphosphate; CPEU, cyclopentenyl uridine; ODN, oligodeoxynucleotide; DMEM, Dulbecco's modified Eagle's medium; IC_{50}, drug concentration resulting in 50% inhibition of growth; CDA, cytidine deaminase; DNMT, DNA methyltransferase; PDE-1, snake venom phosphodiesterase-1; AP, alkaline phosphatase; UCK, uridine/cytidine kinase

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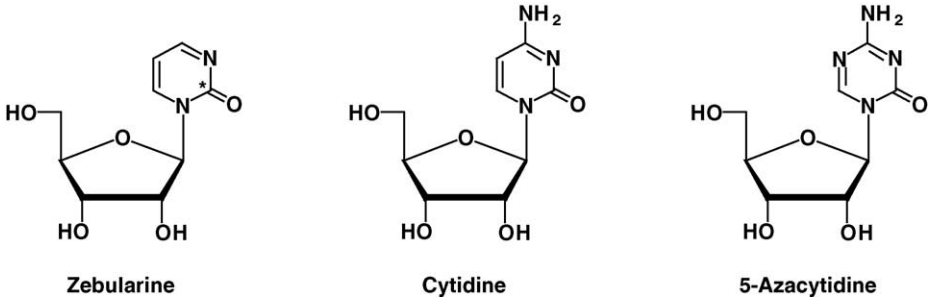

Fig. 1. Chemical structures of zebularine, cytidine and 5-azacytidine. The asterisk indicates the position of the [^{14}C]radiolabel in zebularine.

reactivation of a dormant *p16* tumor suppressor gene [9]. Although not as potent as 5-aza-C or 5-aza-dC, Zeb is attractive as an orally administered agent because of its chemical stability [10], its apparent bioavailability [9] and the low cytotoxicity observed during continuous treatment in vitro [11].

Zebularine was originally synthesized and evaluated as an inhibitor of cytidine deaminase (EC 3.5.4.5, CDA) [12,13]. The 2-(1H)-pyrimidinone ring of Zeb (Fig. 1) lacks an amino group at position 4 and this makes it susceptible to nucleophilic addition of water across the 3,4-double bond to form a covalent hydrated C-4 adduct at the active site of CDA [14–16]. Zebularine was found to be a good inhibitor of this enzyme ($K_i \approx 2$ μM), although about 10-fold less potent than the prototypic inhibitor tetrahydrouridine (THU) [12]. However, because of its acid stability in contrast to that of THU, it was considered potentially useful as an adjuvant for oral administration with drugs which were substrates for CDA such as arabinofuranosyl cytosine (cytarabine) and 5-aza-dC [17]. Zebularine was also found to possess modest antitumor activity against murine B16 melanoma, P388 leukemia and L1210 leukemia, and showed activity against the later when administered either i.p. or orally [18]. The relatively high doses (400–1600 mg/kg) employed in this study as well as the small differences in weight between drug-treated and control mice were additional indications of the low toxicity of Zeb.

In the case of 5-azacytidine and its analogues, it has been shown that inhibition of DNA methylation results from the incorporation of these compounds into DNA with the subsequent formation of stable complexes with DNA methyltransferase (EC 2.1.1.37) to effect enzyme inactivation [19,20]. In vitro experiments with synthetic oligodeoxynucleotides (ODNs) containing the 2-(1H)-pyrimidinone ring of Zeb at the target site for methylation have demonstrated the formation of tight complexes between the ODNs and bacterial methyltransferases [21,22]. These results suggest a mechanism-based inhibition of DNMT resulting from the formation of a covalent bond between the enzyme and the 6-position of the pyrimidine ring in the same manner as that postulated for 5-azacytosine-containing ODNs. Recently, X-ray crystallography has confirmed the formation of this expected covalent complex in an ODN duplex containing the 2-(1H)-pyrimidinone ring of Zeb with bacterial DNMT [23]. In addition, a short ODN containing 2-(1H)-pyrimidinone at the *HhaI* DNMT target site was shown to inhibit methyl transfer to virtually the same extent as a similar ODN substituted with 5-azacytosine [24]. Despite the fact that both Zeb and 5-aza-C have equal inhibitory potency towards DNMT as components of small ODN fragments, 5-aza-C is about 10-fold more potent than Zeb in inducing demethylation when used as a single agent [9]. Since DNA incorporation appears to be a requirement for activity, it is hypothesized that Zeb must first undergo phosphorylation followed by subsequent conversion to the corresponding 2′-deoxynucleotide before it can be incorporated. Thus, this observed disparity might be attributable to a less efficient metabolic activation of Zeb compared to 5-aza-C, and a subsequent diminished incorporation into DNA. Accordingly, we have investigated the in vitro and in vivo metabolic activation of Zeb in the same T24 human bladder carcinoma and tumorigenic EJ6 variant in which demethylation activity was recently demonstrated [9]. We report herein the results of these studies and show that the metabolism of Zeb is complex and results in limited DNA incorporation.

2. Materials and methods

2.1. Chemicals and reagents

Zebularine (1-(β-D-ribofuranosyl)1,2-dihydropyrimidin-2-one, 2(1H)-pyrimidinone riboside, NSC 309132) and [2-^{14}C]Zeb (51 mCi/mmol) were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (NCI). [Methyl-^{3}H]choline chloride (80 Ci/mmol) and [1-^{3}H]ethan-1-ol-2-amine hydrochloride (31 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. [5-^{3}H]Uridine (16.2 Ci/mmol) and [5-^{3}H]cytidine (28.1 Ci/mmol) were supplied by Moravek Biochemicals. Choline chloride, ethanolamine HCl, Tri-reagent® kits and selected nucleoside and nucleotide standards were obtained from Sigma Chemical Co. The enzymes