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POTENT INHIBITION OF HHAI DNA METHYLASE BY THE AGLYCON OF 2-(1H)-PYRIMIDINONE RIBOSIDE (ZEBULARINE) AT THE GCGC RECOGNITION DOMAIN

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INTRODUCTION

In 1960 Ikehara reported the synthesis of a compound he described as 6-deoxyuridine (**5a**).[1] However, the structure of this compound —prepared by the coupling of the chloromercuri salt of 2-(1*H*)-pyrimidinone (**2**) with 1-chloro-2,3,5-tri-*O*-benzoyl-D-ribofuranoside (**1**)— was disputed by several workers who synthesized the correct compound by the same chloromercuri method,[2-4] or by other methods including the desulfurization of 4-thiouracil derivatives,[5,6] and the silyl variant of the Hilbert-Johnson reaction using **3**, a procedure more commonly known as the Vorbrüggen reaction (Scheme 1).[7,8] Compound **5a**, which we later named zebularine,[9]is the structural counterpart of the purine natural product nebularine (**6**).[10] Earlier biochemical investigations identified zebularine as a bacteriostat,[11] and later we and others established that it was a potent inhibitor of cytidine deaminase (CDA).[8,12-14] The transition-state hypothesis that led us to postulate zebularine as a mechanism-based inhibitor of CDA was examined in detail[15] and later confirmed by X-ray crystallography showing the

hydrated 2-(1*H*)-pyrimidinone ring of zebularine (**7**) complexed with zinc at the active site of the enzyme.[16,17]

Scheme 1

Structures 6-8

As a CDA inhibitor zebularine was about 10-fold less potent than the prototypic inhibitor tetrahydrouridine (THU, **8**) with a K_i of 2 μM.[8] However, in contrast to THU it showed good stability, particularly to acid (*vide infra*), making it a useful adjuvant for oral administration with drugs readily deaminated by CDA, such as arabinofuranosyl cytosine (ara-C),[9,18] and 2'-deoxy-5-azacytidine.[19] Our observation that zebularine was additionally cytotoxic to culture L1210 and P388 leukemia cells prompted the examination of its antitumor properties in vivo. Employing a standard NCI testing protocol,[20] intraperitoneal tumor implantation and intraperitoneal drug treatment (ip/ip) produced increases in life span (ILS) as high as 93% at 800 mg/Kg (Table 1).[9] Following oral treatment, good activity (81%-112% ILS) was also obtained with either ip or subcutaneous (sc) L1210 tumor implantation.[9] The relative high doses employed were indicative of the low toxicity of zebularine, which was ascertained by the difference in weight between drug-treated and untreated control mice. In contrast to zebularine, the 2'-deoxy and ara derivatives showed essentially no cytotoxicity against L1210 cells at concentrations of > 100 μM.[21] Of the few 5-substituted zebularine analogues synthesized, the 5-fluoro analogue (**5b**) was the most potent inhibitor of CDA with a K_i value equivalent to that of THU.[8] This in vitro potency carried over to in vivo antitumor activity which was about the same as zebularine's (50-50% ILS) against murine P388 leukemia but 100 times more potent under the same experimental conditions.[9] Against

L1210 leukemia, it was also significantly more active than zebularine (140% ILS versus 90%) with the same 100-fold increase in potency.[9] The down side was that 5-fluorozebularine appeared to be more toxic and less stable than zebularine.

Table 1

STRUCTURE-ACIVITY RELATIONSHIP: FROM A CDA INHIBITOR TO A CYTOSINE-C5-METHYLTRANSFERASE INHIBITOR

As described 1991,[9] we understood that zebularine had antitumor activity, but could not identify or propose a suitable molecular target. It was through our long-standing collaboration with Sheldon Greer of the University of Miami, who had been studying the effect of the radiosensitizer agent, 5-chloro-2'-deoxycytidine (CldC, cytochlor)[22] in combination with CDA inhibitors, that zebularine was tested as a DNA methylase inhibitor. Since it was possible that cytochlor when incorporated into DNA could induce hypomethylation of tumor DNA in the same manner as reported for 5-fluoro-2'-deoxycytidine,[23] he proposed to test separately both agents of the combination, CldC and zebularine, as DNA methylase inhibitors. The initial discovery in the laboratory of Eric Selker at the University of Oregon showing that zebularine reactivated a silenced hph gene in *Neurospora* by inhibiting DNA methylation was immediately corroborated in Peter Jone's laboratory at USC, who recently confirmed that indeed zebularine was capable of reactivating a silenced tumor suppressor gene (p16) both in vitro and in vivo.[24] In chemical terms, provided that zebularine gets incorporated into DNA, the results can be explained by the increased electrophilic character of the 2-(1*H*)-pyrimidinone ring of zebularine relative to cytosine brought about by the absence of the electron donating amino group. We had already shown that water could nucleophilically

add across the 3,4-double bond of zebularine to give a covalent hydrated C4-adduct (**7**) at the active site of CDA.[16,17] Independently, we also observed the formation of a stable intramolecular C6-adduct (**9**), which provided direct chemical evidence that the 2-(1*H*)-pyrimidinone ring of zebularine was also susceptible to nucleophilic attacked at the C6 positions by a hydroxyl group (Scheme 2).[25,26] Attack by water at C4 would then lead to CDA inhibition (**5c**, Nuc = OH), whereas attack at C6 by other nucleophiles could explain the antibacterial activity of zebularine against *E. coli* via inhibition of thymidylate synthase,[27] and the now observed inhibition of cytosine-C5-methyltransferase, since both catalytically similar reactions are initiated by nucleophilic attack at C6 by a thiol group of an invariant cysteine residue (**5d**, Nuc = SH).[28] In vitro experiments with synthetic oligodeoxynucleotides (ODNs) containing the 2-(1*H*)-pyrimidinone ring of zebularine at the target site for methylation have been shown to form tight complexes with bacterial methyltransferases.[29-31] These results support a mechanism-based inhibition of the enzyme resulting from the formation of a covalent bond between a conserved cysteine residue and the C6 carbon of the pyrimidine ring. In agreement with our simple chemical observation that 'harder" oxygen nucleophiles can also react with the ring to form a compound such as **9**, serine and threonine amino acids can replace the active cysteine residue of the M.*Msp*I DNA methylase and still form a covalent complexe with the enzyme.[31] Mechanistically, these reactions are facilitated by an enzyme-mediated base-flipping of the 2-(1*H*)-pyrimidinone ring at the target site for methylation in the same manner as described for 5-fluorocytosine.[32] This base-flipping mechanism is further enhanced by the weaker hydrogen bonding of the 2-(1*H*)-pyrimidinone:guanine base pair relative to the native C:G base pair.[33] Recently, a 13-

mer ODN containing a 5'-GXGC-3'/5'-GCGC-3' with the 2-(1*H*)-pyrimidinone ring of zebularine (X) at the target site was shown by X-ray crystallography to appear with the base rotated out of the helix and into the catalytic pocket forming the expected covalent complex of as in **5d** (Nuc = SH) with the enzyme M.*Hha*I.[34]

Scheme 2

DIRECT COMPARISON OF ODNs CONTAINING 5-AZACYTOSINE AND 2-(1*H*)-PYRIMIDINONE AT THE SAME TARGET POSITION FOR METHYLATION

Although 5-azacytidine (**10a**) and 2'-deoxy-5-azacytidine (**10b**) are structurally different from zebularine (**5a**), they share similar chemical properties affiliated with the increase reactivity at the C6 position of the heterocyclic ring relative to cytosine. However, the question of how these compounds compare with zebularine in terms of their enhanced electrophilic character remained unanswered. In the case of **10a** and **10b**, the C5 $\rightarrow$ N5 substitution is responsible for the enhanced electrophilicity, whereas in the case of zebularine it is the removal of the exocyclic 4-amino group from cytosine that brings about a similar change. Since an identical serine mutant described above for zebularine was also capable of interacting covalently with 5-azacytosine,[35] one would expect comparable potencies for both compounds. However, the only way to unequivocally answer this question was to compare identical ODN sequences differing only in the nature of the base at the target site and measuring the inhibition of methyl transfer to a standard ODN substrate. Since a small hemimethylated 13-mer ODN with 5-fluorocytosine substituted for the target cytosine (underlined) in the M.*Hha*I recognition sequence 5'-G<u>C</u>GC-3' had been shown to act as a strong DNA methylase inhibitor,[32] identical 13-mer sequences were selected for comparing 5-azacytosine and 2-

(1*H*)-pyrimidinone. The synthesis of an ODN containing 5-azacytosine at the same target site has already been reported.[36] The equivalent synthesis of the 2-(1*H*)-pyrimidinone-ODN was performed in a 1 μmol scale using commercially available 2-cyanoethyl phosphoramidites of the natural bases and the 5'-*O*-DMT-2'-deoxyzebularine-3'-*O*-2-cyanoethyl-N,N-diisopropylphosphoramidite which was synthesized and fully characterized. The final condition for deprotection and cleavage of the 13-mer from the solid support, which minimized the decomposition of zebularine, was a brief, 30 min treatment with concentrated ammonium hydroxide at 50 °C, which is much simpler than previous methods reported in the literature.[33,37] Mass spectrometry analysis confirmed the mass expected for the desired 13-mer.

Structures 10a and 10b

Herein we compare vis-à-vis the relative inhibitory potency of a double-stranded ODN containing 5-azacytosine (X)[36] and the corresponding double-stranded ODN containing 2-(1*H*)-pyrimidinone (Y) in the presence of a 24 bp long ODN substrate with a single hemimethylated 5'-GCGC-3' target site [5-methylcytosine (M) opposite to the target C]. The substrate OND was mixed with increasing concentrations of the inhibitor ODNs (15-120 nM) and the methylation reactions were initiated by adding the substrate-inhibitor mix to a standard reaction mixture containing 8.6 nM M.*Hha*I and 2.43 μM AdoMet as the methyl donor. The rate of incorporation of radiolabeled methyl groups from [methyl-^{3}H]AdoMet (Figure 1) indicated that the inhibitory potency of the 2-(1*H*)-pyrimidinone-containing ODN was virtually identical to that of the ODN substituted with 5-azacytosine.[36]

Figure 1

Gel shift assays were also used to evaluate the influence of cofactors and thermal stability on the covalent interaction between M.*Hha*I and the 2-(1*H*)-pyrimidinone-ODN under denaturating conditions. The same double-stranded 2-(1*H*)-pyrimidinone-ODN with ^{32}P-radiolabel at the 5'-end was incubated with M.*Hha*I for 30 min at various temperatures in the presence of AdoMet, AdoHcy or in the absence of cofactor. Complexes maintained at 4 °C and 22 °C were found to have the same electrophoretic mobility in both native (data not shown) and denaturating gels regardless of whether cofactor was present or not (Figure 2, lanes 1-6). When heated to 50 °C, the presence of the high mobility complex without cofactors was significantly reduced, while cofactors were able to stabilize the complex against dissociation by heat in the presence of 1% SDS and β-mercaptoethanol (lanes 7-9). The enhanced capacity of AdoMet over AdoHcy to stabilize the complex at higher temperature was evidenced when the same experiment was conducted at 75 °C (lanes 10-12). The experiments shown in Figures 1 and 2 confirmed that the inhibitor capacity of an ODN with the 2-(1*H*)-pyrimidinone substitution is virtually identical to the equivalently substituted ODN with 5-azacytosine.[36]

Figure 2

POTENCY: THE ROLE OF METABOLISM AND CHEMICAL STABILITY

The chemical instability of 5-azacytosine-containing nucleosides (**10a** and **10b**), even under neutral conditions, is well documented.[38] On the other hand, zebularine appears to be quite stable with a half-lives of 44 and 68 hours at pH 1.0 and 2.0, respectively. Furthermore, zebularine is completely stable at pH 5, and at pH 7.4 the half-life is 508 hours. This enhanced chemical stability is perhaps responsible for the

oral activity of zebularine in the reactivation of silenced p16 expression[24] and general antitumor activity.[9] At higher, non-physiological pH values the drug does decompose and its decomposition products have been thoroughly characterized.[39] Despite the fact that both classes of drugs have equal inhibitory potency as DNA methylase inhibitors when compared as components of small ODN fragments, 5-azacytidine (**10a**) and 2'-deoxy-5-azacytidine (**10b**) are ca. 30-fold and 300-fold more potent, respectively, than zebularine in inducing demethylation when used as single drugs.[24] This disparity can be attributed to a more inefficient metabolism of zebularine compared to both 5-azacytidine and 2'-deoxy-5-azacytidine. The metabolism of the latter two drugs is fairly well understood (Figure 3),[40] while that of zebularine is still under investigation. Tentatively, as indicated in Figure 4, we presume that zebularine will behave as a substrate for uridine/cytidine kinase. Because only 2'-deoxyzebularine is expected to be incorporated into DNA, the conversion of zebularine-5'-diphosphate to 2'-deoxyzebularine-5'-diphosphate by ribonucleotide reductase represents an additional rate-limiting step. This metabolically costly cross-over is probably the reason why 2'-deoxy-5-azacytidine is active at one-tenth the concentration of 5-azacytidine.[24] For that reason, we tested 2'-deoxyzebularine with the intent to overcome the reduced potency of zebularine. Unfortunately, 2'-deoxyzebularine did not inhibit DNA methylation.[24] Perhaps deoxycytidine kinase, the most likely enzyme to phosphorylate 2'-deoxyzebularine, does not recognize or bind well to this compound because it lacks the 4-amino group on the pyrimidine ring. The metabolic activation of zebularine to 2'-deoxyzebularine-5'-triphosphate and the determination of levels of incorporation into DNA and RNA are currently under investigation.

Figure 3

Figure 4

CONCLUSION

The identical level of inhibition of methyl transfer achieved with short ODNs containing either 5-azacytosine or 2-(1*H*)-pyrimidinone at the target site for methylation leads us to propose that once incorporation into DNA has been achieved, zebularine and 5-azacytidine (or 2'-deoxy-5-azacytidine) are similarly potent against DNA (cytosine-C5) methyltransferase. Despite the favorable chemical stability of zebularine that facilitates even oral administration of the drug, there appears to be a metabolic rate-limiting step for zebularine, either at the level of phosphorylation by uridine/cytidine kinase or at the level of ribonucleotide reductase, that reduces considerably the potency of the drug. However, despite this shortcoming zebularine presents itself as a drug that may be clinically useful to reverse DNA methylation with minimal toxicity. Perhaps the toxicity of zebularine reflects incorporation into RNA.

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Table 1. Antitumor Activity of zebularine[a]

Tumor (implantation)[b]	Dose (route of administration) mg/Kg	Weight change difference (g)	% ILS
P388 (ip)	400 (ip)	-2.3	52
L1210 (ip)	400 (ip)	-2.1	75
L1210 (ip)	800 (ip)	-2.5	93
L1210 (ip)	800 (po)	-0.6	77
L1210 (ip)	1600 (po)	-2.3	112
L1210 (sc)	800 (po)	-0.8	81

[a]Adapted from reference 9. [b]ip = intraperitoneal; po = oral treatment; sc = subcutaneous.

Figure legends:

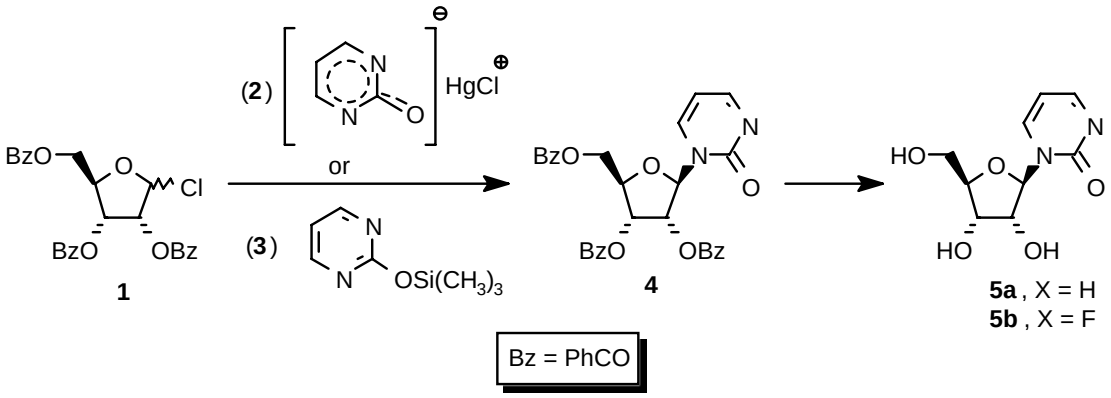
Figure 1. Relative Inhibition of M.*Hha*I

Figure 2. Binding of Zebularine 13-mer to M.*Hha*I

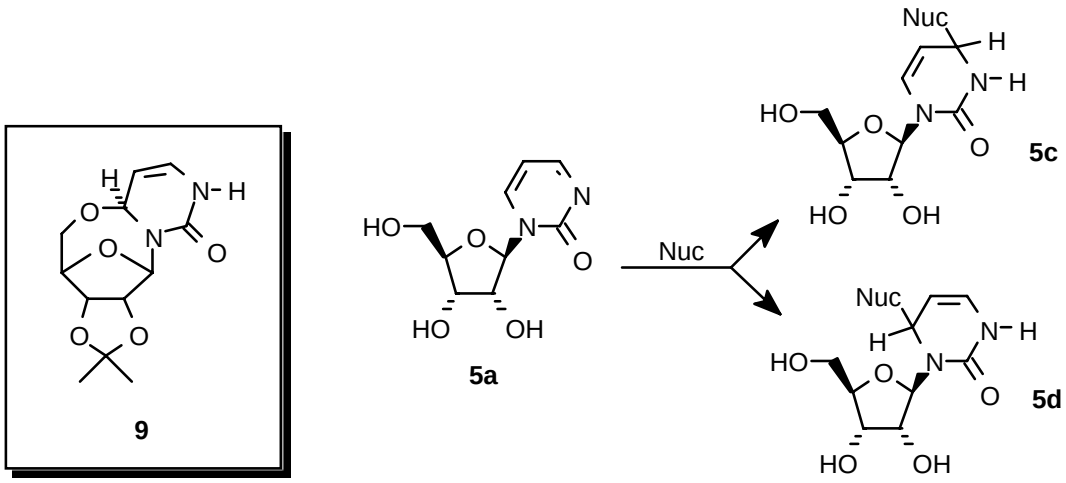
Figure 3. Metabolism of 5-Aza Nucleosides

Figure 4. Metabolism of Zebularine

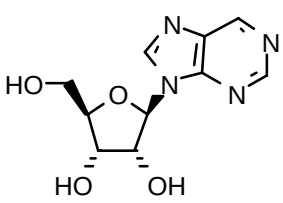
Scheme 1



Scheme 2



Structures 6-8



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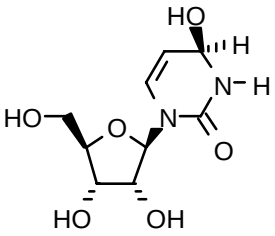

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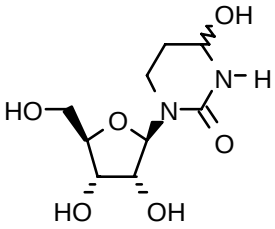
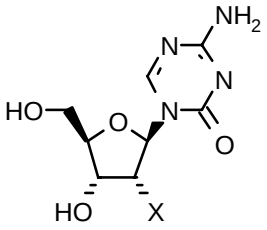


8

Structures 10a,b



10a , X = OH
10b , X = H