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Antiproliferative activity of purine nucleoside dialdehydes against leukemia L1210 in vitro

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Summary. Sixteen purine nucleoside dialdehydes were assayed for antiproliferative activity against murine leukemia L1210 grown in vitro. These compounds either lacked the terminal hydroxymethyl group that is necessary in most cases for phosphorylation, and/or had stereochemically different configurations at one or two positions, or had some alteration in the purine ring structure. Among the latter were two lipophilic N^6-benzyladenine containing dialdehydes, and two nucleoside dialdehydes with a bromine atom at C-8 of the purine. These nucleoside dialdehydes, unlike most clinically useful anticancer nucleosides, did not require enzymatic phosphorylation to become activated. The most interesting agent in this group of compounds was the lipophilic nucleoside dialdehyde obtained from N^6-benzyladenosine after periodate oxidation. It had an IC_{50} of 1.0 ± 0.2 μM, and appears to function by limiting the formation of deoxyguanosine diphosphate (dGDP) by inhibition of ribonucleoside diphosphate reductase, the rate limiting step in the biosynthesis of deoxyribonucleotides.

Introduction

Nucleoside dialdehydes are obtained from nucleosides by periodate oxidation. Although nucleoside dialdehydes do not actually exist in aqueous solution as aldehydes, but are instead complex mixtures of various cyclic and acyclic hemiacetals and hydrates [12, 13, 17, 20], they do afford the familiar reactions of aldehydes. This behavior is similar to the behavior of free sugars, such as D-galactose, which in aqueous solution exist in various hemiacetal forms, but still react as straight-chain aldehydes.

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Nucleoside dialdehydes were first demonstrated to have antitumor activity by Dvonch et al. [9]. This finding encouraged other investigators to prepare additional nucleoside dialdehydes for the purpose of introducing new clinically useful antitumor agents. Most prominent of these compounds was inosine dialdehyde, which was shown to have significant antiproliferative activity both in vitro and in vivo for a number of animal tumor models [5–7, 26, 27]. It also elicited a clinical response in human seminomas, oat cell carcinomas and malignant melanomas [18]. The rationale for the synthesis of the nucleoside dialdehydes as potential anticancer agents was based on the property of aldehydes to form adducts with amino groups, such as carbinolamines and Schiff bases, and thus interact with proteins in a variety of ways, including as cross-linking reagents [6, 7, 22, 27, 33]. An apparent advantage of nucleoside dialdehydes over other dialdehydes not of nucleoside origin is that the nucleoside characteristics retained are expected to confer site-specificity so that only enzymes involved in the biosynthesis of nucleic acids and their precursors would be affected [24, 36]. In accord with this concept, a number of metabolic sites in purine and pyrimidine metabolism have been demonstrated to be sites of action for various nucleoside dialdehydes. These sites include the inhibition of thymidylate kinase [21], DNA-dependent RNA polymerase [21, 32], ribonucleotide reductase [5, 25], *S*-adenosylhomocysteine hydrolase [25], and deoxycytidylate aminohydrolase [26]. Another important proposed site is the biosynthesis of glycosaminoglycans, which are important for cellular recognition, adhesion, and tumor proliferation and metastasis [22]. The formation of cross-links between protein-protein chains [7] and DNA-DNA chains [3] is also well-recognized.

The purpose of this current study is to report on the antileukemic activity and pharmacological properties of certain dialdehydes derived from purine nucleosides, which have structural features different from any previously studied. Among these structural variants are dialdehydes which lack the terminal hydroxymethyl group necessary for phosphorylation, and/or have changes in their stereochemical configuration at one or more sites, or

have some alterations in the purine ring, including the presence of a lipophilic group.

Materials and methods

Antiproliferative agents. The nucleoside dialdehydes illustrated in Table 1 were prepared from the parent nucleosides by oxidation with periodic acid, column chromatography on Bio-Rad AG1-X8 (200–400 mesh, acetate form) resin, evaporation under reduced pressure at 30° C, and lyophilization [12, 13]. Care was taken not to heat any nucleoside dialdehyde having a hydroxymethyl group linked to the same carbon atom as an aldehyde group in order to prevent dehydration to the unsaturated dialdehyde [14]. Dialdehyde *1* was prepared by oxidation of N^6-benzyladenosine [10]. However, for this compound, the column chromatography was performed somewhat differently than for the other preparations. Because of its low solubility in water, *1*, would precipitate out on the resin beads, therefore, it was necessary to elute with a solution of 80% aqueous acetone. Dialdehyde *2* was prepared by oxidation of 6-benzylamino-9-(β-D-ribopyranosyl)purine and purification accomplished as described for *1*. The parent nucleoside, a known compound [2], was prepared in this case by benzylation of 9-β-D-ribopyranosyladenine [8] in a manner based upon the benzylation of adenosine [2]. Nucleoside dialdehydes *3–16* were supplied by Dr. Alan Grant [12], who synthesized these compounds as part of the requirements for a Ph. D. dissertation in one of our laboratories (L. M. L.). It should be noted that each nucleoside dialdehyde can be prepared from more than one parent nucleoside. For example, dialdehyde *6* can be prepared from 9-β-D-xylopyranosyladenine or from 9-α-L-arabinopyranosyladenine, as well as from 9-β-D-ribopyranosyladenine, from which it was actually prepared. Therefore, it would be improper to name these nucleoside dialdehydes as if they were derived from a specific parent nucleoside.

Cell line and culture. All conditions for the growth and maintenance of the L1210 murine leukemia cell line are described in previous publications from our laboratory [23, 28, 29]. To determine the antiproliferative activity of the nucleoside dialdehydes, 2×10^5 L1210 cells/ml were incubated with varying concentrations of individual drugs in 25 cm^2 flasks in a humidified atmosphere of 5% CO_2-95% O_2 for 48 h. The IC_{50} values were determined from dose-response curves.

Ribonucleoside and deoxyribonucleoside triphosphate pools. The triphosphate pools were determined by a high-pressure liquid chromatography (HPLC) assay as described in a previous publication from our laboratory [23]. A Beckman Model 334 HPLC was employed. HPLC grade methanol and acetonitrile were purchased from Aldrich (Milwaukee, Wis.). Buffer solutions were filtered through 0.45 μm filters (Millipore, Belford, Mass.) and deaerated before use. The ribonucleotide and deoxyribonucleotide standards were purchased from Sigma (St. Louis, Mo.) and Boehringer Mannheim (Indianapolis, Ind.). Briefly, control and treated L1210 cells (1×10^7 cells incubated with nucleoside *1* to produce 50% inhibition) were harvested after 48 h and then homogenized in 6% trichloracetic acid. The nucleotides were extracted using a modification of the freon-amine method [19, 30]. The ribonucleotides in the cell extract, which exist in concentrations 6-50 times the concentrations of the deoxyribonucleotides, were determined first. Aliquots of cell samples, the appropriate standards, and cell samples mixed with the standard solutions were neutralized with NaOH, then injected onto a 4 mm × 25 cm column packed with Partisil SAX (Whatman, Hillsboro, Ore.) and subsequently eluted with 0.3 M KH_2PO_4, pH 3.0. The retention times of the peaks obtained from the L1210 cells corresponding to the known standards, were compared with the retention times of the peaks obtained from the standards [1, 35]. The peak heights were then used to quantitate the ribonucleoside triphosphates. It should be noted that the presence of deoxyribonucleotides in the L1210 cell extract did not interfere with the quantitation of the cellular ribonucleotides [1, 15]. The deoxyribonucleoside triphosphates were determined by first degrading the ribonucleotides in the L1210 cell extract by periodate oxidation, then assaying the samples by HPLC in a similar manner to that described for the ribonucleoside triphosphates [1, 11].

Nucleoside Dialdehyde-induced inhibition of macromolecular synthesis. Pulse-labelling experiments using [methyl-^{3}H]thymidine, 55 Ci/mmol, ICN Pharmaceuticals (Irvine, Calif.); [5-^{3}H] uridine, 25.6 Ci/mmol, ICN Pharmaceuticals Inc.; and D L-[1-^{14}C]leucine, 50 mCi/mmol, New England Nuclear Corp. (Boston, Mass.) were employed to determine the inhibitory action of nucleoside dialdehyde *1* on DNA, RNA and protein synthesis, respectively. L1210 cells (4×10^5 cells/2 ml) were incubated with either 2×10^{-6} M compound *1* or without any nucleoside dialdehydes (controls) for 6 h. Individual cultures then received a 0.1 ml aliquot of the appropiately labelled precursors to give final concentrations of 1.25 microcuries/ml for either [^{3}H]-thymidine or [^{3}H]-uridine, or 0.25 μCi/ml for [^{14}C]-leucine. After incubating the cells for an additional 10, 20, or 30 min, the cells were harvested, washed, and the radioactivity determined using a Beckman LS-1800 liquid scintillation counter. These procedures are described in detail in a previous publication from one of our laboratories [31].

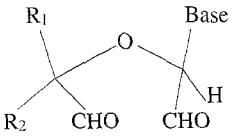
Results

Effect of purine nucleoside dialdehydes on inhibition of cellular proliferation

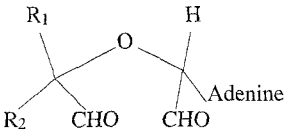
The antiproliferative activity of sixteen purine nucleoside dialdehydes listed in Table 1 was determined. It should be noted that the synthesis of dialdehydes *1–4* have not been previously reported. As illustrated in Table 1, all of the nucleoside dialdehydes except *7*, *15* and *16* had antiproliferative activity against leukemia L1210 cells in vitro at a concentration of 50 μM. Other experiments demonstrated that the activity of the nucleoside dialdehydes was irreversible. L1210 cells exposed to either compound *1* or *6* for 24 h did not retain their capacity to proliferate after the cells were thoroughly washed with Ringer's solution and then reincubated for up to 72 h in fresh media without nucleoside dialdehydes.

Henceforth, in this paper when considering the reference structures of the dialdehydes as illustrated in Table 1, the carbon atom that is bonded directly to the purine nitrogen and which was originally the anomeric carbon atom of the nucleoside will be termed the *proximal* carbon, and the carbon atom attached to the oxygen bridge, and which is also bonded to one of the aldehyde groups, will be termed the *distal* carbon. As shown in Table 1, there does not appear to be much preference for one configuration over another at the *proximal* carbon among the group of compounds that can be directly compared, namely compounds *6–9* (*R* configuration) versus compounds *11–14* (*S* configuration). Only dialdehyde *9* with an *R* configuration was more active than *12* by a significant factor of 10. The configuration at the *distal* carbon did not appear to matter. What was clearly important, regardless of the configuration at the *proximal* carbon, was the presence or absence of a group bonded to the *distal* carbon. The best activity, in general, was found when hydrogen atoms were substituted for the terminal hydroxymethyl group despite the fact that the single most active compound was *1*, which did have the hydroxymethyl group. When other bases or adenine derivatives were substituted for adenine, such as aldehydes *1–4* and *10*, the hydroxymethyl group at the *distal* carbon made no difference, as can be seen by comparison of compounds *1* and *2*. The presence of a bromine atom at position 8 of hypoxanthine (compound *3*) did not alter the

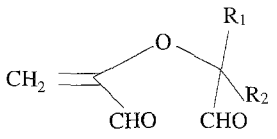
Table 1. Effect of nucleoside dialdehydes on L1210 cell proliferation



Compund no.	Base	R_1	R_12	IC_{50} (μM)
1	6-Benzylaminopurine	$HOCH_2$	H	1.0
2	6-Benzylaminopurine	H	H	1.2
3	8-Bromohypoxanthine	$HOCH_2$	H	1.2
4	8-Bromoadenine	$HOCH_2$	H	6.4
5	Adenine	$CH_3CO_2CH_2$	H	2.2
6	Adenine	H	H	3.3
7	Adenine	CH_3	H	>10
8	Adenine	H	CH_3	10
9	Adenine	$HOCH_2$	H	1.2
10	Hypoxanthine	$HOCH_2$	H	1.2



Compound no.	R_1	R_2	IC_{50} (μM)
11	H	H	1.20
12	$HOCH_2$	H	10
13	CH_3	H	10
14	H	CH_3	10



Compound no.	R_1	R_2	IC_{50} (μM)
15	Adenine	H	>10
16	H	Adenine	>10

All assays were performed in duplicate or triplicate, and the IC_{50}(s) determined from dose response curves obtained from one to four separate experiments; (depending on the interest in the individual compound). Compounds *1, 2, 5, 12–16* were dissolved in 10% DMSO. All of the dialdehydes were sterilized before use by passing through a 0.22 μm filter. All data shown are mean values ± SEM

antitumor activity in comparison to compound *10*. However, a similar substitution in compound *9* to yield compound *4* decreased the antiproliferative activity considerably. It should also be noted that dialdehyde *3* derived from 8-bromoinosine had an IC_{50} which showed it to be considerably more active (1.2 ± 0.3 μM) than dialdehyde *4* which was derived from 8-bromoadenosine (6.4 ± 0.2 μM).

Because of the low solubility in water of the new 6-benzylaminopurine nucleoside dialdehydes *1* and *2*, it was necessary to dissolve them first in dimethylsulfoxide (DMSO) and adjust the final concentration to 10% in DMSO. This concentration of DMSO was shown to have no effect on the proliferation of L1210 cells. As an additional control, dialdehyde *6* was dissolved either in water or 10% DMSO and shown to have equivalent antitumor activity.

Table 2. Ribonucleoside and deoxyribonucleoside triphosphate pools in L1210 leukemia cells before and after incubation with nucleoside *1*

Nucleoside	Concentration (pmol/10^6 cells)	
	Untreated L1210 cells	Inhibited L1210 cells
UTP	760 ± 21	710 ± 18
CTP	362 ± 15	362 ± 34
ATP	2284 ± 143	2638 ± 380
GTP	574 ± 34	810 ± 26
dTTP	61 ± 3	54 ± 3
dCTP	23 ± 2	26 ± 3
dATP	55 ± 2	41 ± 4
dGTP	8 ± 3	2 ± 1

[a] Two separate experiments were performed, each done in duplicate. All data represent mean values ± SEM

Table 3. Effect of nucleoside *1* on macromolecular synthesis

Parameter	Percent inhibition from control[a]		
	10 min	20 min	30 min
DNA	27 ± 2	50 ± 2	71 ± 3
RNA	8 ± 2	17 ± 3	24 ± 4
Protein	5 ± 2	10 ± 3	17 ± 2

[a] All assays were performed in triplicate on two separate samples. All data represent values ± SEM

Mechanism of action of purine nucleoside dialdehydes

In order to delineate the mechanism of action of the antileukemic purine nucleoside dialdehydes, two separate experiments were performed. Firstly, the intracellular levels of ribonucleoside and deoxyribonucleoside triphosphate pools in L1210 cells before and after incubation with nucleoside 1 were determined. Nucleoside dialdehyde *1* was chosen to represent this class of nucleoside derivatives since it was the most active compound and possessed lipophilic properties which would conceivably increase its uptake by mammalian tumor cells. Table 2 shows that compound *1*, which has an IC_{50} of 1.0 ± 0.2 μM, elicited a significant increase in the concentration of guanosine triphosphate (GTP) and a significant decrease of 75% in the concentration of deoxyguanosine triphosphate (dGTP), compared with control L1210 cells. No significant increases or decreases were detected in the triphosphate pool sizes of the other nucleosides, which constitute RNA and DNA, in L1210 cells incubated with compound *1*. Secondly, other studies, as illustrated in Table 3, demonstrate that nucleoside dialdehyde *1* had a more pronounced effect on the inhibition of DNA synthesis after 10 min of incubation with an appropriate labelled precursor (27 ± 2%) than it did on the inhibition of RNA synthesis (8 ± 2%), and protein synthesis (5 ± 2%). In addition, as the time of incubation with the labelled precursors progressed to 20 and 30 min, there ensued approximately equivalent proportional increases in the percent inhibition of DNA, RNA and protein synthesis.

In an attempt to determine whether increasing the dGTP pools in L1210 cells would reverse the antiproliferative activity of N^6-benzyladenosine dialdehyde, varying concentrations of deoxyguanosine and guanosine were added to the cell culture media. Deoxyguanosine was found to be ineffective in reversing the inhibition, while 2.0 μM guanosine, which had no demonstrable effect on cellular proliferation by itself, produced a 35% reversal of the N^6-benzyladenosine dialdehyde (1.0 μM) induced inhibition of L1210 cell proliferation. However, concentrations of guanosine greater than 2.0 μM, without the presence of the dialdehyde, were found to have antiproliferative activity. Therefore, we are unable to conclude unequivocally whether or not guanosine could reverse the inhibition caused by the nucleoside dialdehyde.

Discussion

Sixteen purine nucleoside dialdehydes were assayed for antiproliferative activity against the L1210 murine leukemia grown in cell culture. Some generalizations concerning structure-activity relationships can be made from the data presented in Table 1. Given the R configuration for the *proximal* carbon (dialdehydes *1–10*), the structure of the purine does not appear too important. Only when a substituent, a bromine atom in this case, was placed at C-8 of adenine was there a significant loss in antiproliferative activity. However, this loss in activity was not apparent between the hypoxanthine derivatives, compounds *3* and *10*, which had the same IC_{50} values. The use of the benzylamino group at the 6-position of the purine (dialdehydes *1* and *2*) was an attempt to improve activity by increasing the lipophilic properties of compounds *6* and *9*, and thus possibly facilitate transfer through cell membranes. The results indicate that with these two compounds there was no improvement in activity. What appears more important, in some cases, is the substituent at the *distal* carbon. There was not much difference in activity by changing a hydroxymethyl group to an hydrogen atom, but substitution of a methyl group in either the *R* or *S* configuration considerably decreased the activity. Even more dramatic was the introduction of unsaturation at the *distal* carbon, which led to a loss of activity. It is also interesting that a change in the configuration at the *proximal* carbon to the *S* form did not seem to alter the above conclusions, except for the presence of the hydroxymethyl group where the *R* configuration confers significantly greater activity on compound *9* than the *S* configuration on compound *12*. Several of the nucleoside dialdehydes (*2*, *6* and *11*) do not have the hydroxymethyl at the *distal* carbon; yet, they are as active as the compounds that do, suggesting that these compounds do not have to be activated by phosphorylation, as do the presently clinically useful anticancer nucleosides. The inability of many types of tumor cells to phosphorylate the anticancer nucleosides is a primary mechanism of drug resistance [4]. Based on this data, the nucleoside dialdehydes present a distinct pharmacological advantage over the presently used nucleoside analogues.

In order to determine the pharmacologically active site of the nucleoside dialdehydes, the effects of the most active lipophilic dialdehyde derived from N^6-benzyladenosine by periodate oxidation (compound *1*) on DNA, RNA and protein synthesis was evaluated. Data from these studies showed that this dialdehyde appears to exert its initial effect mainly on DNA synthesis, which is then followed by a generalized inhibition of RNA and protein synthesis. This conclusion was reached from the data showing that there was a significantly greater inhibition of DNA synthesis during progressively increasing incubation periods of L1210 cells with compound *1* than there was for the inhibition of RNA and protein synthesis.

Additional experiments demonstrated that L1210 cells incubated with compound *1* had a significant increase in intracellular levels of GTP and a significant decrease of intracellular levels of dGTP. However, it should be noted that there are difficulties in interpreting data from the determined size of the nucleoside triphosphate pools due to the complexities arising from the involvement of at least sixty different enzyme reactions (including the salvage pathways) in maintaining the nucleoside triphosphate levels [16]. Nevertheless, since there was a significant increase in the concentration of GTP in L1210 cells incubated with dialdehyde *1*, and a significant decrease in the concentration of dGTP in these same cells, it appears that the antiproliferative action of the purine nucleoside dialdehydes may be due, at least in part, to the inhibition of a ribonucleotide diphosphate reductase, the rate-limiting enzyme for biosynthesis of deoxyribonucleotides [16, 34]. Inhibition of ribonucleotide diphosphate reductase would also explain the other data discussed above, which demonstrated that nucleoside dialdehyde *1* preferably inhibits DNA synthesis as compared to RNA and protein synthesis.

Hydroxyurea, the only clinically useful inhibitor of ribonucleotide diphosphate reductase also decreases the intracellular level of dGTP while increasing the intracellular level of GTP [34]. However, clinically, hydroxyurea has a number of negative properties. It is not very active on a concentration basis, it is not too stable in solution, it has a short half-life, and rapid resistance develops to its use [37]. Therefore, it would be desirable to introduce another inhibitor of ribonucleotide diphosphate reductase as a clinically useful anticancer agent. Previous investigators have demonstrated the potential clinical importance of nucleoside dialdehydes as antitumor agents. Unfortunately, phase I trials demonstrated that inosine dialdehyde, which at that time was considered the most promising of this class of compounds, proved to be too toxic [6, 18]. New information gleaned from the present study, concerning the antiproliferative activity and structure-activity relationships of some new purine nucleoside dialdehydes with more favorable pharmacological properties, may lead to the development of a new efficacious nucleoside dialdehyde as an important antitumor agent.

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