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5-Amino-4-imidazolecarboxamide Riboside Potentiates the Metabolism and Anti-Human Immunodeficiency Virus Activity of 2′,3′-Dideoxyinosine

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SUMMARY

The antiviral activity of the purine dideoxynucleosides 2′,3′-dideoxyadenosine (ddA) and 2′,3′-dideoxyinosine (ddI) is dependent on their conversion into ddA triphosphate *in vivo*. 5-Amino-4-imidazolecarboxamide riboside (AICA riboside), a natural metabolite in purine biosynthetic pathways, is converted into IMP, a substrate for the biosynthesis of adenine and guanine nucleotides, and enhances the intracellular purine nucleotide pools. Because IMP also serves as a phosphate donor in the anabolic phosphorylation of ddI (and ddA) into ddI monophosphate by the cytosolic enzyme 5′-nucleotidase, we investigated the effects of AICA riboside on the phosphorylation and antiretroviral activity of these purine nucleoside analogs. At an AICA riboside concentration of 0.5 mM, there was a ~2-fold increase in the intracellular ATP and GTP levels, whereas a nearly 8-fold increase was observed for the phosphorylation of ddA (or ddI). A marked reduction in intracellular pools of the pyrimidine nucleotides CTP and UTP was observed in AICA riboside-treated cells and inhibited cell proliferation. However, this growth inhibition was prevented by the addition of uridine to the cultures. Cells pretreated with AICA riboside and ddI were less susceptible to human immunodeficiency virus (HIV) infection and synthesized reduced levels of HIV proviral DNA. A 10-fold potentiation of the effectiveness of ddI against both wild-type HIV (HIV_{IIIB}) and a ddI-resistant variant HIV was observed in the presence of 0.5 mM AICA riboside. These results show that AICA riboside modulates the anabolism and antiviral activity of ddI, and they have implications for possible therapies with dideoxynucleosides.

A large family of nucleoside analogs, particularly the ddNs, exhibit selective activity against the replication of HIV, the etiological agent of AIDS. Among this class of agents, the pyrimidine nucleoside analogs AZT (1, 2) and ddC (3, 4) and the purine nucleoside analog ddI (5, 6) are the only antiretroviral drugs currently approved for the treatment of AIDS. Administration of AZT and ddI has been shown to result in clinical and immunological improvement and to confer increased survival to HIV-infected patients with advanced immunodeficiency.

The antiretroviral activity of ddNs is thought to derive from their inhibition of viral reverse transcriptase. Metabolic studies have shown that the activation of ddNs into their corresponding ddNTPs by cellular enzymes is a major determinant of their antiviral activity. Different ddNs require different cellular enzymes for their phosphorylation; for example, the initial step of AZT phosphorylation is catalyzed by thymidine kinase (7), whereas ddC is phosphorylated by deoxycytidine kinase (8). However, ddA, ddI, ddG, and carbocyclic 2′,3′-didehydro-2′,3′-ddG all utilize cytosolic 5′-nucleotidase (9, 10). The 5′-monophosphates are further phosphorylated into their corresponding triphosphates by other cellular enzymes (11). The ddNTPs inhibit reverse transcription by competition with their deoxynucleoside triphosphate counterparts for binding to reverse transcriptase and/or by their chain-terminating activity (12, 13). Because IMP serves as an efficient donor in the enzymatic phosphorylation of ddI and its analogs, it was postulated that IMP may act as an intracellular modulator for the activation of these compounds (14). Therefore, agents that modulate intracellular IMP pools may be useful as part of a combination with ddI for the therapy of AIDS. We show here that AICA

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ABBREVIATIONS: ddN, dideoxynucleoside; AICA riboside, 5-amino-4-imidazolecarboxamide riboside; AZT, 3′-azido-3′-deoxythymidine; ddA, 2′,3′-dideoxyadenosine; ddATP, 2′,5′-dideoxyadenosine 5′-triphosphate; ddADP, 2′,5′-dideoxyadenosine 5′-diphosphate; ddC, 2′,3′-dideoxycytosine; ddG, 2′,3′-dideoxyguanosine; ddI, 2′,3′-dideoxyinosine; ddNTP, dideoxynucleoside triphosphate; HIV, human immunodeficiency virus; ZMP, 5-amino-4-imidazolecarboxamide riboside monophosphate; ZTP, 5-amino-4-imidazolecarboxamide riboside triphosphate; XTT, 2,3-bis-2-methyoxy-4-nitro-5-sulfophenyl-5-(phenylamino)carbonyl-2*H*-tetrazolium hydroxide; PRPP, phosphoribosylpyrophosphate; IMPDH, IMP dehydrogenase; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; AIDS, acquired immunodeficiency syndrome.

riboside, a natural precursor of purine nucleotides, can enhance the anabolic phosphorylation and potentiate the antiviral activity of purine ddNs in human lymphoid cells.

Materials and Methods

Cells. The human T lymphoblast cell line CEM was maintained in modified Eagle's medium (Whitaker Bioproducts, Walkersville, MD) containing 10% (v/v) heat-inactivated (56°, 3 hr) newborn bovine serum and 2 mM glutamine. The human T cell leukemia cell line MT-2 and H9/HTLV-IIIB cells were maintained in RPMI 1640 medium with 20% fetal bovine serum and 2 mM glutamate. All cell lines were obtained from the AIDS Research and Reference Reagent Repository, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). Cell concentrations, sizes, and volumes were checked by Coulter counter, and cell viabilities were tested by the trypan blue exclusion assay.

Chemicals. ddA and ddI were obtained from Dr. David Johns, Developmental Therapeutics Program, National Cancer Institute. AICA riboside and XTT were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]ddA (30 Ci/mmol), labeled at the 2′- and 3′- positions of the dideoxyribose moiety, was obtained from Moravek Biochemicals (Brea, CA).

Analysis of intracellular nucleotide pools. The intracellular levels of different nucleotides were determined according to previously described procedures (9, 10, 14, 15). Briefly, exponential cultures of CEM cells were adjusted to a concentration of 1×10^6 cells/ml. Aliquots of 10-ml cultures were preincubated with 0, 0.25, 0.50, or 1.00 mM AICA riboside for 1 hr. ddA (10 μM) and [^{3}H]ddA (2 μCi/ml) were added to the cultures, and after an additional 6-hr incubation the cells were pelleted and extracted with 70% methanol/25 mM Tris, pH 7.0. The nucleotides were separated by HPLC on a 25-cm Whatman Partisil-10 SAX ion exchange column and were quantitated by UV absorbance (A_{254}) and liquid scintillation counting. AICA riboside, nucleosides, and some bases in culture medium were separated by HPLC on a 10-cm Whatman Partisil ODS reverse phase column.

Virus. H9/HTLV-IIIB cells were cultured in RPMI 1640/fetal bovine serum/glutamine medium and supplemented with fresh H9 periodically. HIV was harvested from culture supernatants and aliquots were kept at −70°. The ddI-resistant HIV isolate MB-48 (16) was obtained from Dr. M. H. St. Clair (Wellcome Research Laboratories, Research Triangle Park, NC) and propagated in MT-2 cells. Virus titers were determined by syncytium formation in MT-2 cells.

XTT assay. The XTT assay was performed as described previously (17). Briefly, 96-well microtiter plates were seeded with uninfected or HIV-infected (multiplicity of infection = 0.015) MT-2 cells at a density of 5000 cells/well. The cells were incubated for 7 days with different chemicals at the indicated concentrations. Fifty microliters of XTT solution (0.1% in 25 mM phenazine methosulfate) were added to each well, and the extent of dye reduction was monitored spectrophotometrically (A_{450}), as a measure of cell viability. The drug concentrations that inhibited the proliferation of uninfected MT-2 cells (IC_{50}) were used as a measure of drug toxicity. Under the experimental conditions used, all HIV-infected MT-2 cells succumb to virus infection and no viable cells remain at the end of culture. Thus, drug concentrations at which the viable cell numbers of HIV-infected cells corresponded to 50% of the viable cell numbers in uninfected cultures were determined as a measure of the effective antiviral doses of the drugs (ED_{50}).

PCR. The extent of reverse transcription occurring in HIV-infected cells was monitored by PCR (18). MT-2 cells pretreated with ddI alone or ddI plus 0.5 mM AICA riboside were infected with HIV at a multiplicity of 0.015. After 24 hr, 1×10^6 cells were lysed in 200 μl of PCR sample buffer containing 0.5% Tween 20, 0.5% Triton X-100, and proteinase K. After a 3-hr incubation at 37°, proteinase K was inactivated by a 10-min incubation at 95°. Amplifications were carried out with 10-μl samples and the *gag* gene-specific primer pair SK-38 and SK-39, in a Perkin-Elmer thermal cycler. The products were detected by hybridization with the ^{32}P-labeled oligonucleotide probe SK-19. The sequences of the primers and probe used in this study have been described before (19); they were purchased from Perkin-Elmer Cetus (Emeryville, CA).

Results

Cellular metabolism of AICA riboside in human lymphoid cells. We first evaluated the metabolism of AICA riboside and its effect on intracellular nucleotide pools in CCRF-CEM cells (Table 1). ZMP, an intermediate in the *de novo* pathway of purine nucleotides, and its triphosphorylated product ZTP were not detected in untreated control cells (i.e., <1 pmol/10^6 cells). However, after exposure to AICA riboside intracellular ZMP levels increased progressively and reached concentrations of 127, 993, and 4046 pmol/10^6 cells with 0.25, 0.5, and 1 mM AICA riboside, respectively. On the other hand, ZTP reached detectable levels in CEM cells only at the highest concentration of AICA riboside (1 mM). Presumably ZTP was synthesized from AICA riboside monophosphate and PRPP in a reversal of the PRPP synthetase reaction, based on earlier studies with cultured mammalian cells and purified enzyme (15, 20). This reaction, however, is rather inefficient (K_m, ~3.2 mM) and requires high concentrations of ZMP (20, 21), which may explain why detectable levels of ZTP were observed only at the highest concentration of AICA riboside. The IMP pool size (~15 pmol/10^6 cells) increased progressively, by 2-, 5-, and 52-fold with 0.25, 0.5, and 1 mM AICA riboside, respectively. Increasing amounts of hypoxanthine were excreted into the medium with increasing amounts of AICA riboside. This accumulation of hypoxanthine is probably due to increased hydrolysis by cytosolic 5′-nucleotidase of accumulated IMP to inosine and its subsequent conversion to hypoxanthine via purine nucleoside phosphorylase.

The levels of ATP, GTP, CTP, and UTP in CEM cells maintained in the presence of different concentrations of AICA riboside were also determined by HPLC (Table 1). Levels of ATP and GTP increased with increasing concentrations of AICA riboside up to 0.5 mM and then decreased slightly with 1 mM AICA riboside. A maximal, nearly 2-fold increase in ATP and GTP levels was observed with 0.5 mM AICA riboside. The increases in the intracellular IMP pools, which serve as substrate for purine nucleotide biosynthesis, may be directly responsible for the observed increases in ATP and GTP pools in

TABLE 1

Nucleotide pools in AICA riboside-treated CEM cells

Each value represents a mean of two experiments, and the variation between experiments were less than 10%. The numbers in parentheses show the fold-increase over untreated control.

	AICA riboside			
	0 mM	0.25 mM	0.5 mM	1.0 mM
	nmol/10^6 cells			
Extracellular				
Hypoxanthine	<0.01*	21.7	19.1	34.4
AICA riboside	<0.01	17.4	19.3	42.5
Intracellular				
IMP	0.01 (1)	0.02 (2)	0.05 (5)	0.12 (12.2)
ZMP	<0.01	<0.01	0.03 (>3)	0.42 (>42)
ZTP	<0.01	<0.01	<0.01	0.06 (>5)
ATP	6.9 (1)	10.2 (1.5)	12.5 (1.8)	8.9 (1.3)
GTP	0.64 (1)	0.83 (1.3)	0.99 (1.5)	0.79 (1.2)
CTP	0.75 (1)	0.17 (0.2)	0.17 (0.2)	0.27 (0.4)
UTP	1.8 (1)	0.28 (0.2)	0.23 (0.1)	0.42 (0.2)

AICA riboside-treated cells. In contrast to the purine nucleotides, a significant reduction (approximately 70%) in the pools of the pyrimidine nucleotides UTP and CTP was seen after addition of AICA riboside. The same pattern of an increase in purine nucleotide pools and a decrease in the pyrimidine nucleotide pools was previously seen in Chinese hamster lung fibroblasts after administration of 0.2 mM AICA riboside (22). Also, in those studies a significant decrease was seen in the level of PRPP and in the activity of orotate phosphoribosyl transferase-orotidylic decarboxylase, an enzyme complex that converts orotate to UMP. Thus, it seems likely that the depletion of pyrimidine nucleotides in the cultured T lymphoid cells is the result of a similar inhibitory effect, due to the combination of a decrease in PRPP level, an increase in purine nucleotides, and accumulation of ZTP during AICA riboside treatment. The intracellular pools of different deoxynucleoside triphosphates in cells maintained in the presence of different concentrations of AICA riboside along with different ddNs (10 μM ddA, ddI, or ddG) were essentially similar to those observed in cells treated with AICA riboside alone (data not shown).

Stimulatory effects of AICA riboside on the intracellular accumulation of ddNTPs. The effect of AICA riboside on anabolic phosphorylation of ddA was examined. CEM cells were incubated with 10 μM [^{3}H]ddA in the presence or absence of different concentrations of AICA riboside, and the levels of intracellular 5′-phosphates were quantitated by HPLC (Fig. 1; Table 2). As seen in Table 2, the levels of intracellular ddADP and ddATP showed a substantial increase (approximately 5–8-fold) in the presence of AICA riboside, compared with untreated controls. The same pattern of accumulation of ddA nucleotides in the presence of AICA riboside was also observed in cells treated with ddI, as well as in another T lymphocytic cell line,

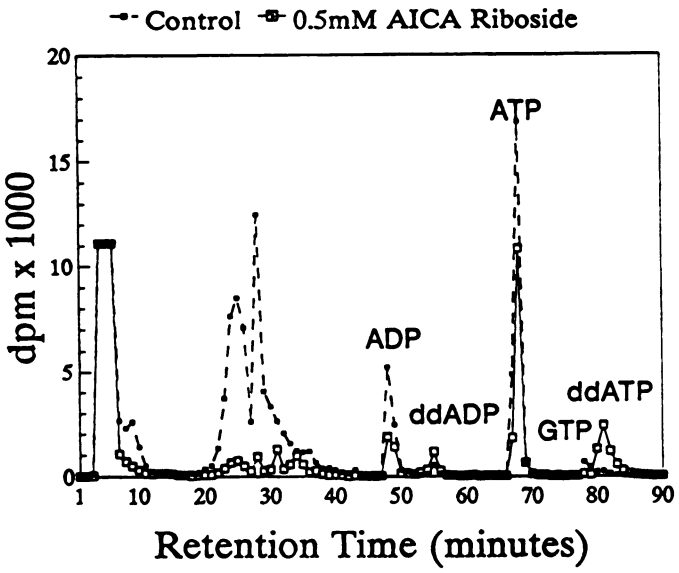

Fig. 1. Anabolic phosphorylation of ddA in CEM cells in the presence of 0.5 mM AICA riboside. CEM cells preincubated with 0, 0.25, 0.50, or 1.00 mM AICA riboside for 1 hr and 10 μM ddA (containing 2 μCi/ml [^{3}H] ddA) were added. After 6 hr, the cell extracts were prepared and analyzed by HPLC and the fractions were monitored by liquid scintillation counting. The identities of the peaks were determined by comparison with the elution profile of standards. The presence of radioactivity in peaks corresponding to ADP, ATP, and GTP is due to the presence of trace levels of radioactive contaminants in the labeled ddA preparation (14) that can undergo phosphorolysis and reutilization in subsequent purine biosynthesis.

TABLE 2

Stimulation of ddA phosphorylation by AICA riboside

CEM cells growing in exponential phase (1 × 10^6 cells) were incubated with 10 μM [^{3}H]ddA (5 μCi/ml) in the presence or absence of the indicated concentrations of AICA riboside. Cells were incubated with AICA riboside for 1 h before the addition of radiolabeled ddA. After 6 h of incubation, cells were extracted with 60% cold methanol and the extracts were analyzed by ion exchange HPLC as described (6, 14). Each value represents the average of duplicate values, with each value obtained varying by <10%. ddIMP and ddAMP were found to elute ~15 to 20 min, but could not be evaluated with accuracy in this procedure.

AICA riboside	Concentration		Increase	
	ddADP	ddATP	ddADP	ddATP
mM	pmol/10^6 cells		-fold	
0	0.32	0.26	1.0	1.0
0.25	0.50	1.30	1.6	5.0
0.50	0.68	1.97	2.1	7.6
1.00	0.70	0.84	2.1	3.2

MT-2. In either case, the fold stimulation of ddATP formation was comparable in magnitude to that seen in CEM cells incubated with ddA (data not shown), a result compatible with the notion of ddA acting mainly as a prodrug of ddI (23).

We also investigated the effect of AICA riboside on the catabolism of the analog triphosphates in cells. CEM cells were pretreated with 10 μM [^{3}H]ddA in the absence or presence of 0.5 mM AICA riboside, washed, and then reincubated in ddA-free medium in the absence or presence of AICA riboside. The $t_{1/2}$ of ddATP in CEM cells was estimated to be ~17 hr for both control and AICA riboside-treated cells (data not shown), indicating that increased anabolism of ddA, rather than decreased catabolism of ddATP, was responsible for the potentiation observed.

HIV reverse transcription in cells pretreated with AICA riboside or AICA riboside and ddI. The antiviral effect of ddI is thought to be mediated via ddATP, which functions as a competitive inhibitor and/or a chain terminator during HIV reverse transcription (6). We therefore investigated whether the modulation of intracellular nucleotide pools and increased anabolism of ddI seen in the presence of AICA riboside affected the extent of HIV reverse transcription and proviral DNA synthesis. MT-2 cells pretreated with different concentrations of ddI for 24 hr, in the absence or presence of 0.5 mM AICA riboside, were infected with HIV-1 at a multiplicity of 0.015, and 24 hr later the levels of HIV proviral DNA were assayed by PCR (Fig. 2). Preincubation of cells with AICA riboside alone resulted in a significant decrease in the levels of proviral DNA. Also, decreasing amounts of proviral DNA were detected in cells incubated with increasing amounts of ddI. However, for any given concentration of ddI appreciably smaller amounts of proviral DNA were detected when the cells had been pretreated with both ddI and AICA riboside. The marked reduction in HIV proviral DNA synthesis seen in cells pretreated with AICA riboside alone was surprising and suggests that reverse transcription is strongly affected by the size of intracellular nucleotide pools.

Anti-HIV activity of ddI in combination with AICA riboside in lymphoid cells. AICA riboside was next evaluated both for its inhibitory effects on cell proliferation and its ability to increase the anti-HIV activity of ddI. Cytotoxicity assays were carried out in MT-2 cells over a 7-day period under the same experimental conditions as used for the evaluation of anti-HIV activity. Under these conditions, AICA riboside inhibited the growth of MT-2 cells (as determined by cell count-

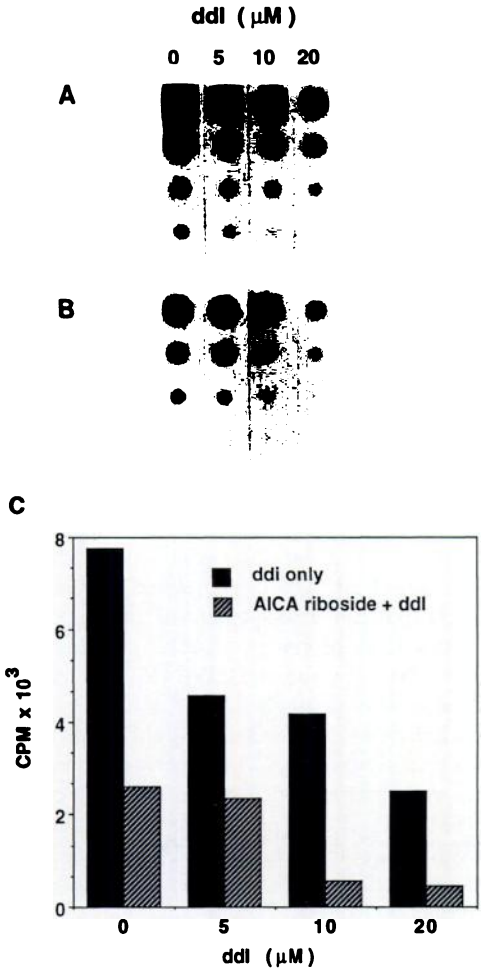

Fig. 2. PCR analysis of HIV proviral DNA. MT-2 cells pretreated with different concentrations of ddI in the presence or absence of 0.5 mM AICA riboside for 24 hr before HIV infection were analyzed by PCR, performed as described in Materials and Methods. Serial 2-fold dilutions were spotted (from *top* to *bottom rows*) on nitrocellulose filters and analyzed by hybridization with a ^{32}P-labeled probe followed by autoradiography. Also, the nitrocellulose membranes were cut and analyzed by liquid scintillation counting. A and B, Autoradiograms; A, cells treated with ddI; B, cells treated with ddI and AICA riboside. C, Quantitative analysis of the results from liquid scintillation counting. Uninfected MT-2 cells did not show any hybridization signal (data not shown).

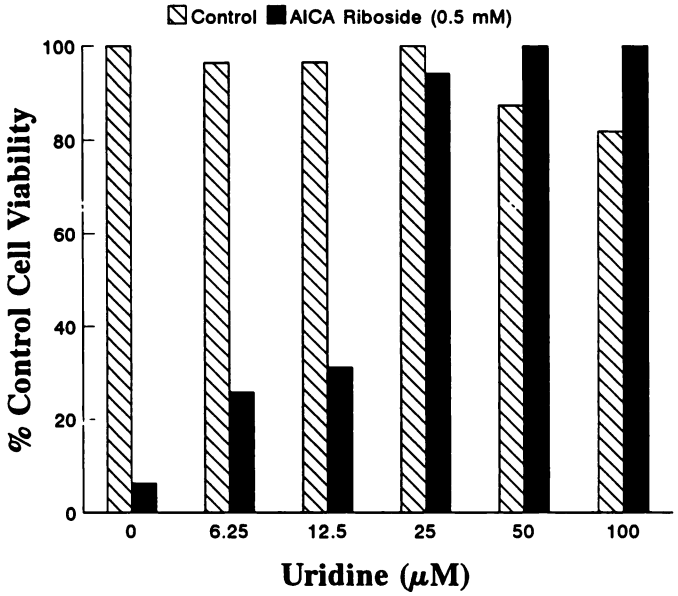

Fig. 3. Protection of AICA riboside-induced cytotoxicity by uridine. MT-2 cells (5×10^4 cells/well in 96-well microtiter plates) were incubated with the indicated concentrations of uridine in the absence or presence of 0.5 mM AICA riboside. After 7 days, the number of viable cells was monitored by a colorimetric XTT assay.

TABLE 3

ED_{50} of ddI in the presence of AICA riboside and/or uridine

The ED_{50} values of ddI against HIV-1_{MB} in presence of uridine and/or AICA riboside was determined in MT-2 cells by XTT assay as described under "Methods." The fold potentiation in the antiviral activity of ddI in presence of uridine alone, or a combination of varying combinations of uridine and AICA riboside, compared with ddI alone is shown in parentheses. Uridine, (25 or 50 μM) alone or a combination of 25 μM uridine and 250 or 500 μM AICA riboside, did not exert any antiviral activity in HIV-infected MT-2 cells.

AICA riboside	Uridine		
	0	25 μM	50 μM
μM		μM	
0	7.26 (1)	7.43 (1)	7.30 (1)
250	T[a]	0.98 (7)	1.15 (6)
500	T	0.51 (14)	0.57 (13)

[a] T denotes toxicity of AICA riboside (IC_{50} ~ 313 μM). Uridine (25 or 50 μM) abolished the growth-inhibitory effects of AICA riboside, but did not exert any effect on untreated or HIV-infected MT-2 cells.

ing) with an IC_{50} of 300 μM. The viability of the cultures, as determined by trypan blue dye exclusion, was not affected by AICA riboside treatment, suggesting that AICA riboside has a cytostatic rather than a cytocidal effect in MT-2 cells. However, addition of the natural nucleoside uridine (25 μM) to the cell culture completely prevented this growth inhibition by AICA riboside (Fig. 3).

The effect of AICA riboside on the protective effect of ddI against HIV activity was evaluated in the MT-2 cell culture system. The ddI concentration that caused 50% inhibition of HIV replication (ED_{50}) was 7.6 μM in MT-2 cells (Table 3). AICA riboside, by itself or in the presence of uridine, had no selective effect against HIV-1 replication in MT-2 cells. When combined with ddI (at 0.25 mM), AICA riboside caused a slight (1.5-fold) increase in the antiviral activity of ddI. However, AICA riboside and uridine added together to the cell culture significantly enhanced the anti-HIV activity of ddI in MT-2 cells. Thus, in the presence of 25 or 50 μM uridine 0.25 and 0.50 mM AICA riboside caused a 7- and 14-fold increase in the anti-HIV activity of ddI, decreasing the ED_{50} value from 7.6 μM to 0.9 μM and 0.5 μM, respectively (Fig. 4; Table 3). By comparison, the maximal potentiation of the anti-HIV activity of ddI by ribavirin, which we observed previously, was about 6-fold (24).

Effect of the ddI/AICA riboside combination on the replication of ddI-resistant HIV in lymphoid cells. We also investigated whether a combination of ddI and AICA riboside could inhibit the replication of ddI-resistant HIV isolated from a patient undergoing long term treatment with ddI. As shown in Table 4, the ED_{50} value of ddI for MB-48 was ~20 μM, which was reduced 10-fold to ~2.1 μM in the presence of 0.25 mM AICA riboside. Interestingly, appreciable enhancement of ddI activity was observed even with lower concentrations of AICA riboside, and a 2–4-fold potentiation was observed with 0.05 and 0.1 mM AICA riboside.

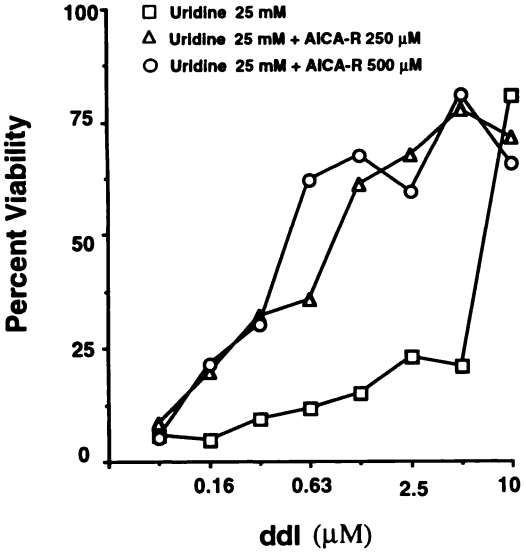

Fig. 4. Enhancement of antiviral activity of ddI by AICA riboside. HIV-1-infected MT-2 cells were incubated with different concentrations of ddI in the presence of 25 μM uridine (□), 0.25 mM AICA riboside and 25 μM uridine (△), or 0.5 mM AICA riboside and 25 μM uridine (○). After 7 days, the cultures were monitored for HIV-induced cytotoxicity by XTT assay. The results are expressed as percentage of cell viability, compared with parallel untreated MT-2 cell controls. Under these conditions, the antiviral effects of ddI resulted in increased cell viability due to inhibition of HIV-induced cell killing.

TABLE 4

Effect of AICA riboside and ddI combination on the replication of ddI-resistant HIV in lymphoid cells

The ED_{50} values of ddI against $HIV\text{-}1_{MB\text{-}46}$ in presence of varying concentrations of AICA riboside (and 25 μM uridine) were determined in MT-2 cells by XTT assay as described under "Methods."

AICA riboside concentration	Uridine	ED_{50} of ddI	Activation
mM	*μM*	*μM*	*-fold*
0	25	20	1
0.05	25	10	2
0.10	25	4.73	4.2
0.25	25	2.10	9.5

Discussion

We have investigated the antiviral effects of AICA riboside, either alone or in combination with ddI, in cultured human lymphoid cells. Analysis of HIV proviral DNA synthesis by PCR showed a reduction in the levels of proviral DNA in cells treated with AICA riboside alone, compared with untreated controls. In contrast, AICA riboside alone showed only a modest protection against HIV infection in the XTT assay. Because the PCR analyses were done in the absence of exogenous uridine, whereas the XTT assay was carried out in the presence of uridine, the inhibitory effects observed by PCR may be in part due to decreased nucleotide pools required for viral DNA replication, rather than a direct effect of AICA riboside on the virus. Although more studies are needed to definitively resolve this point, PCR may be a very useful technique to supplement the available methods of drug evaluation. It is a direct measure of viral DNA replication and a very sensitive method for detection of biochemical effects produced by drugs or their combinations.

The combination of ddI and AICA riboside was shown here to enhance the intracellular accumulation of ddATP from ddA or ddI by as much as 13-fold, and the elevated intracellular levels appeared to correlate with increased potency of the parent nucleosides against the cytopathic effect of HIV in MT-2 cell cultures. AICA riboside is a natural nucleoside that is activated to the physiological purine nucleotides via adenosine kinase (20). It has been well documented that the addition of AICA riboside to cultured fibroblasts or animals is effective in increasing the rate of flux of the purine nucleotide pathways (15, 21). In addition, this compound is undergoing extensive phase II/III clinical trials for the treatment of certain cardiovascular disorders such as myocardial ischemia (25). In this study, we showed that AICA riboside caused a stimulation of purine nucleotide synthesis and markedly stimulated the accumulation of IMP in cultured human lymphoid cells. In previous work with IMPDH inhibitors, we (14, 24) and others (26, 27) observed a positive correlation between anabolism of ddA (and ddI) and the intracellular IMP level. IMP, rather than ATP, serves as a phosphate donor for phosphorylation of ddI via the cytosolic 5′-nucleotidase, which is responsible for the initial activation of ddI and other related purine ddNs. Thus, it seems likely that the enhancement of purine ddN anabolism by AICA riboside is produced through activation of the 5′-nucleotidase activity by the expanded IMP pool in the cells.

A potential drawback to the use of AICA riboside for combination with purines ddNs is its apparent inhibition of pyrimidine nucleotide synthesis. A 70% decrease in both CTP and UTP levels was apparent within 6 hr of AICA riboside treatment in CEM cells. It is important to note, however, that the state of pyrimidine starvation appears to be limited to cultured cells and is reportedly not observed in animals given large doses of AICA riboside (18, 24). The possible explanations for the differential effects on pyrimidine starvation *in vivo* versus *in vitro* are that either there are sufficient concentrations of pyrimidine nucleosides in the circulation to circumvent the inhibition induced by AICA riboside or, alternatively, AICA riboside does not significantly affect PRPP levels or *de novo* pyrimidine nucleotide synthesis *in vivo*. Complete protection from AICA riboside toxicity against CEM and MT-2 cells was observed when the culture medium was supplemented with 25 μM uridine. Under these experimental conditions, the stimulatory effect of AICA riboside on the purine nucleotides was not affected and an approximately 14-fold reduction in the effective dose of ddI against HIV-1 in MT-2 cells was observed in the presence of 0.5 mM AICA riboside (the highest concentration tested), without any significant toxicity.

Recently, it has been demonstrated that antimetabolites, such as ribavirin, tiazofurin, and mycophenolic acid, that inhibit IMPDH potentiate the metabolism of the purine ddNs ddI, ddG, and 2′,6-dideoxy-2,6-diaminopurine riboside in T lymphocytes and significantly enhance the anti-HIV activity of these ddNs *in vitro* (14, 24, 26). In addition, Balzarini *et al.* (26) demonstrated a synergy between ribavirin and ddI (or 2′,3′-dideoxydiaminopurine riboside) against Moloney murine sarcoma virus-induced tumors in mice. This potentiation was related to an enhancement of drug anabolism and was found to correlate with an increase in the intracellular IMP level. In this respect, AICA riboside is similar to the IMPDH inhibitors, except that the increases in IMP pools are achieved by increased anabolism. AICA riboside is at least as effective, if not more active, but without some of the potential drawbacks associated with IMPDH inhibitors. Inhibition of IMPDH dis-

rupts guanine nucleotide production. GTP, the end-product of this pathway, is an essential substrate for RNA and DNA synthesis and a regulator of various critical cellular metabolic pathways. In contrast, AICA riboside increases the flux of the purine nucleotide pathways and leads to an expansion of both ATP and GTP (Table 1). This compound inhibits pyrimidine biosynthesis *in vitro* but, as pointed out earlier, this inhibitory effect may be circumvented *in vivo* by circulating pyrimidine nucleosides. In support of this notion, clinical studies indicate that this compound is well tolerated when administered to individuals in a single dose of up to 100 mg/kg (25). Thus, if phosphorylation is a rate-limiting step in anabolic activation of ddI to its putative active analog triphosphate *in vivo*, as it is *in vitro*, then the use of an agent like AICA riboside, with the capacity to modulate the phosphorylation reaction, might significantly enhance the anabolism of ddI and consequently its potency in patients with AIDS. However, the safety and long term toxicity, if any, of chronic AICA riboside treatment, as might be required for modulating ddI anti-HIV activity, remains to be determined.

As with all studies involving combinations of chemotherapeutic agents, the question arises of whether modulation of the type described in the current study would also bring about a similar or even greater potentiation of toxicity, thus nullifying the advantage of such therapy. Only clinical studies can answer the question of safety and efficacy. However, it is worth noting that there is no evidence at present to suggest that pancreatitis or the peripheral neuropathy that has been associated with ddI therapy is related directly to drug anabolite formation. A potential advantage in the use of potentiating drugs is that lower doses of the active drugs may be used in therapy. The peak plasma concentrations of ddI achieved with the currently recommended dose of ddI (500 mg/kg/day) is around ~6 μM (28). The sensitivity to ddI of clinical isolates of HIV varies widely, and ED_{50} values ranging from 0.5 to 14 μM have been reported (5, 6, 29). Also, with the increasing use of ddI, HIV isolates with reduced sensitivity to ddI are being recovered from patients undergoing therapy (16). A potentiation of ddI anabolism by AICA riboside may provide enhanced virus suppression at concentrations below toxic doses. Consistent with this idea, a 10-fold increase in the antiviral activity of ddI was observed in the presence of AICA riboside.

AICA riboside enhanced the antiviral activity of ddI against MB-48, a ddI-resistant HIV isolate, as well. The ddI resistance of MB-48 has been associated with a Leu^{74} to valine mutation in the viral reverse transcriptase (16). Studies using site-specific mutagenesis indicate that this change is responsible for cross-resistance between ddI and ddC, as well as increased susceptibility to AZT (16, 30). Recently, an additional mutation, Met^{184} to valine, which is associated with resistance to ddI and ddC (but not AZT), has been described (31). The drug resistance of these mutants may therefore be due to a reduced affinity of the mutant viral enzymes for nucleotide analogs with 2′,5′-dideoxy moieties. However, *in vitro* studies with HIV resistant to different ddNs (e.g., AZT) indicate that the enzymes from mutant viruses are indistinguishable from wild-type HIV reverse transcriptase in their affinity for or their sensitivity to inhibition by the corresponding ddNTP (i.e., AZT triphosphate). Likewise, mutant enzymes selected *in vitro* for their resistance to ddNTPs (e.g., AZT triphosphate or ddG triphosphate) do not result in expression of AZT- or ddG-resistant viruses when introduced into the viral genome (32, 33). Presently, it is not clear whether this anomalous behavior is observed with ddI-resistant isolates as well. Regardless of the mechanisms involved, AICA riboside potentiated the effect of ddI against both wild-type and resistant HIV and thus may prove to be useful in investigations concerning the mechanisms of anti-HIV action and resistance to ddI.

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