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Enzymatic formation of potential anticancer and antiviral inosine analogues

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Abstract. Theoretically, inosine analogues should act as effective inhibitors of tumor cell proliferation and viral replication. To acquire a broad spectrum of new candidate inosine analogues, a rapid, facile, quantitative and stereoselective method for deaminating potential antitumor and antiviral adenine analogues previously synthesized in our laboratory was developed. A novel 5′-adenylic acid deaminase, with relaxed substrate requirements, from *Aspergillus* species was utilized to deaminate four hexofuranosyladenine nucleosides and five adenine nucleoside dialdehydes to their corresponding inosine analogues. The fastest rates of deamination for the hexofuranosyl nucleosides were for the compounds where the vicinal hydroxyl groups on the sugars are oriented in the erythro configuration. For rapid deamination of the adenine nucleoside dialdehydes, the *R* configuration at the proximal carbon atom is preferred, while the nature of the group on the distal carbon atom has no significant effect on the rate or extent of deamination.
Key words. Inosine analogues; adenine analogues; fungal 5′-adenylic acid deaminase.

Theoretical considerations dictate that inosine analogues might prove clinically useful as inhibitors of malignant cell proliferation and viral replication. This concept is based, in part, on data which show that nucleic acid polymerases from a variety of sources display a relaxation in stringency as they readily utilize deoxyinosine triphosphate in lieu of deoxyguanosine triphosphate during the synthesis of DNA and RNA, respectively [1–4]. However, since hypoxanthine-cytosine base pairs, which are formed in inosine-containing nucleic acids, are linked by two hydrogen bonds [5] rather than the three that occur in guanine-cytosine base pairs, the secondary structures of the inosine-containing nucleic acids are relatively weak from a thermodynamic point of view.

In order to determine if it is possible to take advantage of the concept of inosine-containing destabilized DNA to design inhibitors of malignant cell proliferation, several hexofuranosyladenine nucleosides, originally synthesized in one of our laboratories [6–10], were enzymatically deaminated to form their corresponding inosine analogues. The compounds chosen had a hydroxyl group at the 5′-position of the sugar so that enzymatic phosphorylation by tumor kinases could be achieved prior to the incorporation of the analogues into newly synthesized tumor nucleic acids. Another possible mechanism that might justify the use of inosine analogues as inhibitors of malignant cell proliferation and viral replication would be their ability to act as metabolic inhibitors of the synthesis of nucleic acids or their precursors. This idea is based on previous investigations which demonstrated that inosine dialdehyde had significant anticancer activity in a number of animal tumor models in vitro and in vivo [11, 12], and elicited a significant clinical response in human seminomas, oat cell carcinomas and malignant melanomas [13]. In addition, dideoxyinosine, an inhibitor of DNA chain elongation, is now widely used for the treatment of the human immunodeficiency virus (HIV).

In previous studies, some adenine nucleoside dialdehydes, originally synthesized in one of our laboratories, had displayed promising antileukemic activity [14]. These adenine nucleoside dialdehydes either lacked the terminal 5′-hydroxyl group necessary in most cases for phosphorylation, which made them especially interesting, and/or had stereochemically different configurations at one or two positions, or had some alteration in purine structure. They were shown to preferably limit DNA synthesis by inhibiting in vivo ribonucleotide diphosphate reductase [13, 14], the rate-limiting enzyme in the biosynthesis of deoxyribonucleotides [15, 16]. In the present report, a number of different analogues of inosine were enzymatically prepared from hexofuranosyladenine nucleosides and adenine nucleoside dialdehyde analogues in anticipation that they may either

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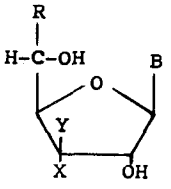
cause increased destabilization of the secondary structure of tumor DNA, resulting in aberrations in DNA transcription, and/or serve as inhibitors of DNA synthesis or a precursor of DNA synthesis.

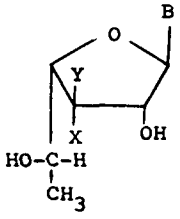
Materials and methods

Adenine nucleoside analogues. The hexofuranosyladenane adenine nucleoside dialdehydes illustrated in table 1 were described in previous publications [6–10, 14]. Stock solutions of 10^{-2} M or 10^{-3} M compounds were prepared by adding the compounds to distilled water and heating in a microwave oven on high power for approximately 5 s. This procedure eliminated the use of dimethyl sulfoxide (DMSO) as a solvent and extended periods of vigorous mixing. The individual compounds had the same ultraviolet (UV) spectra, molar extinction coefficients and R_f values using thin-layer chromatography (TLC) whether they were prepared by heating in a microwave oven or using DMSO as a solvent. Compound **17**, an unsaturated adenine dialdehyde, was prepared by heating compound **15** for 3 h at 100 °C, then rapidly cooling. The compound was then purified by passage over a Dowex 1- X2 (OH) column, 2.5 × 20 cm, washed with 10 ml of water and eluted with 20 ml of 0.03 M aqueous triethylammonium bicarbonate buffer, pH 7.5. Fractions containing 0.5 ml were collected. Compound **17**, and all other compounds which were further purified in this manner, were located in fractions 4–10. These compounds were concentrated by lyophilization. The inosine analogue of compound **17** was prepared either by enzymatically deaminating compound **17** or by heating compound **16** for 3 h at 100 °C.

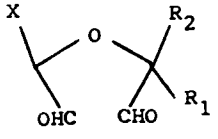
Table 1. Structures of hexofuranosyl nucleosides and nucleoside dialdehydes.



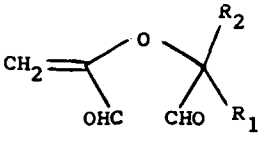
	B	*R*	*X*	*Y*
1	Adenine	CH_2OH	OH	H
2	Hypoxanthine	CH_2OH	OH	H
3	Adenine	CH_3	H	OH
4	Hypoxanthine	CH_3	H	OH
5	Adenine	CH_2OH	H	OH
6	Hypoxanthine	CH_2OH	H	OH



7	Adenine	—	OH	H
8	Hypoxanthine	—	OH	H



	R_1	R_2	*X*
9	Adenine	H	H
10	Hypoxanthine	H	H
11	H	Adenine	H
12	H	Hypoxanthine	H
13	Adenine	H	CH_3
14	Hypoxanthine	H	CH_3
15	H	Adenine	CH_2OH
16	H	Hypoxanthine	CH_2OH



17	H	Adenine	-
18	H	Hypoxanthine	-

TLC. TLC was performed on 0.25-mm layers of silica gel G, Type 60 (E. Merck, Darmstadt). Five to ten microliters of solution containing 5–10 µg of each compound was spotted on the TLC plate and developed using two different solvent systems: solvent A, isopropyl alcohol:NH_4OH:H_2O (60:30:10); and solvent B: 0.1 M EDTA:NH_4OH:H_2O:isobutyric acid (0.93:2.45:35.08:61.54). After being developed with solvent B, the plates were exposed to ammonia fumes prior to viewing. The spots were located with a Mineralight lamp (254 nm).

Enzymatic deamination. Deamination kinetics and the preparation of the inosine analogues were carried out at room temperature or at 37 °C in cuvettes using a Perkin-Elmer recording spectrophotometer, Model Lamda 2S. The formation of the inosine analogues from their corresponding adenosine analogues was monitored by the UV spectrum: hypsochromic shift, λ_{max} 259 → 248 [17] and by TLC. Deamination was measured by the decrease in absorption of the parent adenine analogue at 265 nm [17]. The enzyme reaction incubation mixture, 1.5 ml, contained HEPES buffer, 0.05 M, pH 7.3, 10^{-4} M compound and 0.0434 U of 5′-adenylic acid deaminase from *Aspergillus* species (Sigma, A-1907, Lot 120H0234, 0.096 U/mg).

Results

UV λ_{max} and molar extinction coefficients (ϵ). A hypochromic shift was observed from a UV λ_{max} of 259 nm for the adenine nucleosides to a UV λ_{max} of 247–249 nm for the corresponding inosine analogues. The molar extinction coefficients for the adenine nucleosides

Table 2. Rate of enzymatic deamination of certain hexofuranosyladenine and adenine nucleoside dialdehyde analogues with 5′-adenylic acid deaminase from *Aspergillus* sp.

Compound	Percent deaminated								
	Time 4 min	8	20	30	1 h	2	4	6[a]	22[b]
1	53	87	100	100	-	-	-	-	100
3	5	8	19	24	36	57	77	90	100
5	0	1	9	12	20	36	57	75	100
7	29	47	79	88	98	100	-	-	100
9	3	6	13	15	22	28	41	60	74
11	28	38	63	85	96	100	-	-	100
13	15	20	32	36	40	43	46	58	73
15	43	62	98	100	-	-	-	-	100
17	45	62	91	100	-	-	-	-	100
S-adenosyl-methionine	1	3	-	10	-	-	-	20	25
2′-ethylthio-adenosine	70	100	-	-	-	-	-	-	100

The enzyme reaction incubation mixture assayed at 26 °C contained HEPES buffer, 0.05 M, pH 7.3, 10^{-4} M compound and 0.043 U of 5′-adenylic acid deaminase from *Aspergillus* sp. (0.096 U/mg). The enzyme reactions were performed in duplicate on two separate occasions except for compounds **15** and **17**, for which the extent of deamination was repeated in duplicate three separate times. The individual results of all the separate assays did not vary by more than 15%. Product concentrations were determined by UV and TLC.

[a]After 6 h fresh enzyme (0.043 U) was added to the incubation mixtures.

[b]After 22 h fresh enzyme (0.043 U) was again added to the incubation mixtures of compounds **9**, **13** and *S*-adenosylmethionine. No increase in the extent of deamination of these compounds was observed at 36 h.

ranged from 13,200 to 14,600, while the molar extinction coefficients of the inosine analogues had a range of 11,300–12,400. In all cases the ϵ of the inosine analogues was approximately 80–85% of the corresponding adenosine analogues. This also holds true for inosine as compared to adenosine.

Enzymatic deamination. Initial experiments showed broad pH optima of 6.8–7.4 for the deamination reaction. In addition, there were no significant differences in enzyme kinetics when the enzyme reaction was performed either at 26 °C or 37 °C. Table 2 shows that the hexofuranosyl nucleosides with the fastest deamination rates were **1** and **7**. Nucleoside **3** was deaminated slightly faster than **5**. The nucleoside dialdehydes **15** and **17** were deaminated as fast as compound **1**, with **11** being somewhat slower. Nucleosides **9** and **13** were not completely deaminated (70–75% after 22 h); therefore, the corresponding inosine dialdehyde products **10** and **14** had to be separated by Dowex 1-X2 column chromatography. Following column chromatography, the fraction containing the inosine analogue was lyophilized and redissolved so that the final concentration of the nucleoside was 10^{-4} M. Spectrophotometric and TLC data indicated that separation from the corresponding adenosine dialdehyde analogue was complete and that 80–85% of the inosine dialdehyde was recovered. Additional experiments demonstrated that the inosine dialdehyde analogues **10** and **14** had no effect on the rate or extent of deamination of compound **15**, which was completely deaminated. Thus, the inability of the fungal enzyme to quantitatively deaminate the adenosine dialdehyde analogues **9** and **13** was not due to a product-feedback inhibition mechanism. When Tris buffer in lieu of HEPES was used, the free amino group of Tris reacted with the dialdehyde moiety of compounds **9**, **11**, **13**, **15** and **17**, forming a complex mixture of compounds that included a cyclic adenosine-like nucleoside (results not presented). In order to determine if scale-up purification procedures can be utilized so that sufficient amounts of purified inosine analogues can be isolated, a proportional amount of enzyme was utilized for 24 h at 37 °C to deaminate 10–25 mg of compounds **1** and **9**. After deamination, the compounds were further purified by Dowex 1-X2 column chromatography, and the resultant fractions containing the hypoxanthine analogue were pooled and concentrated by lyophilization. Monitoring of product formation was done by TLC and UV absorption. Scale-up procedures will be used for the formation of select compounds which first show significant antiproliferative activity in in vitro cell culture tumor models.

Biological activity. All of the compounds were assayed for antileukemic activity in vitro using the murine L1210 cell line [14]. Adenosine dialdehyde and inosine dialdehyde had substantial, equivalent antileukemic activity ($IC_{50} = 1 \pm 0.2$ μM). However, substitution of hypoxanthine for adenine in the dialdehyde analogues did not confer any increase in the antileukemic activity of these nucleoside dialdehydes. The antiproliferative activity of the enzymatically formed inosine dialdehyde analogues as either equivalent to or less (10–50%) than the parent adenosine dialdehydes. The hexofuranosyladenine and hexofuranosyl hypoxanthine analogues had no significant antileukemic activity ($IC_{50} > 100$ μM).

Discussion

It was postulated that inosine analogues have the structural configuration necessary to be effective inhibitors of DNA synthesis or DNA transcription, and thus have the potential to be clinically useful antitumor agents and/or antiviral agents. In order to achieve our goal, a number of adenine analogues that had previously been synthesized in one of our laboratories [9, 10, 14] were enzymatically deaminated to their corresponding inosine analogues. Deamination was performed enzymatically, since chemical deamination would result in incomplete deamination and the formation of unwanted by-products [18]. The enzymatic deamination method utilized a novel 5′-adenylic acid deaminase prepared from *Aspergillus* species, which was shown to have less

stringent substrate requirements than mammalian adenosine deaminase [18–22]. Some general observations concerning the kinetic parameters of the fungal enzyme are as follows: The fastest rates of deamination for the hexofuranosyl nucleosides were for compounds **1** and **7**, where the vicinal hydroxyl groups on the sugar are oriented in the erythro configuration. Nucleoside **1** is deaminated slightly faster than **7** probably because the 'downward' orientation of carbons 5′ and 6′ on compound **7** do not allow an optimal interaction, such as an interaction of a hydroxyl group. Nucleosides **3** and **5** may be deaminated more slowly either because the hydroxyl group at C-3′ is on the opposite side and is not available for binding or because of an unfavorable conformational change due to the dipole-dipole interaction with the ring oxygen atom. Nucleoside **3** may be enzymatically deaminated slightly faster than **5** because of less steric strain due to the smaller methyl group in the terminal position, or due to its interaction with a hydrophobic region on the enzyme. Nucleoside dialdehydes **9** and **13** are slow-reacting and were not completely deaminated due to the *S* configuration at the proximal atom, i.e. the adenine is oriented in an opposing direction. There is no significant difference in the rates of deamination of compounds **11**, **15** and **17**, since the nature of the group on the distal carbon atom does not appear to be of significance.

It should also be noted that 5′-adenylic acid deaminase from *Aspergillus* species was able to deaminate adenosine compounds lacking a 5′-OH group and their corresponding sulfoxides, but had limited ability to deaminate adenosine sulfonium compounds such as *S*-adenosylmethionine. Additional data also demonstrated that the enzyme reaction can be scaled up so that with purification procedures utilizing Dowex 1-X2 chromatography, as outlined in the 'Results' section, sufficient amounts of purified inosine analogues (80–85% yield) can be isolated to perform in vivo antitumor and antiviral studies. Although initial experiments demonstrated no increase in antileukemic activity when inosine was substituted for adenine in the compounds employed in these studies, further antileukemic screening using human solid tumors will have to be performed. This is a reasonable supposition, since clinical phase I trials demonstrated that inosine dialdehyde was more active against human oat cell carcinoma, malignant melanoma and seminomas in comparison with the liquid human lymphomas [13]. In addition, it will be prudent to screen all of the adenine and inosine analogues against various viruses, expecially HIV, since dideoxyinosine is a useful drug used clinically to inhibit HIV replication [23].

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