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Two Approaches that Increase the Activity of Analogs of Adenine Nucleosides in Animal Cells[1]

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SUMMARY

Deamination of many analogs of adenine nucleosides results in the loss of their chemotherapeutic efficacy. Two approaches have been used in this study to overcome this problem. First, some adenine nucleotides, which are resistant to mammalian adenosine deaminase, are more toxic to animal cells than are the respective nucleosides. For example, 9-β-D-arabinofuranosyladenine 5'-phosphate, a molecule that penetrates the cell without degradation, has a more sustained toxicity against mouse fibroblasts (L-cells) than does 9-β-D-arabinofuranosyladenine (ara-A). Furthermore, L-cells treated with 2',3'-dideoxyadenosine 5'-phosphate are extensively killed after 48 hr, whereas 2',3'-dideoxyadenosine is almost nontoxic to L-cells.

Specific inhibition of adenosine deaminase by nontoxic concentrations of erythro-9-(2-hydroxy-3-nonyl)adenine greatly potentiates the biological activity of both ara-A and 3'-deoxyadenosine (cordycepin). Simultaneous administration of cytostatic concentrations of ara-A and the inhibitor of adenosine deaminase to L-cells killed greater than 99.9% of the cells in 36 hr. A similar concentration of ara-A plus the deaminase inhibitor also markedly extended the mean survival of mice bearing Ehrlich ascites carcinoma as compared to ara-A alone. A cytostatic concentration of cordycepin (1×10^{-4} M), administered in the presence of deaminase inhibitor, killed greater than 99.9% of cultured L-cells in only 8 hr. During the latter incubation, accumulation of uridine in acid-insoluble material reached a maximum after 30 min, and incorporation of thymidine into acid-insoluble material was almost totally arrested after 2 hr.

INTRODUCTION

Toxic adenine nucleosides such as ara-A,[2] 9-β-D-xylofuranosyladenine, ddA, and 3'-deoxyadenosine (cordycepin) are rapidly deaminated by adenosine deaminase (1, 4, 5, 11, 42). The deamination products, *i.e.*, the hypoxanthine nucleosides, are relatively nontoxic (8, 9, 14, 19, 31). The rapidity and extent of this deamination are probably major factors limiting the toxicity of these compounds to cells and, in some instances, the chemotherapeutic effectiveness of the analogs. It has been suggested that the biological activity of these compounds may be potentiated by the simultaneous administration of an inhibitor of adenosine deaminase (3, 8, 21, 39). Such synergistic action has been demonstrated for 1-β-D-arabinofuranosylcytosine, a drug whose activity is greatly potentiated by specific inhibition of the deaminating enzyme by the nontoxic tetrahydrouridine (25). However, potent inhibitors for adenosine deaminase have become available only recently.

We have used 2 different approaches to minimize deamination and each of these results in increased toxicity of some analogs of adenine nucleosides. ara-AMP is not deaminated by adenosine deaminase, and its addition to culture medium produces a sustained cytotoxic effect against fibroblasts. Study of the differences between the cellular metabolism of ara-A (29) and ara-AMP (30) had revealed that small amounts of the nucleotide slowly penetrated the cells, leaving the bulk of the external nucleotide to bathe the cells and to restock internal ara-ATP. ddA is an adenine nucleoside that is virtually nontoxic to L-cells; phosphorylation of this nucleoside is very slow or nonexistent. However, the phosphorylated derivative, ddAMP, kills 90% of the cells in 48 hr.

We have used a powerful inhibitor of adenosine deaminase, erythro-9-(2-hydroxy-3-nonyl)adenine (33), to increase the survival of both ara-A and cordycepin in mouse fibroblasts. When administered with the deaminase inhibitor, ara-A becomes at least 20 times more toxic to L-cells, and 3'-deoxyadenosine (cordycepin) becomes a highly lethal agent and an effective inhibitor of DNA synthesis. The combination of ara-A and the deaminase inhibitor also prolongs the life of tumor-bearing mice significantly beyond that of ara-A alone.

MATERIALS AND METHODS

Materials. All chemicals were reagent grade. ara-A was purchased from Pfanstiehl Laboratories, Inc., Waukegan, Ill. The Cancer Chemotherapy National Service Center provided ddA. Cordycepin was obtained as gifts from Dr. R. J. Suhadolnik, Albert Einstein Medical Center, Philadel-

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[2] The abbreviations used are: ara-A, 9-β-D-arabinofuranosyladenine; ddA, 2',3'-dideoxyadenosine; ara-AMP, 9-β-D-arabinofuranosyladenine 5'-phosphate; ara-ATP, 9-β-D-arabinofuranosyladenine 5'-triphosphate; ddAMP, 2',3'-dideoxyadenosine 5'-phosphate; ara-Hx, 9-β-D-arabinofuranosylhypoxanthine; 3',5'-cyclic ara-AMP, 9-β-D-arabinofuranosyladenine cyclic 3',5'-phosphate; araIMP, 9-β-D-arabinofuranosylhypoxanthine 5'-phosphate; PCA, perchloric acid; dAdo, 2'-deoxyadenosine; PBS, phosphate-buffered saline.

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phia, Pa., and Dr. J. R. Tata, National Institute of Medical Research, London, England, and was also purchased from Sigma Chemical Co., St. Louis, Mo.

ara-Hx, 3'-deoxyinosine, and 2',3'-dideoxyinosine were produced enzymatically by deamination of ara-A, 3'-deoxyadenosine, and ddA, respectively, with calf intestinal mucosa adenosine deaminase (Type I; Sigma) as previously described for ara-Hx (4). The reaction mixtures were heated in a boiling water bath for 5 min and lyophilized. After solution in a minimal volume of H_2O, the reaction mixtures were streaked on Whatman no. 3MM paper and separated by descending chromatography in 94.6 ml water-saturated *n*-butyl alcohol:5.4 ml NH_4OH (Solvent 1). The deamination products of both ara-A and 3'-deoxyadenosine possessed the spectrophotometric and chromatographic properties described for ara-Hx (4) and 3'-deoxyinosine (31), respectively. 2',3'-Dideoxyinosine had absorbance maxima at 248 nm (pH 2) and 254 nm (pH 12). When chromatographed on Avicel F thin-layer chromatography plates (Analtech, Inc., Newark, Del.), 2',3'-dideoxyinosine had R_F values of 0.12 to 0.17 and 0.74 to 0.81 in Solvent J and 66 ml isobutyric acid:33 ml H_2O:1 ml NH_4OH (Solvent 2), respectively. Dr. H. J. Schaeffer (Wellcome Research Laboratories, Research Triangle Park, N. C.), generously supplied erythro-9-(2-hydroxy-3-nonyl)adenine (33).

ara-AMP was synthesized by L. Lapi in this laboratory (4, 12, 30). Dr. M. Hubert-Habart synthesized 9-β-D-arabinofuranosyladenine cyclic 2',5'-phosphate (15). Dr. R. K. Robins of the ICN Nucleic Acid Research Institute, Irvine, Calif., kindly provided 3',5'-cyclic ara-AMP, ara-IMP, 9-β-D-arabinofuranosyladenine-N^1-oxide 5'-phosphate, and ddAMP. ara-IMP was also prepared from ara-AMP by deamination with adenylic deaminase (Sigma). The product behaved similarly to authentic ara-IMP in several thin-layer chromatographic systems, thin-layer electrophoresis, and high-pressure liquid chromatography.

[*methyl*-^{3}H]Thymidine (2.2 and 10.6 Ci/mmole) was purchased from New England Nuclear, Boston, Mass., and Schwartz/Mann, Orangeburg, N. Y., and purified by paper chromatography in *tert*-butyl alcohol:methyl ethyl ketone:H_2O:formic acid (44:44:11:0.26) before use. [*G*-^{3}H]Uridine (9.5 Ci/mmole; New England Nuclear) was purified by thin-layer chromatography in Solvent 2. Tritiated dideoxyadenosine was prepared and purified on Dowex 1-X2 (OH^-) before use (37). [2-^{3}H]ara-A (11 Ci/mmole) (New England Nuclear) was recrystallized from H_2O before use.

Cell Culture Methods. Strain L mouse fibroblasts (8) were maintained in suspension culture as described earlier (29). Unless otherwise indicated, exponentially growing cells were harvested, washed free of normal growth medium, and resuspended in medium containing 10% fetal calf serum that had been heated for 20 hr at 56°. The heating procedure markedly reduced various activities in the sera, *i.e.*, ara-A deaminase activity was found to be less than 6 nmoles/ml/4 hr (29), and ara-AMP phosphatase activity was less than 1 nmole/ml/4 hr.

Cell number was determined with a Model B Coulter Counter. Cell viability was measured (13) after dilution of 0.5 ml of culture in 2 ml normal growth medium and centrifugation at 190 × *g* for 5 min. After removal of the supernatant, the cells were resuspended and diluted in normal growth medium (37°) so that approximately 100 viable cells were added in 5 ml to 60-mm Falcon Petri dishes. Following 7 days of incubation at 37° in 5% CO_2-95% air, the dishes were washed with PBS (30) and stained with Giemsa stain, and colonies were counted with the aid of a 20× dissecting microscope. Plating efficiencies of untreated cultures were 70 to 90%. All viability data presented represent the mean of triplicate plates.

Mouse Tumor Studies. Ehrlich ascites carcinoma cells, obtained from Dr. W. K. Roberts, Department of Microbiology, University of Colorado Medical Center, were maintained in C57BL/6J × DBA/2J F_1 mice (The Jackson Laboratory, Bar Harbor, Maine) by i.p. injection of approximately 5 × 10^6 cells at weekly intervals. Seven days after injection, a mouse was sacrificed; the ascitic fluid was removed from the peritoneal cavity by sterile syringe and diluted in 10 volumes of PBS. Tumor cells were sedimented and washed twice in PBS to remove red blood cells, and the cell concentration was determined with a Coulter Counter. Mice (16 to 19 g) were given i.p. injections of 2 × 10^6 tumor cells in 0.2 ml PBS. After 1 day, the tumor-bearing mice were given daily injections for 5 days with the appropriate compounds. Each mouse was weighed before injection, and stock solutions of compounds were adjusted so that each mouse was given an injection of 0.04 ml/g. Daily observations were made of tumor progress and mouse mortality.

Enzymatic Deamination of Adenine Nucleosides. The deamination of adenine nucleosides by calf intestinal adenosine deaminase (Type I, lot 1036-2490-1; Sigma) was followed spectrophotometrically at 265 nm, as described by Kaplan (18). The reaction mixture contained 45 μmoles K_2HPO_4 (pH 7.5), 100 μmoles of the appropriate adenine nucleoside, and 0.023 unit of enzyme in 1.0 ml. Adenosine was deaminated at a rate of 0.91 mmole/min/unit at 21°.

Homogenates of L-cells and Ehrlich ascites carcinoma cells were assayed for deaminase activity by the same method. Exponentially growing L-cells or 7-day Ehrlich ascites carcinoma cells were harvested, washed with PBS, incubated briefly in hypotonic medium, and disrupted in a Potter-Elvehjem homogenizer. Following centrifugation at 37,000 × *g* for 15 min, the supernatant was stored in multiple portions at −70°. Adenosine was deaminated at a rate of 4.6 μmoles/min/mg extract protein and 1.3 μmoles/min/mg by extracts of L-cells and Ehrlich ascites cells, respectively.

[^{3}H]ara-A is rapidly deaminated by exponentially growing L-cells (29), and over 95% of the ara-Hx appears in the medium. After varying periods of incubation in the presence of 1 × 10^{-4} M [^{3}H]ara-A (specific activity, 1.7 × 10^7 cpm/μmole), cells were removed by centrifugation. PCA (10 N) was added to the medium to a final concentration of 0.4 N PCA. The acid-insoluble material was removed by centrifugation and the supernatant was neutralized with KOH. Ten μl of the supernatant were added to 25 nmoles each of ara-A and ara-Hx. The mixture was spotted on Avicel F and chromatographed in Solvent 1. The areas on the thin-layer plates containing the nucleosides, which were

visualized under UV light (254 nm), were scraped into tubes. The compounds were eluted with 1.0 ml 0.01 N HCl for 1 hr, and 0.5-ml portions were counted in precounted scintillation vials containing 10 ml scintillation fluid (3a70; Research Products International Corp., Elk Grove Village, Ill.).

Incorporation of Thymidine and Uridine. Exponentially growing cells were harvested and resuspended in growth medium without serum at a final cell density of 2.5×10^6 cells/ml. Control experiments showed that a 5-fold concentration of the cells did not significantly affect their ability to incorporate thymidine into acid-insoluble material. Aliquots of cells were transferred to separate spinner flasks containing a final concentration of 0.01 μmole/ml of either [^{3}H]thymidine (specific activity, 4.0×10^7 cpm/μmole) or [^{3}H]uridine (specific activity, 4.3×10^6 cpm/μmole). Cordycepin (0.10 μmole/ml) and erythro-9-(2-hydroxy-3-nonyl)adenine (0.01 μmole/ml) were added as noted. Following incubation for the appropriate times, 10-ml portions were removed from each spinner and diluted in 3 volumes of room-temperature PBS. The cells were harvested and washed twice in 25 ml of PBS before being extracted twice for 30 min in 1.0 ml 0.4 N PCA at 4°.

Acid-insoluble pellets were washed twice with 10 ml of 0.4 N PCA at 4° and suspended in 1.0 ml H_2O. The pH was adjusted to 7.5 with 0.1 N KOH, and the entire clear sample was added to 10 ml scintillation fluid and counted. Nonspecific absorption of labeled compounds was estimated. The cells were removed before addition of the labeled nucleoside, washed in PBS, and extracted with 0.4 N PCA. The cellular residue was resuspended in 10 ml of growth medium minus serum; the labeled nucleoside was added and these samples were fractionated as described above. Radioactivities in the acid-insoluble fractions, averaging 10 pmoles/2.5×10^7 cells for thymidine incorporation and 20 pmoles/2.5×10^7 cells for uridine incorporation, were subtracted from the values of the samples taken during the experiment.

RESULTS

Lethality of ara-A Nucleotides. We have reported that ara-AMP is more toxic to exponentially growing L-cells than is ara-A at 0.04 and 0.10 μmoles/ml (26). At these concentrations of ara-A, cell growth is inhibited but there is little or no net decrease in viable cells in the cultures. As shown in Chart 1, ara-A at 0.20 μmoles/ml is unequivocally lethal, with about 75% loss of viable cells in the 1st 24 hr, followed by the multiplication of surviving cells. In contrast, cultures treated with ara-AMP show a slight increase in viable cells during the 1st 12 hr, followed by a decrease of viability that is sustained throughout the experiment. After 24 hr, essentially all of the ara-A is deaminated to ara-Hx in this system (8, 29), whereas 99% of the ara-AMP remains as such. Thus phosphorylation of the nucleoside to ara-AMP appears to be an effective mechanism by which ara-A, the nucleoside moiety essential for cytotoxicity, is preserved from inactivation by deamination in this cellular system. As shown elsewhere, ara-AMP is taken up at only a few % of the rate of incorporation of ara-A (29). Nevertheless, it was also shown that ara-ATP derived from ara-AMP can attain a concentration inhibitory to DNA polymerase (30).

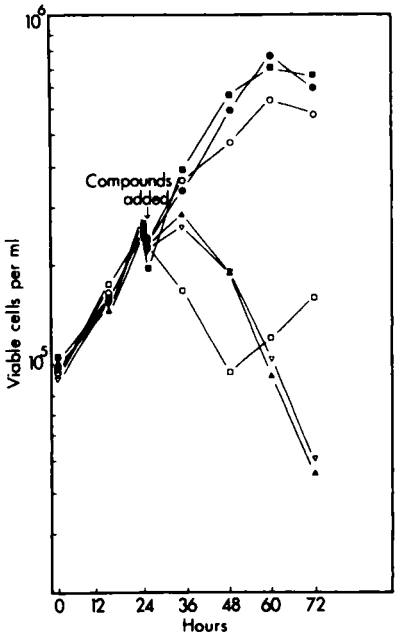

Chart 1. Viability of L-cells during incubation with arabinosyl nucleotides. All compounds were added to a final concentration of 2×10^{-4} M. ■, control; ●, 9-β-D-arabinofuranosyladenine cyclic 2',5'-phosphate; ○, adenosine cyclic 3',5'-phosphate; □, ara-A; ▽, 3',5'-cyclic ara-AMP; ▲, ara-AMP.

3',5'-Cyclic ara-AMP is as toxic to L-cells as is ara-AMP (Chart 1). An extract of L1210 cells has been shown to convert the cyclic nucleotide to ara-AMP (16). The toxicity of the cyclic compound in L-cells may be due to its conversion to ara-AMP. In this and 2 other experiments, it has been observed that cell growth rate and the final cell density in cultures treated with 2×10^{-4} M adenosine cyclic 3',5'-phosphate are significantly less than the control. The 9-β-D-arabinofuranosyladenine cyclic 2',5'-phosphate is not toxic. In separate experiments, we have shown that, at 2×10^{-4} M, neither ara-Hx, ara-IMP, nor 9-β-D-arabinofuranosyladenine-N^1-oxide 5'-phosphate had any effect on cell viability.

Lethality of the 5'-Phosphate of Dideoxyadenosine. ddA is an adenine nucleoside that is lethal to *Escherichia coli* and has been shown to be converted to the triphosphate and incorporated terminally into DNA (7, 37, 38). However, we have found this compound to be almost inactive against L-cells (Chart 2). Other experiments have indicated that ddA is not a good substrate for L-cell nucleoside kinases. Cells were incubated 1 hr with either [^{3}H]ddA or [^{14}C]dAdo. The constituents of the nucleotide pools were extracted (24) and separated by high-pressure liquid chromatography (29), and 2-min fractions were collected and analyzed for radioactivity (not shown). Although approximately equal radioactivity was present as nucleoside in cells treated with either precursor, there was at least 430 times as much radioactive material in the adenine triphosphate fraction of the dAdo-treated cells as in ddA-treated cells. In fact, in no fraction of the ddA-treated extract did the counts outside the nucleoside region equal the background count (25 cpm).

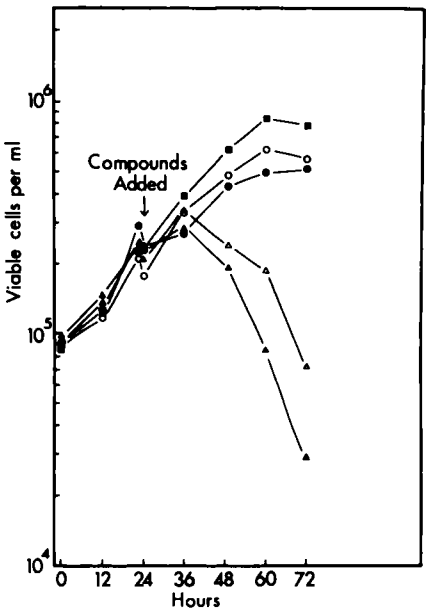

Chart 2. Viability of L-cells during incubation with dideoxyribosyl compounds and an adenosine deaminase inhibitor. ■, control; ○, 4 × 10^{-4} M ddA; ●, 8 × 10^{-4} M ddA; △, 4 × 10^{-4} M ddAMP; ▲, 8 × 10^{-4} M ddAMP.

In earlier experiments with ara-AMP (30), it had been demonstrated that the nucleotide was both resistant to deamination and capable of penetrating the cells intact. Although the cells appeared unable to phosphorylate ddA at a readily detectable rate and were killed when treated with the nucleotide ddAMP (Chart 2), the hypothesis that ddAMP also penetrates L-cells intact requires more direct proof.

Inhibition of Adenosine Deaminase. Deamination of the several adenine compounds was studied using both intestinal deaminase and homogenates of several kinds of animal cells. The relative rates of deamination by the intestinal enzyme of adenosine, cordycepin, ddA, and ara-A were 1.00, 0.70, 0.48, and 0.27, respectively. These values were similar to those reported earlier (1, 5, 11, 42). Deamination of AMP and of ara-AMP by this enzyme was not detected. Only small differences are seen in the relative rates of deamination of the same substrates by homogenates of L-cells: adenosine, 1.00; cordycepin, 0.63; ddA, 0.48; ara-A, 0.28. In homogenates of Ehrlich cells, ara-A is deaminated at a rate of 13% of that of adenosine.

Recently, Schaeffer and Schwender (33) synthesized erythro-9-(2-hydroxy-3-nonyl)adenine and showed it to be an extremely potent inhibitor of the calf intestine adenosine deaminase. This has been confirmed and extended to the deaminase activity in homogenates of L-cells and Ehrlich ascites carcinoma. When erythro-9-(2-hydroxy-3-nonyl)adenine is added at a final concentration of 1 × 10^{-5} M to deaminase assays, using either the intestinal enzyme or L-cell homogenates, no deamination of adenosine, cordycepin, ddA, and ara-A is detectable. Similarly, no deamination of adenosine or ara-A by Ehrlich cell extracts is detectable in the presence of 1 × 10^{-5} M deaminase inhibitor. In contrast, 1 × 10^{-3} M deaminase inhibitor did not affect the rate of adenosine deamination by extracts of *E. coli* strain 15 TAUAd (7).

L-cells growing exponentially in spinner culture at 4.6 × 10^5 cells/ml deaminate ara-A at a rate of 11 μmoles/hr over 6 hr. However, in the presence of 1 × 10^{-5} M deaminase inhibitor, only 6% of the ara-A is deaminated after incubation for 24 hr. Since the viability of L-cells is dependent on the concentration of ara-A, this system was used to test the biological efficacy of the deaminase inhibitor.

Potentiation of ara-A Toxicity by erythro-9-(2-hydroxy-3-nonyl)adenine. Cells were incubated with 1 × 10^{-4} M ara-A and varying concentrations of the deaminase inhibitor. This concentration of ara-A alone is virtually cytostatic (Chart 3; Ref. 26), but in the presence of 1 × 10^{-5} M deaminase inhibitor and 2 × 10^{-5} M ara-A, a concentration that evokes little or no growth inhibition alone, the increase in viable cells stops, and after a lag of 22 hr, viability in the culture decreases exponentially for the duration of the experiment. ara-A at 1 × 10^{-5} M is also highly lethal in the presence of the inhibitor. The inhibitor clearly increases the toxicity of ara-A more than 20-fold. Concentrations of ara-A less than 1 × 10^{-5} M are only very slightly toxic in the presence of the deaminase inhibitor.

The lag time prior to decline of cell viability is seen to increase (Chart 4) as the concentration of ara-A is decreased in the presence of 1 × 10^{-5} M erythro-9-(2-hydroxy-3-nonyl)adenine. This lag may represent the time necessary to accumulate toxic concentrations of ara-ATP in the cells.

The Effect of ara-A and erythro-9-(2-Hydroxy-3-nonyl)adenine on Tumor-bearing Mice. The synergistic

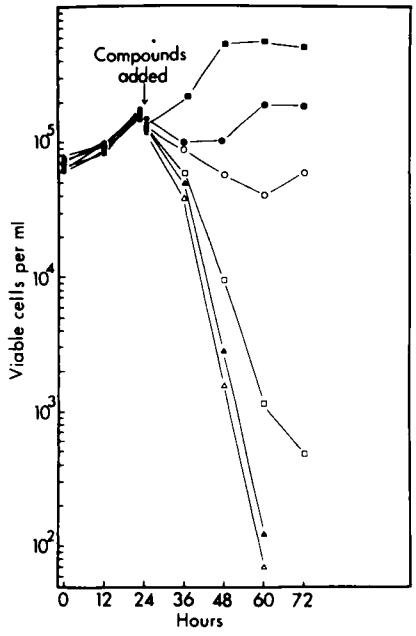

Chart 3. Viability of L-cells during incubation with ara-A and various concentrations of erythro-9-(2-hydroxy-3-nonyl)adenine. ■, control; ●, 1 × 10^{-4} M ara-A; ○, 1 × 10^{-4} M ara-A plus 1 × 10^{-8} M erythro-9-(2-hydroxy-3-nonyl)adenine; □, 1 × 10^{-4} M ara-A plus 1 × 10^{-7} M erythro-9-(2-hydroxy-3-nonyl)adenine; ▲, 1 × 10^{-4} M ara-A plus 1 × 10^{-6} M erythro-9-(2-hydroxy-3-nonyl)adenine; △, 1 × 10^{-4} M ara-A plus 1 × 10^{-5} M erythro-9-(2-hydroxy-3-nonyl)adenine.

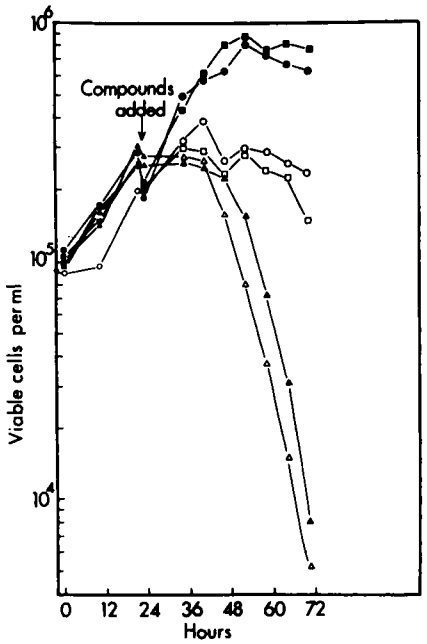

Chart 4. Viability of L-cells during incubation with erythro-9-(2-hydroxy-3-nonyl)adenine and various concentrations of ara-A. ■, control; ●, 1×10^{-5} M erythro-9-(2-hydroxy-3-nonyl)adenine; ○, 2.5×10^{-6} M ara-A plus 1×10^{-5} M erythro-9-(2-hydroxy-3-nonyl)adenine; □, 5.0×10^{-6} M ara-A plus 1×10^{-5} M erythro-9-(2-hydroxy-3-nonyl)adenine; ▲, 1×10^{-5} M ara-A plus 1×10^{-5} M erythro-9-(2-hydroxy-3-nonyl)-adenine; △, 2×10^{-5} M ara-A plus 1×10^{-5} M erythro-9-(2-hydroxy-3 nonyl)adenine.

effect of erythro-9-(2-hydroxy-3-nonyl)adenine on ara-A toxicity is also seen in the protection of mice carrying tumors. In 2 experiments (Table 1), the average survival time of $BD2F_1$ mice bearing Ehrlich ascites carcinoma cells was extended beyond that of control mice by ara-A, 50 mg/kg, administered i.p. daily for 5 days. However, tumor-bearing mice treated simultaneously with ara-A and the deaminase inhibitor survived significantly longer than did those treated with ara-A alone.

Potentiation of Toxicity of Cordycepin by erythro-9-(2-Hydroxy-3-nonyl)adenine. It is known that 3'-deoxyadenosine (cordycepin) is inactivated by deamination (19, 31). Exponentially growing L-cells incubated with 1×10^{-4} M cordycepin stopped multiplying and showed no further increase in the number of viable cells in the culture (Chart 5). More than 99.9% of the cells treated with the same concentration of cordycepin plus 1×10^{-7} M erythro-9-(2-hydroxy-3-nonyl)adenine lost viability in the 1st 12 hr of incubation, after which there was no further loss of viable cells. This degree of lethality, although equal in magnitude, is attained in one-fourth the time required by 1×10^{-4} M ara-A plus 1×10^{-7} M deaminase inhibitor (Chart 3), suggesting that cordycepin may act on a particularly sensitive site essential to cell viability. Lower concentrations of cordycepin in the presence of the deaminase inhibitor also had their greatest effects in the early stages of the incubation (Chart 5).

To study the kinetics of this rapid killing effect more closely and to maximize the toxicity at potentially obtainable therapeutic doses, exponentially growing cells were incubated with 1×10^{-5} M erythro-9-(2-hydroxy-3-nonyl)adenine and either 1×10^{-4} or 1×10^{-5} M cordycepin. The number of viable cells in the culture treated with the higher cordycepin concentration remains stable for barely 1 hr before undergoing a rapid exponential decrease (Chart 6). After 8 hr in the presence of the mixture, greater

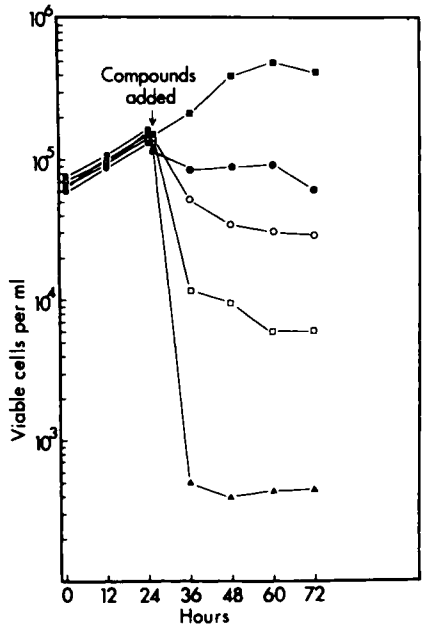

Chart 5. Viability of L-cells during incubation with erythro-9-(2-hydroxy-3-nonyl)adenine and various concentrations of cordycepin. ■, control; ●, 1×10^{-4} M cordycepin; ○, 2.5×10^{-5} M cordycepin plus 1×10^{-7} M erythro-9-(2-hydroxy-3-nonyl)adenine; □, 5×10^{-5} M cordycepin plus 1×10^{-7} M erythro-9-(2-hydroxy-3-nonyl)adenine; ▲, 1.0×10^{-4} M cordycepin plus 1×10^{-7} M erythro-9-(2-hydroxyl-3-nonyl)adenine.

Table 1

Effect of ara-A and erythro-9-(2-hydroxy-3-nonyl)adenine on the survival of $BD2F_1$ mice bearing Ehrlich ascites carcinoma

Treatment	Days surviving	
	I	II
PBS	14.1 ± 2.1[a] (10)[b]	13.8 ± 2.1 (10)
ara-A (50 mg/kg)	20.3 ± 3.8 (9)	16.2 ± 2.0 (8)
ara-A (50 mg/kg) + inhibitor (3.1 mg/kg)	31.8 ± 6.9 (10)	29.4 ± 11.2 (9)
Inhibitor (3.1 mg/kg)	15.2 ± 1.9 (5)	

[a] Mean ± S.D.

[b] Numbers in parentheses, number of mice tested.

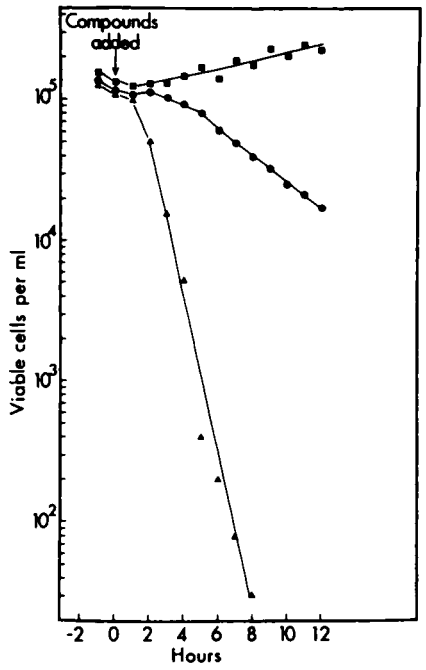

Chart 6. Viability of L-cells during incubation with cordycepin and erythro-9-(2-hydroxy-3-nonyl)adenine. ■, control; ●, 1 × 10^{-5} M cordycepin plus 1 × 10^{-5} M erythro-9-(2-hydroxy-3-nonyl)adenine; ▲, 1 × 10^{-4} M cordycepin plus 1 × 10^{-5} M erythro-9-(2-hydroxy-3-nonyl)adenine.

than 99.9% of the cells are unable to form colonies, in contrast to the relatively cytostatic effect of 1 × 10^{-4} M cordycepin alone (Chart 5). In the absence of deaminase inhibitor, 1 × 10^{-5} M cordycepin has no effect on cell growth over several days (not shown). However, in the presence of 1 × 10^{-5} M deaminase inhibitor, 1 × 10^{-5} M cordycepin kills 90% of the cells in only 12 hr (Chart 6).

The Effect of Cordycepin and the Deaminase Inhibitor on Nucleic Acid Synthesis. The increased toxicity of cordycepin in the presence of the deaminase inhibitor suggested that this combination would produce more marked effects on macromolecular synthesis than had previously been observed. Fairly high concentrations of this nucleoside (1 × 10^{-4} M) alone gave incomplete inhibition of synthesis of RNA and very little of DNA (35). In the presence of cordycepin alone (1 × 10^{-4} M), uridine incorporation into RNA is about 50% of the control at 30 min (Chart 7*A*). This rate continues for 45 min, when uridine accumulation stops. In the presence of the nucleoside (1 × 10^{-4} M) plus deaminase inhibitor (1 × 10^{-5} M), the initial rate of uridine incorporation is very low and attains 31% of the control at 30 min, at which time the uptake of uridine into nucleic acid is completely arrested.

Of particular interest is the question of the possible effect on synthesis of DNA, which, in many systems, is initiated on newly synthesized RNA primer chains (10). It might be anticipated that a termination of RNA by cordycepin would lead to an absolute arrest of DNA synthesis. As shown in Chart 7*B*, 1 × 10^{-4} M cordycepin has little effect on the incorporation of thymidine into acid-insoluble material during the 1st hr of incubation. Thereafter, incorporation is inhibited by approximately 35% for each hr. In contrast, 1 × 10^{-4} M cordycepin plus 1 × 10^{-5} M deaminase inhibitor inhibits thymidine incorporation to only 40% of the control value during the 1st 2 hr, after which incorporation is essentially blocked.

DISCUSSION

The biological activity of analogs of adenine nucleosides is essentially destroyed by deamination. Although this may occur after the compound has been phosphorylated to the nucleotide (30), the most damaging activity appears to come from deamination of the nucleoside by adenosine deaminase, an enzyme of widespread occurrence (21, 22). In this report, we have presented 2 general approaches for protecting deaminase-sensitive adenine nucleoside analogs and have demonstrated the utility of these approaches in increasing the biological efficacy of several compounds.

Toxic Nucleotides. Ortiz *et al.* (26) originally reported that ara-AMP was more toxic than ara-A to cultured fibroblasts. We have confirmed this observation and extended it to other nucleotides. Of the arabinose-containing nucleotides tested, only 2, ara-AMP and 3',5'-cyclic ara-AMP, showed a greater sustained effect on the viability of L-cells than did ara-A (Chart 1). In contrast to the rapid development of toxicity in ara-A-treated cells, there is an initial increase in the number of viable cells in cultures treated with ara-AMP and 3',5'-cyclic ara-AMP prior to the onset of lethality, a fact probably related to the slower rate of penetration of the nucleotides (30).

Although the rates of killing are similar in ara-A- and ara-AMP-treated cells, differences in the cellular metabolism of the 2 compounds (29, 30) are reflected by the sustained lethality in ara-AMP-treated cultures *versus* the recovery of cells incubated with ara-A (Chart 1). Essentially all of the exogenous ara-A was found to be deaminated to ara-Hx after 24 hr by the deaminase of cells and the slight deaminase activity in the medium. The arrest of the decrease in viability in the ara-A-treated culture appears to

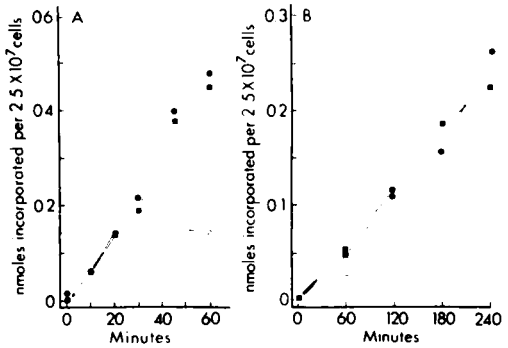

Chart 7. Effect of cordycepin and erythro-9-(2-hydroxy-3-nonyl)adenine on the incorporation of [^{3}H]uridine (*A*) or [^{3}H]thymidine (*B*) into acid-insoluble fractions by L-cells. ■, control; ●, 1 × 10^{-5} M erythro-9-(2-hyroxy-3-nonyl)adenine; □, 1 × 10^{-4} M cordycepin; ○, 1 × 10^{-4} M cordycepin plus 1 × 10^{-5} M erythro-9-(2-hydroxy-3-nonyl)adenine. Each *point* in *A* is from a single experiment. Each *point* in *B* is the average of 2 experiments, except those for 1 × 10^{-5} M erythro-9-(2-hydroxy-3-nonyl)adenine, which are from a single experiment.

be due to conversion of ara-A to the relatively ineffective ara-Hx, followed by the turnover and decrease of cellular ara-ATP. The viable cells then go on to multiply.

Only small amounts of cellular arabinosyl nucleotides could have arisen from extracellular dephosphorylation of ara-AMP, followed by nucleoside penetration and subsequent cellular rephosphorylation (30). We have observed no potentiation of ara-AMP toxicity in the presence of 1×10^{-7} M erythro-9-(2-hydroxy-3-nonyl)adenine, suggesting the absence of such a nucleoside deaminase-sensitive step. The sustained killing effect of ara-AMP may be attributed to its resistance to deamination by virtue of protection by the 5'-phosphate and the slow but continuous uptake of the intact nucleotide.

Dideoxyadenosine can also be deaminated by L-cells. The inability of the cells to phosphorylate the compound also contributes to the lack of toxicity of this nucleoside. The fact that ddAMP is lethal to cells whereas ddA has no effect on viability suggests that direct penetration of the nucleotide may be responsible for the increased lethality. If so, the use of phosphorylated nucleoside analogs may not only serve to protect them from inactivation by deamination but, in some instances, may be a practical method to by-pass cellular resistance to a nucleoside, due to lack of activation by phosphorylation.

Inhibition of Adenosine Deaminase. DNA synthesis is inhibited in ara-A-treated cells (8), an effect thought to be largely the result of the inhibition of DNA polymerase by ara-ATP (12). However, the conversion of the nucleoside to the triphosphate must be very inefficient, because the toxic concentration of ara-A is 100-fold that of the K_i of the ara-ATP for a mammalian DNA polymerase ($K_i = 1 \times 10^{-6}$ M; Ref. 12). As described above, this inefficiency appears to be related mainly to the deamination of ara-A to ara-Hx. Our finding of high toxicity at much lower ara-A concentrations (5 to 10×10^{-6} M) in the presence of the adenosine deaminase inhibitor (Chart 3) confirms the hypothesis that the low toxicity of ara-A alone is indeed the result of deamination of this nucleoside. It will therefore be important to study the effects of ara-A in the presence of the deaminase inhibitor for comparison with studies on ara-A alone (29), both in tissue culture and in animal experiments. Recently, Bryson *et al.* (2) reported an increased antiviral activity of ara-A in the presence of 2'-deoxycoformycin, another inhibitor of adeonsine deaminase (41).

The effect of erythro-9-(2-hydroxy-3-nonyl)adenine on the toxicity of cordycepin is even more remarkable. Under conditions where deamination is prevented, a concentration of cordycepin, which by itself is cytostatic to fibroblasts, becomes capable of killing nearly 4 decades of cells in as little as 8 hr, about 40% of the normal cell cycle (Chart 6).

Cordycepin has antitumor properties (17), but it was generally thought to be cytostatic rather than cytotoxic to mammalian cells (28, 36). Mono-, di-, and triphosphates of cordycepin accumulate in the cells (20) and may inhibit *de novo* purine synthesis (32) as well as terminate RNA chains (34), as a function of the absence of a 3'-hydroxyl group. More recently, cordycepin has been shown to perferentially inhibit the synthesis of ribosomal precursor RNA and tRNA (35, 40) and to interfere with cytoplasmic mRNA production by blocking posttranscriptional poly(A) addition to mRNA molecules (6). Curiously, it appeared to have little effect on synthesis of heterogenous nRNA (28, 35, 40). These results have been attributed to differences in the sensitivity of the various cellular RNA polymerases to cordycepin 5'-triphosphate (23). However, the effects are frequently short-lived (27), probably due to rapid deamination of cordycepin by cell cultures. It seems evident that these phenomena require reexamination under conditions in which the cordycepin is maintained by prevention of deamination.

RNA accumulation is rapidly arrested in the presence of cordycepin and the deaminase inhibitor (Chart 7*A*). DNA synthesis is also greatly inhibited initially and is terminated somewhat more slowly, within 2 hr (Chart 7*B*). Since RNA synthesis in many systems is necessary to provide a primer for the synthesis of DNA (10), inhibition of such RNA synthesis may make initiation of DNA synthesis impossible and thus account for the observed termination of DNA synthesis.

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