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Cytotoxic Action of Adenosine Nucleoside and Dialdehyde Analogues on Murine Neuroblastoma in Tissue Culture: Structure-Activity Relationships[1]

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ABSTRACT

A series of purine nucleoside and dialdehyde analogues was studied to determine their potency as inhibitors of C-1300 murine neuroblastoma cell growth in tissue culture. Tumor cells were incubated with each analogue for 72 h, and the number of viable cells was determined at 24, 48, and 72 h. Dose-response curves were generated (concentration range, 10^{-8} to 10^{-3} M), and the drug concentration producing 50% inhibition of cell growth was calculated for each analogue. The 50% inhibitory concentrations, in ascending order of potency, were as follows: adenosine (inactive); *S*-adenosylhomocysteine (inactive); methylfuryladenine (5.6×10^{-1} M); adenosine 5′-carboxylic acid (2.0×10^{-1} M); 5′-chloro, 5′-deoxy-*ara*-adenosine (3.0×10^{-2} M); sinefungin (1.7×10^{-3} M); 5′-deoxyadenosine (2.2×10^{-4} M); 5′-chloro, 5′-deoxyadenosine (2.1×10^{-4} M); 3′,5′-dichloro, 2′,3′,5′-trideoxyadenosine (1.3×10^{-4} M); 3-deazaadenosine (5.6×10^{-5} M); and adenosine dialdehyde (1.5×10^{-6} M).

Oxidation of the pentose to a dialdehyde increased, whereas reduction of the dialdehyde or substitution of adenine with either hypoxanthine, guanine, uracil, or cytosine decreased the inhibitory potency. The analogues 4′,5′-anhydroadenosine dialdehyde and 5′-deoxyadenosine dialdehyde, which cannot be phosphorylated at the 5′ position, had 50% inhibitory concentrations of 9.1×10^{-6} and 7.6×10^{-6} M, respectively.

These data suggest that the inhibitory action and potency of nucleoside dialdehydes on neuroblastoma growth are independent of their capacity to undergo a kinase-mediated phosphorylation at the 5′ position.

INTRODUCTION

The role of AdoMet[3]-dependent transmethylation reactions as modulators of normal eukaryotic cell function has been actively investigated in recent years (1, 2). They have been shown to exert a regulatory influence on stimulus-secretion coupling (3, 4), leukocyte chemotaxis (5), and cellular differentiation and replication (6). The utilization of AdoMet is governed by a complex series of enzyme-dependent reactions. AdoHcy hydrolase appears to play a pivotal role in this regard by regulating the metabolic conversion of AdoHcy to adenosine and homocysteine. Since AdoHcy acts as a competitive antagonist of transmethylase enzymes, increases in the intracellular concentration of this substrate, secondary to inhibition of AdoHcy hydrolase, will lead to significant changes in cell function.

Many synthetic analogues of adenosine have been shown to indirectly inhibit cellular methylation reactions by suppression of AdoHcy hydrolase or directly by antagonism of methylase enzymes. A variety of tissue culture systems, *i.e.*, L929 murine leukemia (7), chick embryo fibroblast infected by Rous-sarcoma virus (8), and C-1300 murine neuroblastoma (9) cell lines, have been used to demonstrate those effects. In general, analogues have been synthesized by substitution at the 5′-carbon of the pentose (10, 11), replacement of the ribose moiety with a cyclopentene ring (12), or oxidation of the pentose ring to form a dialdehyde (13).

A series of adenosine nucleoside and dialdehyde analogues has been studied to determine their effects on murine neuroblastoma cell growth in tissue culture and to define the relationships between chemical structure and cytotoxic potency.

MATERIALS AND METHODS

MNB Tumor Cell Culture. MNB tumors were grown in A/J mice following the s.c. implantation of 10^6 tumor cells. Tumors were excised under sterile conditions (14 to 21 days after implantation) and placed in culture medium (RPMI-1640) supplanted with 10% fetal calf serum, 10,000 units/ml penicillin, and 100 μg/ml streptomycin. The tumor was minced in a Petri dish containing tissue culture medium and MNB cell suspensions prepared. MNB cells were grown as monolayers in 75-cm^2 Falcon tissue culture flasks containing 20 ml of RPMI-1640 medium in an atmosphere of 7.5% CO_2/92.5% air at 37°C. Subcultures were prepared by addition of 1×10^6 MNB cells to each flask. The media were changed every 3 days or as required in accordance with experimental conditions.

Nucleoside and Dialdehyde Analogues. The compounds investigated in this study were obtained from commercial sources (sinefungin, Lilly Research Lab), as gifts (3-deazaadenosine, Burroughs-Wellcome Research Lab), or synthesized in our laboratories utilizing methods previously described (10, 13). The analogues were categorized as follows.

In Fig. 1 are nucleoside analogues: sinefungin; 3-deazaadenosine; *S*-adenosylhomocysteine; 5′-chloro, 5′-deoxyadenosine; 5′-deoxyadenosine; 5′-chloro, 5′-deoxy-*ara*-adenosine; 3′,5′-dichloro, 2′,3′,5′-trideoxyadenosine; methylfuryladenine; adenosine 5′-carboxylic acid; and adenosine.

In Fig. 2 are dialdehyde analogues: adenosine dialdehyde; reduced adenosine dialdehyde; 5′-deoxyadenosine dialdehyde; 4′,5′-anhydroadenosine dialdehyde; inosine dialdehyde; guanosine dialdehyde; uridine dialdehyde; and cytidine dialdehyde. The dialdehydes were prepared by oxidation of the parent nucleoside with paraperiodic acid using previously described methods (13, 14). This procedure involved oxidation of the nucleoside with the theoretical amount of paraperiodic acid (H_5IO_6) at room temperature, making certain to protect the reactants from light. The solution was then passed through a column of Bio-Rad AG1-X8 (acetate form, 100 to 200 mesh) anion-exchange resin to remove iodate ion. The eluates were checked for iodine by the starch iodide test, and the solutions were evaporated under high vacuum followed by lyophilization. The chemical characterization and purity of each dialdehyde were confirmed by nuclear magnetic resonance spectrometry.

Experimental Design. All nucleoside analogues were added to tissue culture flasks at zero time, and cell counts were performed at 24, 48, and 72 h thereafter. They were dissolved in a diluent consisting of dimethyl sulfoxide (3.3%), ethanol (50%), and 0.9% saline (46.7%). Final concentrations of the diluent ranged from 0.33 to 0.033% for dimethyl sulfoxide and 5 to 0.5% for ethanol, which did not affect MNB cell growth. Control flasks containing only MNB cells, medium, and diluent were set up for each analogue investigated. Cell counts in the control flasks were compared with those of experimental flasks which contained nucleoside analogues ranging in concentration from 10^{-8} to 10^{-3} M. At 24, 48, and 72 h, each flask was agitated, and cells were removed for counting at the same time each day. Cells were

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[3] The abbreviations used are: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; IC_{50}, drug concentration producing 50% inhibition of cell growth; MNB, murine neuroblastoma.

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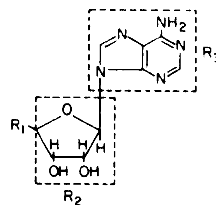

ANALOGUE | R_1 | R_2 | R_3

ADENOSINE

5'-CHLORO-5'-DEOXYADENOSINE

5'-DEOXYADENOSINE

5'-CHLORO-5'-DEOXYARAADENOSINE

3',5'-DICHLORO-2',3',5'-TRIDEOXYADENOSINE

METHYLFURYLADENINE

S-ADENOSYLMETHIONINE

S-ADENOSYLHOMOCYSTEINE

SINEFUNGIN

3-DEAZAADENOSINE

Fig. 1. Chemical structures of adenosine analogues. Constituent substitutions were made at the R_1 (5'-carbon) or R_2 (pentose) and R_3 (purine base) positions.

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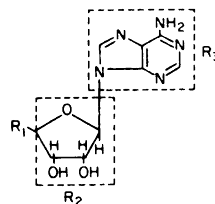

NUCLEOSIDE DIALDEHYDE | R_1 | R_2 | R_3

ADENOSINE DIALDEHYDE

REDUCED ADENOSINE DIALDEHYDE

INOSINE DIALDEHYDE

GUANOSINE DIALDEHYDE

URIDINE DIALDEHYDE

CYTIDINE DIALDEHYDE

4',5'-ANHYDROADENOSINE DIALDEHYDE

5'-DEOXYADENOSINE DIALDEHYDE

Fig. 2. Chemical structures of nucleoside dialdehydes. Constituent substitutions were made at R_1, R_2, and R_3.

counted manually in an eosinophil counting chamber after dilution with 0.1% trypan blue in 0.9% sodium chloride. The average cell viability in control flasks ranged from 95 to 98% as assessed by trypan blue dye exclusion. The number of viable cells present at each time point was determined in duplicate and subjected to regression analysis. From these data, log-linear plots were computer generated, and the IC_{50} of each analogue was determined.

RESULTS

Cytotoxic Action of Analogues on Murine Neuroblastoma Tumor Cell Growth. The cytotoxic potency of nucleoside and dialdehyde analogues varied widely in the MNB tissue culture model. The growth-suppressing activity of each analogue has been expressed as a regression line generated by plotting viable cell number against incubation time (Figs. 3 to 5) and also as the IC_{50} (Tables 1 and 2).

The naturally occurring metabolites, adenosine and *S*-adenosylhomocysteine, were virtually inactive as suppressants of MNB cell growth, even at concentrations as high as 10^{-3} M (Fig. 3). Synthetic analogues modified in the pentose moiety, such as methylfuryladenine or 5′-chloro, 5′-deoxy-*ara*-adenosine, had IC_{50}s of 5.6×10^{-1} M and 3.0×10^{-2} M, respectively. Oxidation of the 5′-hydroxymethyl group of adenosine to a carboxylic acid yielded a low potency inhibitor with an IC_{50} of 2.0×10^{-1} M. Analogues of adenosine substituted with chlorine at the 5′ or the 3′ and 5′ positions, such as 5′-chloro, 5′-deoxyadenosine and 3′,5′-dichloro, 2′,3′,5′-trideoxyadenosine, or those reduced at the 5′ carbon, such as 5′-deoxyadenosine, were more potent inhibitors with IC_{50}s of 2.1×10^{-4} M, 1.3×10^{-4} M, and 2.2×10^{-4} M, respectively. Sinefungin, an analogue of AdoHcy, in which the sulfur is replaced with an aminomethyne group, had modest growth-suppressing activity. In contrast, 3-deazaadenosine and the thiopurine analogue, 6-mercaptopurine, were more potent inhibitors (Table 1).

Cytotoxic potency was greatest in those analogues whose pentose ring had been oxidatively cleaved to form a dialdehyde. The IC_{50} of adenosine dialdehyde was 1.5×10^{-6} M. This was about 100-fold less than that observed with any of the substituted derivatives of adenosine, with the exception of 3-deazaadenosine whose IC_{50} was 5.6×10^{-5} M. The non-dialdehyde

Table 1 *Inhibitory potency of adenosine analogues on murine neuroblastoma cell growth in tissue culture*

Adenosine analogues	Inhibitory concentration (M)[a]
Adenosine	Inactive
S-Adenosylhomocysteine	Inactive
Methylfuryladenine	5.6×10^{-1}
Adenosine 5′-carboxylic acid	2.0×10^{-1}
5′-Chloro, 5′-deoxyaraadenosine	3.0×10^{-2}
Sinefungin	1.7×10^{-3}
5′-Deoxyadenosine	2.2×10^{-4}
5′-Chloro, 5′-deoxyadenosine	2.1×10^{-4}
3′,5′-Dichloro, 2′,3′,5′-trideoxyadenosine	1.3×10^{-4}
3-Deazaadenosine	5.6×10^{-5}
6-Mercaptopurine[b]	4.7×10^{-5}

[a] Drug concentration (IC_{50}) producing 50% inhibition of MNB cell growth over a 72-h period.
[b] Thiopurine analogue.

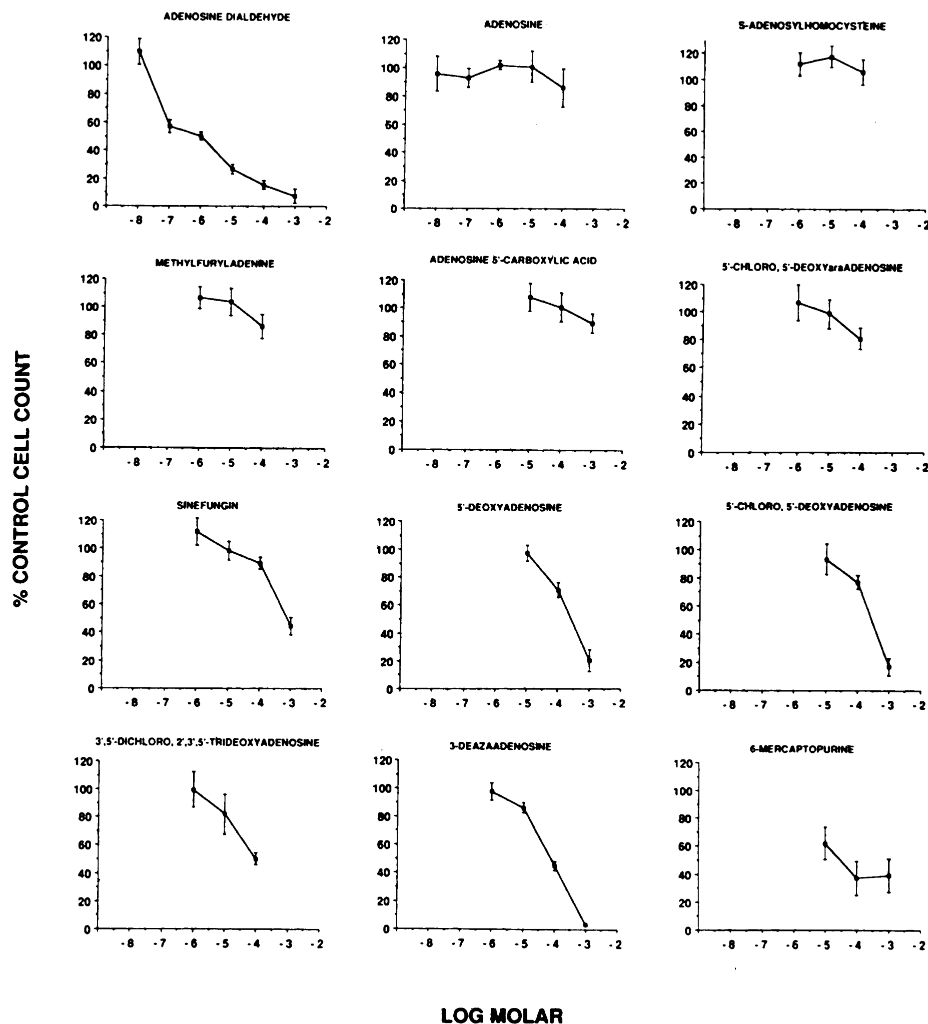

Fig. 3. Effect of nucleoside analogues on murine neuroblastoma cell growth in tissue culture. MNB cell suspensions were incubated with each analogue in concentrations ranging from 10^{-3} to 10^{-8} M for 72 h. Counts of viable cells were performed on control (diluent) and experimental (analogues) tissue cultures. Data are presented as the ratio of cell count (experimental) to cell count (control) × 100 = percentage of control cell count. *Points*, mean of duplicate determinations obtained in 5 to 20 individual experiments; *bars*, SE.

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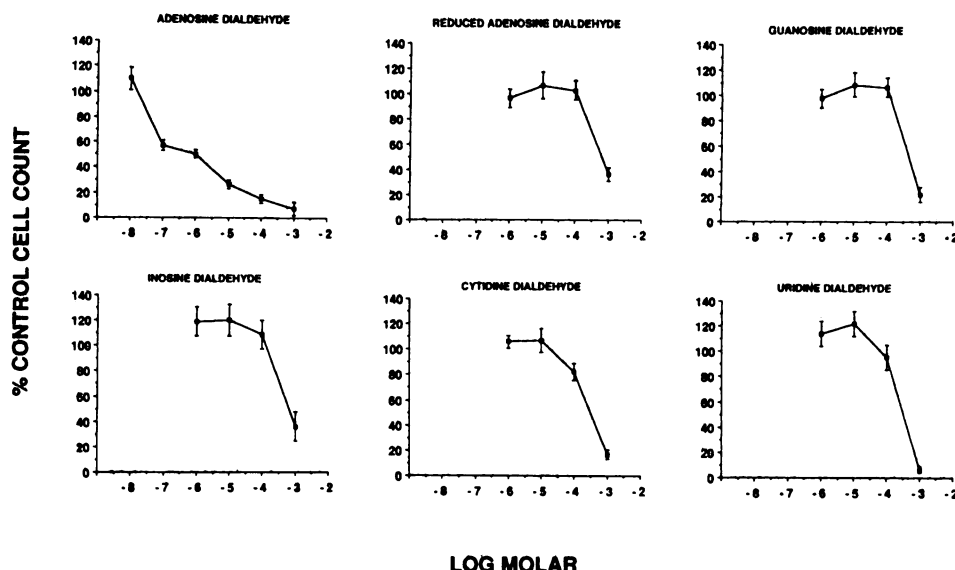

Fig. 4. Effect of nucleoside dialdehydes on murine neuroblastoma growth in tissue culture. The conditions for these experiments were similar to those described in Fig. 3. *Points*, mean of duplicate determinations obtained in 5 to 20 experiments; *bars*, SE.

Table 2 *Inhibitory potency of nucleoside dialdehydes on murine neuroblastoma cell growth in tissue culture*

Nucleoside dialdehydes	Inhibitory concentration (M)[a]	Potency ratio[b]
Reduced adenosine dialdehyde	2.8×10^{-3}	0.00053
Inosine dialdehyde	1.9×10^{-3}	0.00078
Guanosine dialdehyde	9.5×10^{-4}	0.0015
Uridine dialdehyde	3.1×10^{-4}	0.0048
Cytidine dialdehyde	2.9×10^{-4}	0.0051
4',5'-Anhydroadenosine dialdehyde	9.1×10^{-6}	0.16
5'-Deoxyadenosine dialdehyde	7.6×10^{-6}	0.19
Adenosine dialdehyde	1.5×10^{-6}	1.0

[a] Drug concentration (IC_{50}) producing 50% inhibition of MNB cell growth over a 72-h period.

[b] Potency ratio = $\frac{IC_{50}\text{ (adenosine dialdehyde)}}{IC_{50}\text{ (analogue)}}$.

thiopurine analogue, 6-mercaptopurine, had an IC_{50} of 4.7×10^{-5} M. The cytotoxic potency of adenosine dialdehyde was diminished, but not abolished, following reduction of the dialdehyde moieties; reduced adenosine dialdehyde had an IC_{50} of 2.8×10^{-3} M.

The high potency of adenosine dialdehyde when compared to the dialdehydes of other nucleosides suggested an unusual degree of cytotoxic specificity for this compound. This concept was supported by the fact that dialdehydes formed from inosine, guanosine, uridine, and cytidine had IC_{50}s ranging from 1.9×10^{-3} M to 2.9×10^{-4} M, which were approximately 100-fold greater than that of adenosine dialdehyde. In comparison, the 4',5'-anhydro derivative of adenosine dialdehyde as well as 5'-deoxyadenosine dialdehyde was almost equivalent in potency to the parent compound, adenosine dialdehyde. Their respective IC_{50}s were 9.1×10^{-6} M and 7.6×10^{-6} M (Table 2).

DISCUSSION

Nucleoside and dialdehyde analogues have been shown to exert potent cytotoxic effects on a variety of cell lines in tissue culture (11, 15–17) and *in situ*. A primary mechanism by which these agents presumably exert their action is via inhibition of AdoMet-dependent methyltransferase reactions (20). This conclusion is based on data demonstrating a reduction in AdoHcy hydrolase activity (7, 16) and an increase in intracellular AdoHcy concentration following incubation of intact cells with certain nucleoside analogues.

The major nucleoside product of enzyme-catalyzed methyl-transfer, AdoHcy, has traditionally served as a model for the design of analogues that will perturb intracellular methylation reactions. The agents examined in this investigation were designated into two general categories, namely, purine nucleoside and dialdehyde analogues (Figs. 1 and 2).

The former consisted of analogues of adenosine modified in the purine or sugar moieties and a derivative of AdoHcy. The IC_{50}s of these agents ranged from 5.6×10^{-1} M for methylfuryladenine to 5.6×10^{-5} M for 3-deazaadenosine (see Table 1 for IC_{50} of analogues). Adenosine and AdoHcy were both inactive as suppressants of *in vitro* MNB cell growth, whereas the aminomethyne analogue of AdoHcy, sinefungin, exhibited modest growth-inhibiting activity ($IC_{50} = 1.7 \times 10^{-3}$ M). The low potency of AdoHcy and sinefungin as inhibitors of cell replication has been attributed to their poor penetration of mammalian cells and/or metabolic degradation. The data supporting this view are conflicting. Intracellular concentrations of AdoHcy were not elevated following i.v. administration of this substrate to rats and dogs (15). However, other studies have shown that incubation of AdoHcy with intact phytohemagglutinin-stimulated rat lymphocytes (18) and murine neuroblastoma cells (19) decreased methylation in these intact cell models. The inhibitory potency of AdoHcy in intact cells varied for each transmethylation reaction so that incubation with AdoHcy (5×10^{-5} M) diminished tRNA methylation 28% (18) and catechol-*O*-methyltransferase activity 37% below controls (19).

Our study demonstrated decreases in the residual protein carboxylmethyltransferase activity of murine neuroblastoma cells that were only 3 to 7% below control levels after exposure to AdoHcy (9). These data suggest that AdoHcy can enter intact eukaryotic cells and act as a weak suppressant of intracellular methylation. The inability to demonstrate significant elevations in intracellular AdoHcy after incubation with this substrate is probably due to its rapid degradation to adenosine and homocysteine rather than to a lack of intracellular transfer. Since 7-deaza-AdoHcy (*S*-tubercidinylhomocysteine), which is not readily hydrolyzed, can be detected in cells, it is likely that AdoHcy also penetrates to some extent (18, 19).

The other group of compounds evaluated in this study were dialdehydes prepared by periodic acid oxidation of specific nucleosides (Fig. 2; Table 2). The cytotoxic activity of each substrate analogue appeared to correlate best with the following structural determinants.

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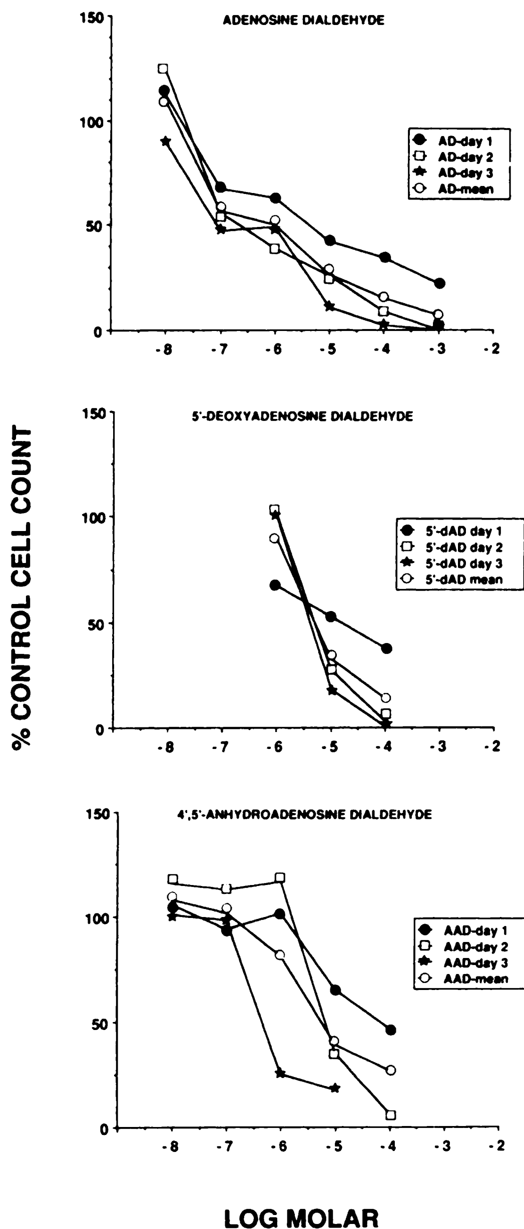

Fig. 5. Effect of adenosine, 5'-deoxyadenosine, and 4',5'-anhydroadenosine dialdehydes on murine neuroblastoma growth in tissue culture. MNB cells were incubated with varying concentrations of each dialdehyde, and counts of viable cells were performed on Days 1, 2, and 3. The data for Days 1, 2, and 3 represent the mean of five individual experiments. The mean value represents the average compiled from cell counts performed on Days 1, 2, and 3. The 5'-deoxyadenosine and 4',5'-anhydroadenosine dialdehyde analogues were selected to demonstrate that cytotoxic potency was not dependent on kinase-mediated phosphorylation at the 5' position (see Table 2). *AD*, adenosine dialdehyde; *5'-dAD*, 5'-deoxyadenosine dialdehyde; *AAD*, 4',5'-anhydroadenosine dialdehyde.

(*a*) The presence of an active 2',3'-dialdehyde moiety was essential for activity, since reduction of adenosine dialdehyde to the 2',3',5'-triol (reduced adenosine dialdehyde) shifted the IC_{50} from 1.5×10^{-6} M to 2.8×10^{-3} M, an 1800-fold decrement in potency.

(*b*) The purine base of each nucleoside influenced the cytotoxic activity of its respective dialdehyde. The dialdehyde prepared from adenosine had the highest potency (IC_{50}, 1.5×10^{-6} M) when compared to the dialdehydes of inosine, guanosine, uridine, and cytidine which had IC_{50}s ranging from 1.9×10^{-3} M to 2.9×10^{-4} M.

(*c*) Phosphorylation of the dialdehyde did not appear to be an essential requirement for cytotoxic activity. The IC_{50}s of 4',5'-anhydroadenosine dialdehyde and 5'-deoxyadenosine dialdehyde which cannot be phosphorylated at the 5' position were 9.1×10^{-6} M and 7.6×10^{-6} M, respectively.

Inhibition of AdoMet-dependent methyltransferase reactions has been shown to suppress a variety of cellular reactions, such as the Rous sarcoma virus-induced transformation of chick embryo fibroblasts (11), the mitogenic response of lymphocytes (10), the growth of Leishmania organisms (17), and in this investigation, the replication of neuroblastoma cells in tissue culture (9). While significant differences between the effects of nucleoside analogues on enzyme activity in cell-free preparations and intact cells have been reported,[4] our data demonstrate that the dialdehyde analogue of adenosine, in particular, exerts a highly potent cytotoxic action on intact murine neuroblastoma cells. The potential utility of this class of compounds as chemotherapeutic agents for the suppression of neural crest tumors *in situ* has been evaluated and appears promising in animal models.[4]

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