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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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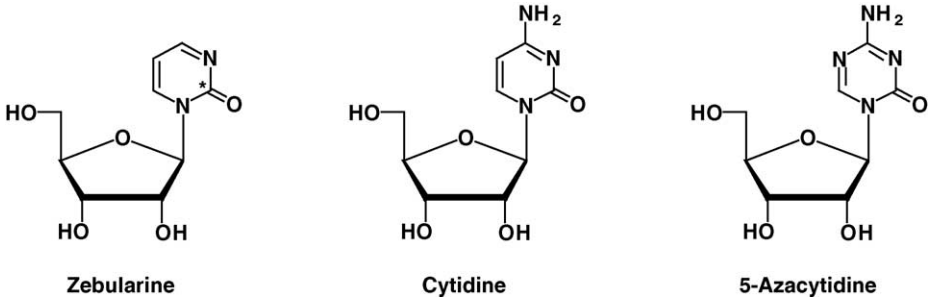


Fig. 1. Chemical structures of zebularine, cytidine and 5-azacytidine. The asterisk indicates the position of the [^{14}C]radiolabel in zebularine.

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

deoxyribonuclease I (DNase I, type II [bovine pancreas], EC 3.1.21.1), ribonuclease A (type I-A, [bovine pancreas], EC 3.1.27.5), PDE-1 (type VII [*Crotalus atrox* venom], EC 3.1.4.1), and AP (type III [*E. coli*], EC 3.1.3.1) were likewise purchased from Sigma. Zeb-TP and 2′-dZeb-TP standards were chemically synthesized by Trilink Biotechnologies, Inc. from the corresponding parent nucleosides and each had an HPLC purity >98%. A Zeb-MP standard was produced by complete enzymatic conversion of Zeb-TP by PDE-1. 2′-Deoxyzebularine [25] and CPEU [26] were synthetic products that were available from previous studies in our laboratories. All other chemicals and reagents were of the highest quality commercially available.

2.2. Cell culture

Human T24 bladder carcinoma and human Molt-4 lymphoid cells were obtained from the American Type Culture Collection. The EJ6 cell line, a tumorigenic derivative of T24 cells, was kindly provided by Dr. Eric J. Stanbridge, Department of Microbiology and Molecular Genetics, College of Medicine, University of California at Irvine, and murine MC38 colon cancer cells were a gift from Dr. Steven A. Rosenberg, Surgery Branch, Center for Cancer Research, NCI. T24, EJ6 and MC38 cells were maintained in culture using DMEM media (high glucose), while Molt-4 cells were grown in RPMI 1640 media. For double-label studies employing [methyl-^{3}H]choline chloride and [1-^{3}H]ethan-1-ol-2-amine hydrochloride, cells were grown in choline chloride free DMEM media purchased from Biological Industries. All media were supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were in logarithmic growth at the time of use and were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Effect of zebularine on T24 cell growth

Exponentially growing T24 cells were cultured in 24-well plates (10^5 cells per well) overnight. Cells were then washed with fresh medium and varying concentrations (0–1000 μM) of 5-aza-C or Zeb were added. After incubation for 48 h, cells were trypsinized, collected and counted in a ZB1 Coulter Counter. The cell growth rate was expressed as a percentage of the increase in cell number of the comparable untreated control cultures. A growth-inhibition curve was generated for each compound by fitting the above data to a sigmoidal dose–response function that allowed variable slope using Prizm 4.0, a scientific graphing and curve-fitting program (GraphPad Software). The respective IC_{50}s were determined directly from the calculated curves and represent an absolute 50% growth inhibition.

2.4. Separation and measurement of zebularine and its cellular metabolites

2.4.1. Preparation of cell extracts

After the appropriate incubations with [2-^{14}C]Zeb, cells were washed three times with cold PBS, treated with trypsin and collected by centrifugation at 1500 × *g* for 10 min. The resulting cell pellet was extracted with 0.50 ml of cold (4 °C) 60% methanol (HPLC grade) by vigorous vortex mixing, following which the methanolic extract was removed and heated at 95 °C for 2.5 min. The heated extract was centrifuged at 12,000 × *g* for 10 min and the clear supernatant fraction was removed and evaporated to dryness under N_2. This sample was reconstituted in 250 μl H_2O and aliquots were subjected to gradient anion-exchange chromatography as described below.

2.4.2. Gradient anion-exchange HPLC

Separations of Zeb and its phosphorylated metabolites were carried out using a Hewlett-Packard 1100 HPLC system with a diode-array ultraviolet absorption detector and controlled by ChemStation software (Version 6.01). A Whatman Partisil-10 SAX column (250 mm × 4.6 mm) certified to have greater than 25,000 plates/m was used with the following elution program: 0–5 min, 100% buffer A (0.01 M ammonium phosphate, native pH); 5–20 min, linear gradient to 25% buffer B (0.7 M ammonium phosphate with 10% methanol); 20–30 min, linear gradient to 100% buffer B; 30–40 min, isocratic buffer B; 40–55 min, linear gradient to 100% buffer A and equilibration. The flow rate was 2 ml/min throughout. One-minute fractions were collected and radioactivity was determined by scintillation spectrometry. Fractions containing radiolabeled Zeb-containing nucleotides were quantified based on the known specific activity of [2-^{14}C]Zeb. To confirm that intact Zeb was present in these radiolabeled nucleotides, aliquots of the cell extract were enzymatically treated to degrade all nucleotides to nucleosides and analyzed by anion-exchange and reverse phase HPLC as described below.

2.4.3. Reverse-phase HPLC

Reverse-phase HPLC was employed for the separation and identification of Zeb nucleosides in cell extracts, for measuring [2-^{14}C]Zeb after enzymatic degradation of its phosphorylated metabolites, and for the DNA and RNA studies. Separations were carried out on a 5-μm Beckman Ultrasphere C_{18} column (250 mm × 4.6 mm) using the following gradient elution program with a flow rate of 2 ml/min throughout: 0–25 min, linear gradient from 1 to 25% methanol/H_2O (native pH); 25–30 min, isocratic with 25% methanol; 30–40 min, linear gradient to 1% methanol, and equilibration. One-minute fractions were collected and radioactivity was determined by liquid scintillation counting. Under these conditions the retention time of Zeb was 5 min and that for 2-deoxyzebularine was 7 min.

2.5. Enzymatic characterization of zebularine metabolites

Characterization of zebularine metabolites was carried out by selective enzymatic degradation of cellular extracts as previously described [27]. Briefly, the lyophilized methanolic cell extract was dissolved in 100 μl of 0.01 M Tris–HCl, pH 9.0 containing 1 mM $MgCl_2$; and 1.5 U AP and/or 0.03 U PDE-1 was added to the appropriate aliquots. Samples were incubated for 6 h at 37 °C, enzymes were inactivated by heating at 95 °C for 2.5 min, and aliquots were then analyzed by anion-exchange and C_{18} reverse phase HPLC as described above.

2.6. Biosynthesis of doubly labeled choline and ethanolamine adducts

T24 cells were cultured in DMEM medium supplemented with 10% FCS and 4 mM L-glutamine but without endogenous choline or ethanolamine. The cells so grown exhibited doubling times (24 h) identical to those of T24 cells in choline replete medium. To determine whether choline and ethanolamine adducts are formed during zebularine metabolism, T24 cells were incubated with 100 μM [2-^{14}C]Zeb (1 μCi/ml) alone and in combination with either 28 μM [methyl-^{3}H]choline (10 μCi/ml) or 50 μM [1-^{3}H]ethanolamine (10 μCi/ml) for 24 h in separate parallel experiments. Thereafter, cells were harvested, washed and extracted with 60% methanol. Zebularine metabolites were determined by gradient ion-exchange HPLC as described above.

2.7. Dose-dependent formation of zebularine metabolites

Triplicate aliquots of logarithmically growing T24 cells (1×10^6 cells) were incubated for 6 h with concentrations of [2-^{14}C]Zeb (1 μCi/ml) ranging from 1–500 μM. At the end of the incubations, methanolic extracts were prepared and amounts of zebularine metabolites were determined by gradient anion-exchange HPLC as described before.

2.8. Rates of accumulation and decay of zebularine metabolites

T24 cells in logarithmic growth were incubated with 10 μM [2-^{14}C]Zeb (1 μCi/ml cell suspension). At predetermined intervals, 1-ml aliquots of the cell suspension were removed and the cells counted, harvested, extracted and analyzed for zebularine metabolites as previously described. After 24 h of exposure to drug, the cells were washed three times with fresh medium and re-incubated in drug-free medium. Aliquots of this incubation mixture were then sampled periodically for the ensuing 24-h period and the same work-up and analysis carried out. Apparent disappearance half-lives after removal of drug were determined for individual metabolites by non-linear least squares analysis of their concentration versus time profiles using a monoexponential decay function.

2.9. Effect of cytidine, uridine and CPEU on zebularine phosphorylation

Logarithmically growing T24 cells (1×10^6 cells) were incubated for 6 h with 10 μM [2-^{14}C]Zeb (1 μCi/ml) alone and in combination with either 10 or 50 μM of cytidine, uridine or CPEU, respectively. Control experiments were also carried out under similar conditions utilizing either 10 μM [5-^{3}H]Cyt (1 μCi/ml) or 10 μM [5-^{3}H]Urd (1 μCi/ml) alone and in combination with either 10 or 50 μM CPEU to assess inhibition of pyrimidine nucleoside salvage. At the end of incubation, duplicate aliquots of cells were harvested and methanolic extracts were prepared and analyzed as previously described.

2.10. Incorporation of zebularine into cellular DNA and RNA

DNA and RNA were isolated using the TRI-reagent® procedure [28,29]. Briefly, 5×10^7 T24 cells were incubated for 24 h with 10 μM [2-^{14}C]Zeb (1 μCi/ml). At the end of the incubation, cells were washed three times with cold PBS, harvested by trypsinization and collected by centrifugation. One milliliter TRI-reagent® (guanidine thiocyanate and phenol in a monophasic solution) was added to the cell pellet, solubilizing the DNA, RNA and protein. Chloroform (0.2 ml) was then added and the mixture was centrifuged at 12,000 × *g* for 15 min. RNA (in the aqueous phase) and DNA (in the interphase) were then separated according to the TRI-reagent® protocol, and the radioactivity in each quantified by liquid scintillation counting.

The isolated DNA and RNA from each time-point were individually hydrolyzed overnight at 37 °C in 1 ml of pH 7.4, 0.1 M HEPES buffer containing 140 μg of either DNase or Rnase and 0.02 U PDE-1 and 5 U AP. One hundred-microliter aliquots of this reaction mixture representing approximately 25 μg of hydrolyzed DNA or RNA were analyzed by reverse-phase HPLC as described above to determine the presence and amount of either [2-^{14}C]2′-dZeb (DNA) or [2-^{14}C]Zeb (RNA).

2.11. Metabolism of zebularine in EJ6-derived tumors

All animal care and experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the Ben-Gurion University of the Negev. Male BALB/c *nu/nu* mice (Harlan) ($n = 6$), 6–8 weeks of age, were implanted s.c. with 5×10^5 EJ6 cells each into both the left and right flanks. Mice were kept at 22 ± 1 °C in 40–60% relative humidity with alternating 12-h periods of light and dark. The animals were maintained on a diet of commercial, pelleted mouse food (Purina chow) and given

free access to food and water. After a period of 2–3 weeks during which time macroscopic tumors (50–200 mm^3) developed, the mice (mean weight = 25 ± 4 gm) were treated i.p. with 500 mg/kg [2-^{14}C]Zeb (500 μCi/kg). EJ6 tumors were removed from mice under ether anesthesia 24 h after drug administration and immediately frozen in liquid N_2 and stored at −70 °C pending sample work-up and analysis. Individual tumors (100–200 mg) were homogenized at 4 °C in 1.0 ml of 60% methanol using a Polytron homogenizer. The homogenate was heated at 95 °C for 3 min and centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was collected and evaporated under a nitrogen stream. The resulting residue was then dissolved in deionized water and appropriate aliquots subjected to anion-exchange HPLC analysis as described by Noy et al. [30].

3. Results

3.1. Effect of zebularine on T24 cell growth

Zebularine exhibited a moderate cytotoxic effect on logarithmically growing T24 cells in culture in contrast to the marked toxicity observed for 5-aza-C. As seen in Fig. 2, a 48-h exposure to Zeb inhibited T24 cell proliferation with an IC_{50} of 120 μM. Interestingly, this antiproliferative effect appeared to reach a plateau at concentrations of 500 μM and above for this exposure time. For 5-aza-C, the IC_{50} under these conditions was 17 μM and the cell growth rate was depressed to a greater extent than that for Zeb at concentrations above 125 μM.

3.2. Cellular metabolism of zebularine

A prerequisite for the biological activation of nucleoside analogues is their anabolic conversion to nucleotides. In the case of Zeb, conversion to the 2′-dZeb-TP appears to be necessary for incorporation into DNA and the ability to function as an inhibitor of DNA methylation. Therefore, the ability of Zeb to be phosphorylated was assessed using one murine and two human cell lines (Table 1). This was accomplished by exposing exponentially growing cells to 10 μM [2-^{14}C]Zeb for 6 h, then extracting the cells with 60% methanol, and subjecting the extract to gradient-elution, ion-exchange chromatography with radiochemical detection. Typical radiochromatograms obtained after Zeb treatment of T24 cells are shown in Figs. 3 and 4A. In addition to the parent drug, which elutes close to the void volume (2 min), five acidic metabolites were observed. Three of these metabolites with retention times of 9, 18 and 29 min, respectively, corresponded to the 5′-mono-, di- and triphosphates of Zeb. These assignments were made by comparison with authentic standards and are supported by enzymatic digestion of the extracts with PDE-1 and AP (Fig. 4B and C). Treatment of the cellular extract with PDE-1 resulted in the disappearance of the two HPLC peaks corresponding to Zeb-DP and Zeb-TP and an increase in the peak corresponding to Zeb-MP (Fig. 4B). AP digestion of another aliquot of this extract eliminated the HPLC peaks corresponding to Zeb-MP, Zeb-DP and Zeb-TP and generated a concomitant increase in parent nucleoside (Fig. 4C).

Table 1
Intracellular levels of zebularine metabolites in human and murine tumor cells after incubation with 10 μM [2-^{14}C]Zeb for 6 h

Metabolite	Concentration[a] (pmol/10^6 cells)		
	T24	Molt-4	MC38
Zeb-MP	13.8 ± 0.84	12.8 ± 2.5	30.6 ± 2.3
Zeb-DP	10.0 ± 0.69	5.2 ± 1.8	26.9 ± 3.2
Zeb-TP	25.8 ± 2.2	22.2 ± 4.1	152 ± 11
Zeb-DP-EA adduct	1.5 ± 2.0	1.1 ± 0.2	1.8 ± 0.4
Zeb-DP-Chol adduct	57.9 ± 2.8	17.4 ± 3.0	103 ± 8.7

[a] Mean ± S.D. from three experiments.

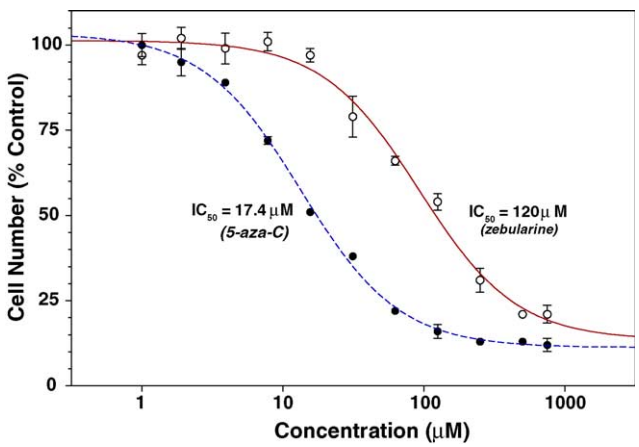


Fig. 2. Effect of 5-azacytidine (●) and zebularine (○) on human bladder carcinoma cell proliferation after treatment for 48 h. Points represent the mean ± S.D. (n = 6). T24 cell number averaged 4 × 10^5 for control at 48 h. Curves were fit ($r^2 > 0.990$) to a sigmoidal dose–response function allowing variable slope. Calculated IC_{50}s are indicated on the graph.

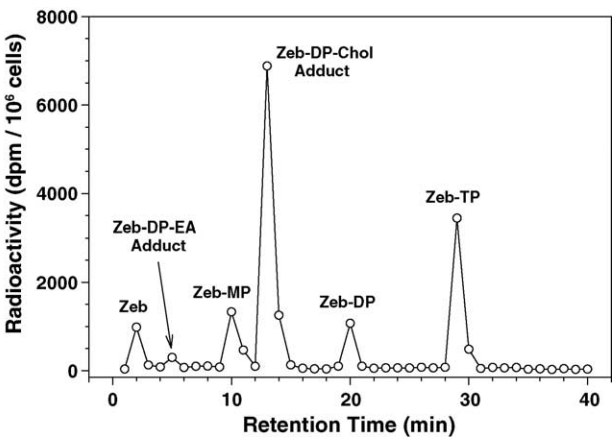


Fig. 3. HPLC radiochromatogram of [^{14}C]metabolites arising from incubation of T24 cells with 10 μM [2-^{14}C]Zeb (5 μCi/ml) for 6 h. Methanolic extracts were subjected to ion exchange HPLC as described in Section 2.

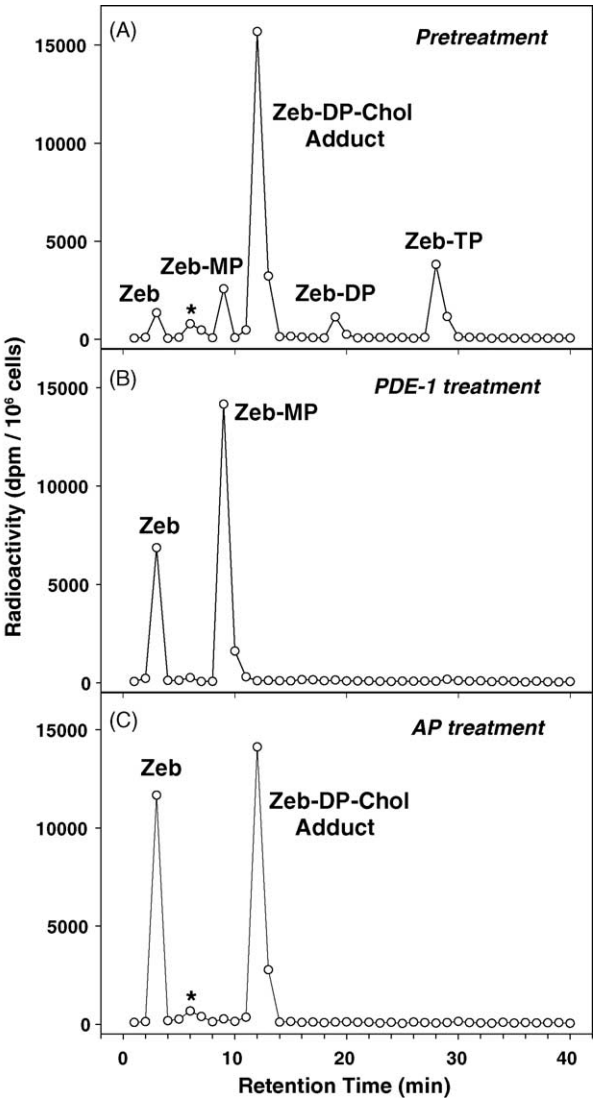


Fig. 4. Enzymatic characterization of zebularine metabolites isolated from T24 cells incubated with 10 μM [2-^{14}C]Zeb (1 μCi/ml) for 6 h. Equivalent aliquots of the methanolic extract from these cells were analyzed by anion-exchange HPLC prior to any enzymatic treatment (A) or following treatment with either PDE-1 (B) or AP (C). The peak corresponding to the Zeb-DP-EA adduct is indicated by an asterisk.

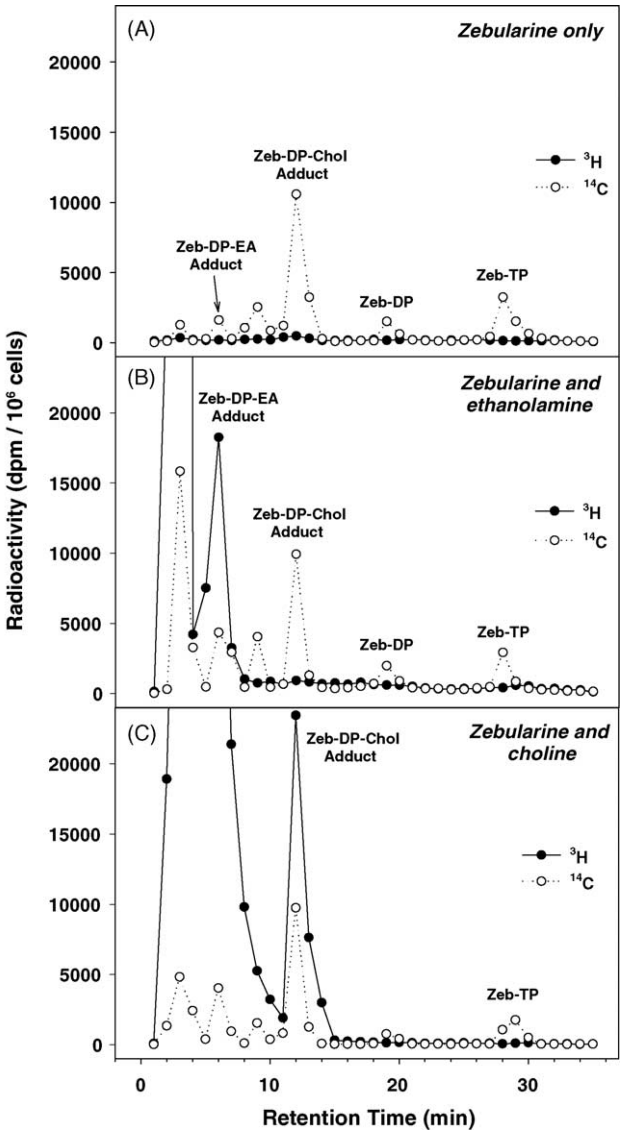


Fig. 5. Anion-exchange HPLC radiochromatograms of zebularine metabolites in cells treated with [2-^{14}C]Zeb and with either [^{3}H]choline or [^{3}H]ethanolamine. T24 cells (5×10^6) were incubated with 100 μM [2-^{14}C]Zeb (1 μCi/ml) for 24 h alone (A) or in combination with either 50 μM [^{3}H]ethanolamine (10 μCi/ml) (B) or 28 μM [^{3}H]choline (10 μCi/ml) (C). Cells were extracted and analyzed for zebularine metabolites as described in Section 2.

The remaining two metabolites, eluting at 5 min and 11 min respectively, were identified as phosphodiester conjugates of ethanolamine and choline based on the following evidence. Quantitative decomposition of these metabolites by PDE-1, with the apparent formation of Zeb-MP (Fig. 4B), strongly suggested that they contained a phosphodiester moiety. Their resistance to hydrolysis by AP (Fig. 4C) corroborated this and implied that a phosphomonoester conjugate of Zeb was unlikely. These results and the metabolic precedent of the formation of phosphodiester conjugates of ethanolamine and choline with other cytidine analogues such as arabinosyl cytosine [31] and 2′,3′-dideoxycytidine [32] led us to explore this possibility further. To assess whether choline and ethanolamine could be directly utilized by T24 cells to form Zeb-DP conjugates, T24 cells were incubated with [2-^{14}C]Zeb and [^{3}H]choline or [^{3}H]ethanolamine. HPLC and radiometric analysis of the cellular extracts resulting from these double-label experiments indicated that the two metabolites eluting with retention times of 5 and 11 min, respectively, did indeed contain both ^{14}C and tritium. When T24 cells were incubated with [2-^{14}C]Zeb and [^{3}H]ethanolamine, the peak eluting at 5 min contained both labels (Fig. 5B). When [^{3}H]choline was used with [2-^{14}C]Zeb, the double label was associated with the peak with a retention time of 11 min (Fig. 5C). Furthermore, no tritium was associated with either peak when [^{3}H]ethanolamine or [^{3}H]choline was incubated with T24 cells in the absence of Zeb (data

not shown). Thus, the two metabolites eluting at 5 and 11 min can be provisionally identified as phosphodiesters of Zeb conjugated with ethanolamine and choline, respectively.

Since the 2′-Zeb-TP and Zeb-TP standards coeluted in the gradient anion-exchange HPLC system that was used, it was only possible to indirectly determine the presence or absence of the deoxyribonucleotide. Selected cytosolic extracts from T-24 cells treated with 10 μM [2-^{14}C]Zeb were digested simultaneously with both AP and PDE-1 to convert all phosphate metabolites to the corresponding nucleosides. Analysis of these enzymatic digests was then carried out on a reverse-phase HPLC system capable of separating Zeb and 2′-dZeb. Only Zeb was detected, indicating that the vast majority of phosphorylated metabolites were ribonucleotides.

Zebularine phosphorylation was also evaluated in Molt-4 human lymphoblasts and in MC38 murine colon carcinoma under conditions comparable to those employed for T24 bladder carcinoma. As can be seen in Table 1, the five previously described zebularine metabolites were also observed in these two cell lines after a 6-h incubation with 10 μM [2-^{14}C]Zeb. For this particular time point, levels of each of the five zebularine metabolites were highest in the MC38 cells with the concentration of Zeb-TP being six-fold greater than in T24 or Molt-4 cells. Although the absolute concentration of phosphorylated metabolites varied in each cell line, Zeb-TP and the Zeb-DP-Chol adduct were always substantially greater than the others, with the former being highest in Molt-4 and MC38 and the latter greatest in T24 bladder carcinoma. Individual metabolite levels were comparable in the two human cells lines except for the Zeb-DP-Chol adduct which was three-fold higher in T24 cells.

3.3. Dose-dependent formation of zebularine metabolites

The formation of the five zebularine metabolites was evaluated as a function of zebularine dose in T24 cells. Cells were incubated with increasing concentrations of [2-^{14}C]Zeb (1–500 μM) for 6 h, at which time the levels of zebularine metabolites were measured. As can be seen in Fig. 6, levels of all metabolites increased with increasing zebularine dose. The rates of this dose-dependent formation were the greatest for Zeb-TP and the Zeb-DP-Chol adduct, the two metabolites present in greatest concentration. At zebularine doses of 100 μM and above, intracellular concentrations of these two major metabolites were more than four-fold greater than the other metabolites and approached or exceeded the nanomole per million cell level. Furthermore, the formation of Zeb-TP did not appear to be saturable in contrast to the other metabolites whose levels begin to plateau at 250 μM Zeb (Fig. 6). Thus, increasing the Zeb concentration to 500 μM produced Zeb-TP levels that exceeded those of the Zeb-DP-Chol adduct.

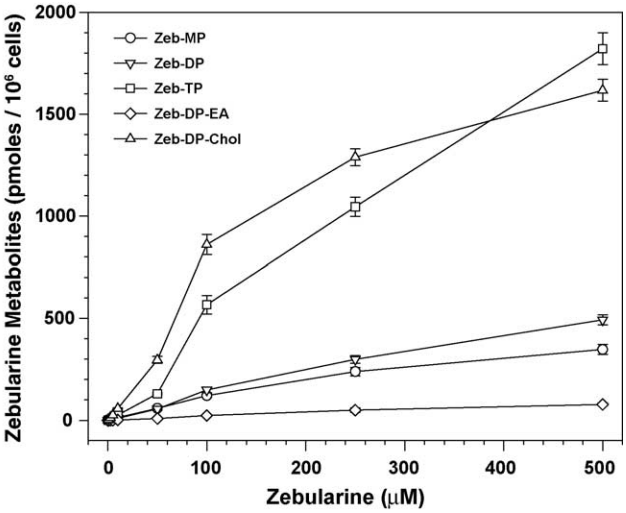


Fig. 6. Dose-dependent formation of phosphorylated zebularine metabolites in T24 cells. Cells were incubated with [2-^{14}C]Zeb for 6 h and then extracted and analyzed as described in Section 2. Data are mean ± S.D. (n = 3).

3.4. Rate of accumulation and decay of zebularine metabolites

The 24-h intracellular accumulation of individual zebularine metabolites was evaluated for T24 cells using a dose of 10 μM [2-^{14}C]Zeb. After incubation with radiolabeled drug for an initial 24-h period, cells were washed three times and reincubated in drug-free media so that the decay rate of these metabolites could be determined over the ensuing 24-h period. As described in Section 2, levels of the various zebularine metabolites were determined at timed intervals over the entire 48-h period. Fig. 7 depicts the concentration versus time profiles of the intracellular accumulation and decay of the individual zebularine metabolites. Zeb-MP, Zeb-DP and Zeb-TP all exhibited an initial rapid rate of intracellular accumulation over the initial 4 h before reaching a more or less constant steady-state level by 8–12 h (Fig. 7A). The rate of accumulation of the phosphodiester adducts was more gradual and sustained, since both the Zeb-DP-EA and Zeb-DP-Chol adducts increased over the entire 24-h period and did not appear to reach a steady-state (Fig. 7B). Upon removal of parent drug, Zeb-DP and Zeb-TP decayed very rapidly with estimated half-lives of 0.6 and 1.2 h, respectively. The intracellular disappearance of the phosphodiester adducts was much more gradual with roughly equivalent half-lives of 5.5 h for the Zeb-DP-EA adduct and 5.9 h for the Zeb-DP-Chol adduct. In contrast to the zebularine 5′-phosphates, these two metabolites could still be detected in the cellular extract 24 h after removal of drug. The intracellular elimination rate of Zeb-MP was intermediate to that of the other metabolites with a half-life of 4.2 h.

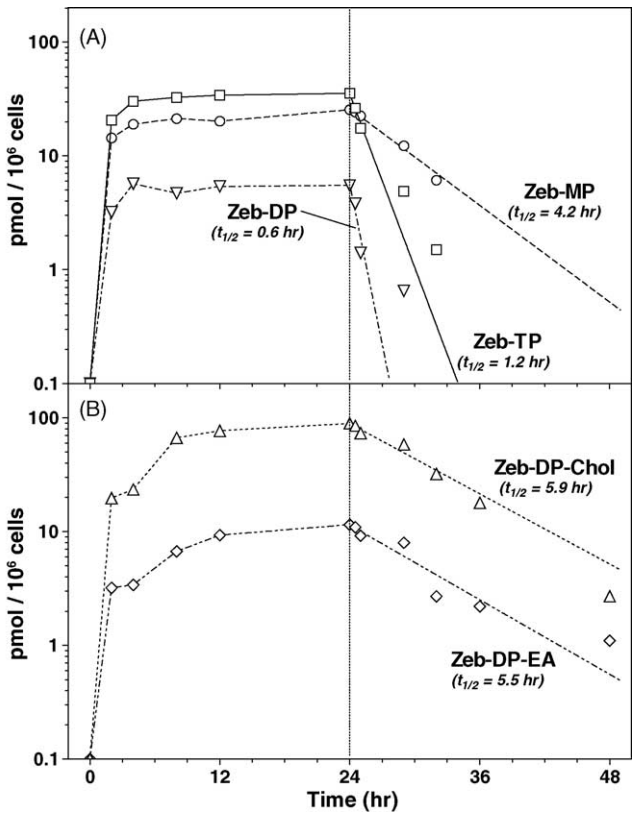


Fig. 7. Concentration versus time profile of zebularine metabolites in T24 cells. Cells were incubated with 10 μM [2-^{14}C]Zeb (1 μCi/ml) for the indicated times before being collected, extracted and analyzed as described in Section 2. The vertical dotted line indicates removal of drug-containing medium and replacement with drug-free media. Data points are the average of duplicate measurements. (A) Zebularine-5′-phosphates. (B) Zebularine-5′-diphosphosocholine and ethanolamine conjugates. Symbols are the same as in Fig. 6.

3.5. Effect of cytidine, uridine and CPEU on zebularine phosphorylation

Since Zeb is an analogue of cytidine (Fig. 1), it was of interest to ascertain whether the initial phosphorylation step was catalyzed by UCK (EC 2.7.1.48). Therefore, the intracellular levels of the various zebularine metabolites were determined in T24 cells incubated with 10 μM [2-^{14}C]Zeb for 6 h in the presence of the natural UCK substrates cytidine and uridine and the UCK inhibitor CPEU. As shown in Table 2, 10 μM cytidine reduced the concentration of all zebularine metabolites except Zeb-DP by more than 50%. Increasing the cytidine concentration to 50 μM led to the almost complete abrogation of zebularine anabolism; Zeb-MP and the two phosphodiester adducts could no longer be detected in the cellular extract. In contrast to cytidine, uridine at either 10 μM or 50 μM had no significant inhibitory effect on zebularine metabolism. Cyclopentenyl uridine, a potent inhibitor of UCK with low cytotoxicity [26], was more effective than cytidine in restricting zebularine phosphorylation at the 10 μM level and at least equally effective at 50 μM. Cyclopentenyl uridine was also capable of inhibiting the phosphorylation of added cytidine and uridine when they were incubated with T24 cells but to a significantly lesser extent than that observed for zebularine.

3.6. Incorporation of zebularine into cellular DNA and RNA

The ability of Zeb to be incorporated into host cell nucleic acids was evaluated by examining DNA and RNA isolates from T24 cells following 24–72 h exposure to 10 μM [2-^{14}C]Zeb. As illustrated in Fig. 8A, the vast

Table 2
Relative effect of cytidine, uridine and cyclopentenyl uridine on zebularine metabolism in T24 cells after incubation with 10 μM [2-^{14}C]Zeb for 6 h

	(% of control)[a]				
	Zeb-MP	Zeb-DP	Zeb-TP	Zeb-DP-EA adduct	Zeb-DP-Chol adduct
Control (10 μM Zeb)	100[b]	100	100	100	100
10 μM Zeb + 10 μM Cyd	34	71	49	44	16
10 μM Zeb + 50 μM Cyd	ND[c]	22	11	ND	ND
10 μM Zeb + 10 μM Urd	134	110	113	103	91
10 μM Zeb + 50 μM Urd	100	115	100	66	71
10 μM Zeb + 10 μM CPEU	38	21	29	26	22
10 μM Zeb + 50 μM CPEU	4	3	6	6	ND
	CMP	CDP	CTP		
10 μM Cyd + 10 μM CPEU	102[d]	99[d]	79[d]	–	–
10 μM Cyd + 50 μM CPEU	20[d]	35[d]	51[d]	–	–
	UMP	UDP	UTP		
10 μM Urd + 10 μM CPEU	46[e]	60[e]	57[e]	–	–
10 μM Urd + 50 μM CPEU	31[e]	38[e]	62[e]	–	–

[a] Control levels (pmol/10^6 cells) were: Zeb-MP, 11.4 ± 2.1; Zeb-DP, 7.9 ± 1.5; Zeb-TP, 31.0 ± 2.7; Zeb-DP-EA adduct, 2.2 ± 0.6; Zeb-DP-Chol adduct, 68.0 ± 7.8.
[b] All values in the table are the average of duplicate measurements.
[c] Not detectable.
[d] Cytosine ribonucleotides; control levels (pmol/10^6 cells) were: CMP, 3.8; CDP, 4.7; CTP, 6.8 (average of duplicate measurements).
[e] Uracil ribonucleotides; control levels (pmol/10^6 cells) were: UMP, 4.9; UDP, 3.0; UTP, 8.7 (average of duplicate measurements).

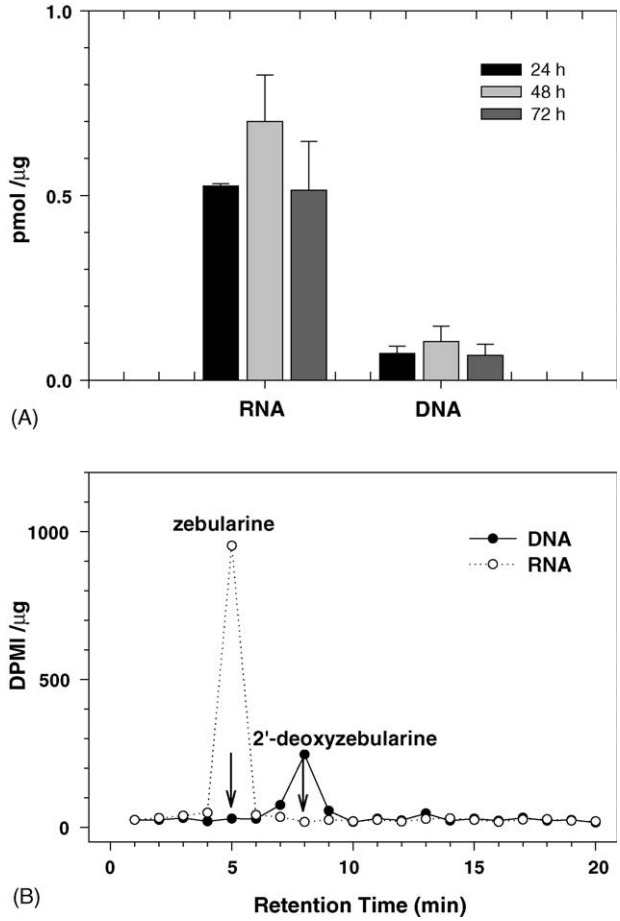


Fig. 8. Incorporation of zebularine into the DNA and RNA of T24 cells after treatment with 10 μM [2-^{14}C]Zeb (1 μCi/ml). (A) DNA and RNA were isolated by the Tri-reagent® procedure and incorporated radioactivity was determined as described in Section 2. Values are the mean ± S.D. (n = 4). (B) Reverse-phase radiochromatograms of DNA (●) and RNA (○) from zebularine-treated cells digested to constituent nucleosides. DNA and RNA isolated from cells treated with drug for 24 h were digested and analyzed as described in Section 2. Arrows indicate the retention times of authentic Zeb and 2′-deoxyzebularine.

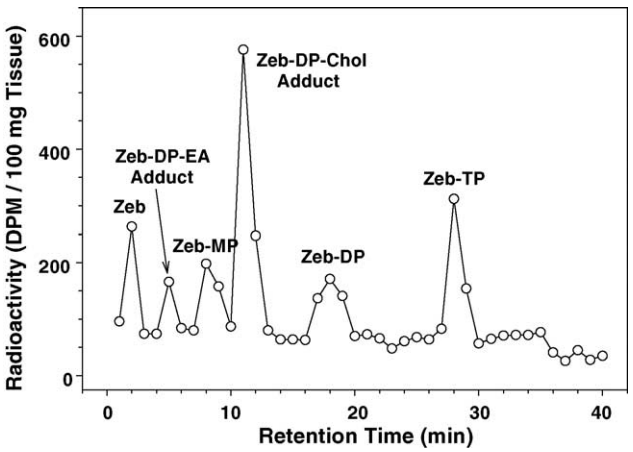


Fig. 9. Anion-exchange HPLC radiochromatogram of zebularine metabolites in EJ6-inoculated tumor from mice treated with zebularine. Nude mice were inoculated s.c. with EJ6 tumor cells, which were allowed to grow for 3 weeks. Mice were then treated i.p. with 500 mg/kg [2-^{14}C]Zeb (500 μCi/kg). Twenty-four hours after treatment, mice were sacrificed and tumors were removed and extracted for analysis.

majority of incorporated radioactivity was found in the RNA of treated cells. DNA incorporation of radiolabel, while observable and significant, was only on average about 15% that of RNA. Subsequent reverse-phase HPLC analysis of the free nucleosides generated from the complete enzymatic digestion of the isolated DNA and RNA revealed that the radiolabel coeluted with Zeb itself in RNA and with authentic 2′-deoxyzebularine in DNA, and that no other radioactive peaks were seen in either case (Fig. 8B). Measurement of radioactivity incorporated into the RNA and DNA isolated from T24 cells treated 10 μM [2-^{14}C]Zeb for 48 and 72 h indicated little change from the levels observed at 24 h.

3.7. Metabolism of zebularine in EJ6-derived tumors

The in vivo phosphorylation of Zeb was assessed following an i.p. dose of 500 mg/kg [2-^{14}C]Zeb to nude mice bearing EJ6-derived T24 tumors. Tumors were excised and examined for the presence of zebularine metabolites 24 h following drug administration. HPLC analysis of the methanolic extract of tumor tissue using radiochemical detection indicated that extensive metabolism had occurred (Fig. 9). Most of the radioactivity was accounted for by six principal peaks, which were identified as parent compound and the five metabolites observed in vitro (Table 3). The relative amounts of the various phosphorylated metabolites were quite similar to that observed for T24 cells in culture after zebularine exposure (Figs. 3 and 4A).

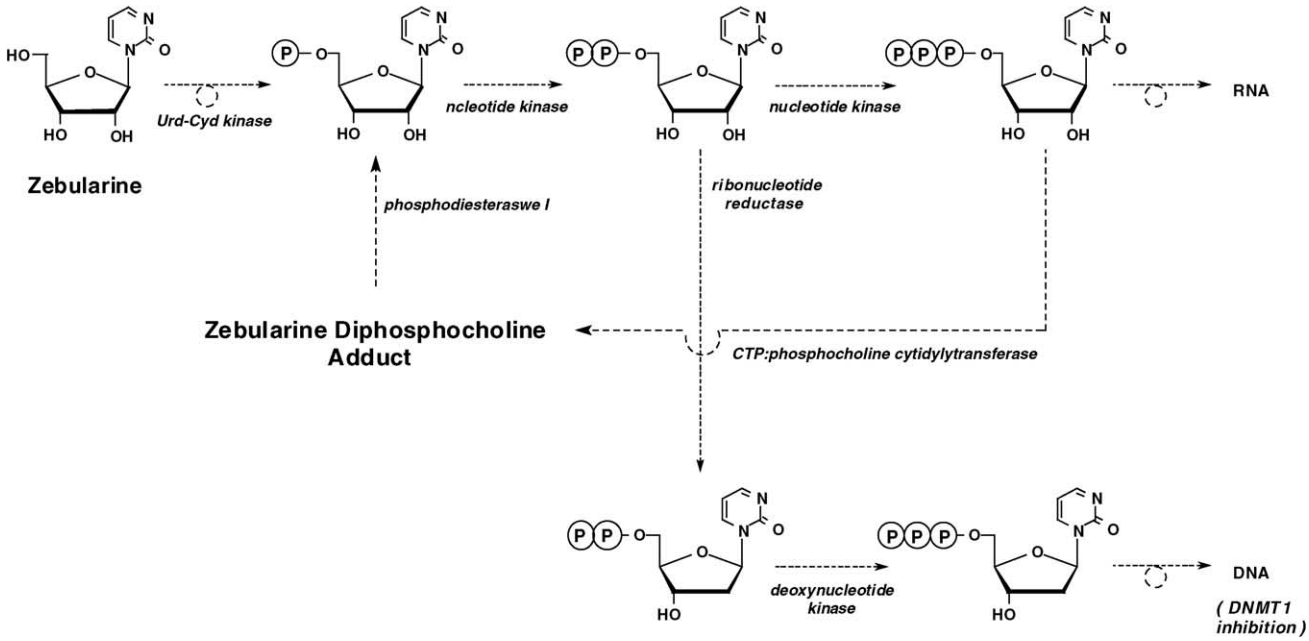
Table 3
Concentrations of zebularine and its metabolites in tumors from nude mice bearing the EJ6 variant of T24 human bladder carcinoma after i.p. treatment with 500 mg/kg [2-^{14}C]Zeb

Metabolite	Concentration[a] (pmol/100 mg tissue)
Zeb	1103 ± 482
Zeb-MP	694 ± 239
Zeb-DP	833 ± 328
Zeb-TP	1068 ± 187
Zeb-DP-EA adduct	671 ± 392
Zeb-DP-Chol adduct	2123 ± 191

Tissue samples obtained 24 h after drug treatment.
[a] Mean ± S.D. from three animals.

4. Discussion

The metabolic activation of zebularine was initially assessed in three cells lines (Table 1). Two of these cells lines, MOLT-4 lymphoblasts, a human T cell line, and murine MC38 colon carcinoma, were standard cells lines that our laboratories have typically used to evaluate the



Scheme 1. Proposed metabolic activation of zebularine.

metabolic activation of nucleoside analogues in vitro [27] and in vivo [30]. The third cell line, the human T24 bladder carcinoma [33], was chosen because of the demonstrated ability of Zeb to reactivate a silenced and hypermethylated *p16* tumor suppressor gene both in vitro and in vivo in these cells with minimal toxicity [9,11]. Of particular note, in this regard, was the ability of orally administered Zeb to reactivate the *p16* gene in the EJ6 variant of these cells grown in nude mice [9]. It can be seen from Table 1 that a similar pattern of anabolic metabolism is observed for Zeb in all three cell lines under equivalent in vitro conditions. Because of this observed metabolic similarity and because of the extensive in vitro and in vivo biological data already available for the effect of Zeb on T24 cells [9,11], we focused on defining the metabolic activation of Zeb in this cell line.

Zebularine is much less cytotoxic in vitro than other nucleoside analogues such as 5-aza-C and 5-aza-2′-dC that are also capable of effecting the demethylation and reactivation of silenced genes. This is well illustrated in Fig. 2 where the calculated IC_{50} for Zeb is almost seven-fold higher than that of 5-aza-C (120 μM versus 17.4 μM). Furthermore, the cytotoxic effect of 5-aza-C is probably understated in this instance, since the effective concentration of drug that the cells are exposed to most likely decreases with time because of the well-documented aqueous instability of this compound [34,35]. By contrast, with a half-life of 508 h under physiological conditions [24], Zeb would have hydrolytically decomposed by less than 7% in 48 h. Also of note is that almost no antiproliferative effect is seen for Zeb against T24 cells for concentrations of less than 20 μM. Accordingly, we chose to conduct the majority of our studies using a non-cytotoxic concentration of 10 μM Zeb.

Reversed-phase chromatographic analysis of selected cellular extracts following enzymatic conversion to the nucleoside level indicated that the vast majority (>95%) of measured radioactivity eluted as [2-^{14}C]Zeb. Since no radioactivity could be measured eluting at the retention time of authentic 2′-dZeb, any of this nucleoside that is present is below the limit of detection of the HPLC method. Thus any deoxyribonucleotides that are formed (as postulated in Scheme 1, vide infra) are of very low concentration. Therefore, the Zeb metabolites that we have observed are exclusively ribonucleotides.

The formation of choline and ethanolamine adducts are well documented for cytidine and its analogues [31,32,36–38]. Endogenous cytidine-5′-diphosphocholine is synthesized from CTP and phosphocholine in a reversible reaction catalyzed by choline-phosphate cytidylyltransferase (EC 2.7.7.15) and plays an important role in the generation of phospholipids involved in membrane formation and repair [36]. The antitumor agents cytarabine (arabinofuranosyl cytosine) and gemcitabine (2′,2′-difluorodeoxycytidine) and the anti-AIDS drug zalcitabine (2′,3′-dideoxycytidine) are known to form diphosphocholine adducts in vitro. Although these metabolites have been hypothesized to contribute to the in vivo toxicity profiles of cytarabine and zalcitabine at high doses [31,32], there is no evidence that these phospholipid conjugates have any significant biological effect. For both cytarabine and zalcitabine, the 5′-diphosphocholine adduct is a major metabolite, but intracellular concentrations never exceed those of the nucleoside triphosphate to the extent the Zeb-DP-Chol adduct does in T24 cells (Fig. 6). Zeb-TP appears to be uniquely able to compete with CTP to form a choline adduct, resulting in a considerable portion (25–50%) of phosphorylated Zeb being sequestered as this metabolite.

In vivo CDP-choline is readily hydrolyzed by phosphodiesterases to CMP and phosphocholine [36]. We have demonstrated that the Zeb-DP-Chol adduct is a substrate for PDE-1 in vitro (Fig. 4), and this is likely also to be the case for phosphodiesterases occurring in vivo. Thus, the Zeb-DP-Chol adduct represents a substantial depot form of already phosphorylated drug that can potentially be recycled metabolically. The complete structural and biochemical characterization of these two Zeb-DP adducts, including their chemical synthesis, will be the subject of a future and more comprehensive report. The formation of these choline and ethanolamine adducts is additional evidence that Zeb functions biologically as an analogue of cytidine.

The initial phosphorylation of Zeb is most likely mediated by uridine-cytidine kinase [39](Scheme 1). This was supported by the fact that 10 μM CPEU, a potent, non-cytotoxic inhibitor of UCK [26] substantially reduced the phosphorylation of Zeb, while 50 μM inhibitor almost completely abrogated this process (Table 2). Interestingly, of the endogenous UCK substrates uridine and cytidine, only cytidine was effective in competing with Zeb and inhibiting its phosphorylation (Table 2). This may be partially due to the intrinsic K_m differences between these two substrates for UCK [40], since cytidine concentrations were much closer to its K_m (45 μM) than were those of uridine (150 μM). There is, in addition, some evidence that CTP is a stronger feedback inhibitor of UCK than UTP [40]. Also, since Zeb is a potent inhibitor of cytidine deaminase [11,12], it is unlikely that cytidine would be catabolized to any extent while uridine is more rapidly utilized in nucleotide biosynthesis and is readily degraded by uridine phosphorylase (EC 2.4.2.3). In regard to the latter observation, it should be noted that zebularine itself was a very poor substrate for pyrimidine phosphorylases (Lai et al., unpublished results), so it is more like cytidine.

Phosphorylation by UCK is usually considered to be rate-limiting [39], and the sensitivity of many cells to various pyrimidine ribonucleoside analogues correlates with the activity of this gateway enzyme [41,42]. Indeed, a recent report evaluating zebularine response in normal fibroblasts and in cancer cells indicated that incorporation of radioactive zebularine into DNA correlated strongly, although not perfectly, with UCK activity which tended to be higher in the cancer cells [43]. In our study, formation of Zeb-MP in T24 cells did not appear to be saturable with zebularine doses below 250 μM, since production of this metabolite was quite linear ($r^2 = 0.9996$) (Fig. 6). Once the ribonucleoside monophosphate is formed, further phosphorylation is usually catalyzed by UMP-CMP kinase (EC 2.7.4.14) and then by nucleoside diphosphate kinases (EC 2.7.4.6) and results in the rapid formation of di- and triphosphates [44]. This also appears to be the case for Zeb phosphorylation in T24 cells where the initial rates of formation of Zeb-DP and Zeb-TP parallel that of Zeb-MP (Fig. 7A), and these zebularine-5′-phosphates appear to reach a steady-state level after 6–8 h. The somewhat slower rate of accumulation of the diphosphocholine and ethanolamine adducts (Fig. 7B) is consistent with their probable formation from Zeb-TP (Scheme 1). Once drug is removed and the T24 cells are washed and reincubated, Zeb-DP and Zeb-TP decay rapidly, indicating that these anabolites are readily utilized. The surprisingly slower intracellular decay rate of Zeb-MP and the persistence of the diphosphocholine and diphosphoethanolamine adducts is consistent with the possibility that the latter are probably functioning as a source of Zeb-MP through the action of intracellular phosphodiesterases (Scheme 1).

It is hypothesized that once Zeb is metabolically activated to form a deoxynucleotide triphosphate (dNTP), incorporation into DNA at the GCGC target site of DNMT is necessary for it to function as a mechanism-based inhibitor of DNA methylation [24]. Thus a primary question of the current study was the extent to which the intact 2(1H)-pyrimidinone base is actually incorporated into DNA. We found that Zeb incorporation into the DNA of T24 cells exposed to non-cytotoxic concentrations of drug occurred, but was minimal and was far outweighed by incorporation into RNA (Fig. 8A). The vast majority of the radioactivity incorporated into DNA corresponded to 2′-deoxyzebularine, just as the much greater amount incorporated into RNA was almost entirely represented by Zeb itself (8B). The 2′-dZeb-TP used as a standard for this study was a substrate for Klenow/DNA polymerase I, *E. coli*, (EC 2.7.7.7) and could be efficiently incorporated into DNA opposite G (Yang A Dept Med, Keck School of Medicine, USC). This suggests that the likely rate-limiting step for zebularine incorporation into DNA is its probable conversion to a deoxyribonucleotide by ribonucleotide reductase. Attempts to inhibit this enzyme with hydroxyurea were unsuccessful, since at the concentrations required for in vitro inhibition in T24 cells (200 μM) significant cytotoxicity was observed (Ben-Kasus et al., unpublished results). It is unknown whether RNA incorporation correlates with either the biological activity or the cytotoxicity of Zeb.

Also of interest was whether Zeb could be metabolically activated in tumor cells growing in vivo. For this, we partially repeated an experiment of Cheng et al. [9] where EJ6 cells, a tumorigenic variant of T24 cells, were inoculated subcutaneously into nude mice and allowed to grow until macroscopic tumors appeared. The mice were then treated with a single i.p. dose of Zeb and after 24 h the tumor was excised and examined. Analysis of the tumor tissue revealed extensive Zeb metabolism with a phosphorylation pattern (Fig. 9 and Table 3) very similar to that observed in vitro. Although this similarity between in vitro and in vivo metabolism in this preliminary study is encouraging, there are many factors (e.g. dose, schedule, route of administration, tumor site and type, etc.) which can potentially influence the rate and extent of the in vivo metabolic activation of Zeb. Accordingly, a more compre-

hensive in vivo investigation of these parameters is currently in progress and will be the subject of a future report.

It can be seen from the foregoing discussion that, biochemically, Zeb behaves like a cytidine analogue, albeit one with unique properties. Its metabolic activation to form a dNTP for incorporation into DNA (Scheme 1) is complex and inefficient. The initial phosphorylation of Zeb appears not to be the ultimate, rate-limiting step for formation of a dNTP as it for the cytidine analogues cytarabine and gemcitabine, which are first activated by deoxycytidine kinase (EC 2.7.1.74) [37,38,45]. In the case of the latter, phosphorylation is concentration dependent and rapidly saturates due to the low concentrations of deoxycytidine kinase [37,45]. In contrast, Zeb exhibits little concentration-dependence in the rates of metabolite formation (Fig. 6), indicating that nucleotide formation is not limited by the kinase. Consequently, the overall rate-limiting step for the incorporation of the zebularine base into DNA is hypothesized to be the ribonucleotide reductase-mediated conversion of Zeb-DP to 2′-dZeb-DP. In this regard, the metabolic activation of Zeb is similar to that of 5-aza-C, likewise an inhibitor of DNA methylation [24], which is also incorporated into RNA and DNA [24,37]. Unfortunately, a more direct route to the deoxyribonucleotides is not currently available, since 2′-deoxyzebularine does not appear to be phosphorylated by dCK and is consequently inactive [24]. The results reported here, therefore, point to several areas that could be investigated as a potential means of modulating the incorporation of Zeb into DNA and therefore, presumably, increasing its potency. One is a prodrug strategy for 2′-deoxyzebularine or some other appropriate derivative [25] to overcome or circumvent the metabolic roadblock imposed by its inability to be phosphorylated. 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 [11]. It is this minimal toxicity of Zeb 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 cancer chemotherapy [4–6,46], chemoprevention [47] and epigenetic therapy in general [48].

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