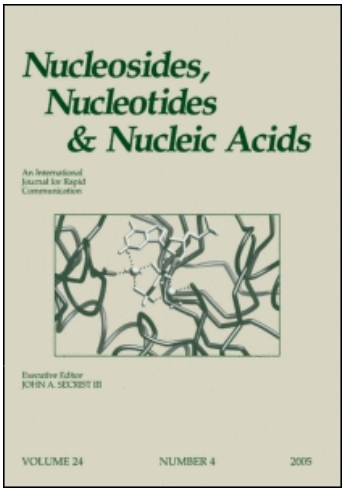

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Srinivas, Ranga V. , Gong, Yi-Fei , Becher, Anna and Fridland, Arnold(1995) 'Modulators of Nucleotide Metabolism Inhibit HIV Replication in Lymphoid Cells and Affect Its Inhibition by Dideoxynucleosides', Nucleosides, Nucleotides and Nucleic Acids, 14: 3, 641 — 644
To link to this Article: DOI: 10.1080/15257779508012441
URL: http://dx.doi.org/10.1080/15257779508012441

NUCLEOSIDES & NUCLEOTIDES, 14(3-5), 641-644 (1995)

MODULATORS OF NUCLEOTIDE METABOLISM INHIBIT HIV REPLICATION IN LYMPHOID CELLS AND AFFECT ITS INHIBITION BY DIDEOXYNUCLEOSIDES[¶]

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ABSTRACT. Hydroxyurea (HU), - a ribonucleotide reductase inhibitor which depletes dNTP pools, inhibited HIV replication, and the extent of inhibition correlated with its antiproliferative effects. HU treatement, prior to, and during the initial 12 h of HIV infection, resulted in a significant reduction in virus yield, without affecting cell proliferation. AICA riboside, an IMP precursor which increases the flux of purine biosynthesis, also inhibited HIV replication suggesting a requirement for optimum ratios of the different dNTPs for efficient reverse transcription and virus replication. The different modulators also affected the antiviral efficacy of various dideoxynucleosides. HU enhanced the antiviral efficacy of both AZT and ddI, whereas AICA riboside potentiated ddI and did not exert any appreciable effect on AZT.

The reverse transcription of viral RNA to proviral DNA is an initial step in HIV replication which is dependent on deoxynucleoside triphosphate (dNTP) levels (1). The intracellular dNTP concentrations are highly regulated, and the concentrations and ratios of the different dNTPs vary both with different cell types, and with cell cycle (reviewed in 2). Exogeneous addition of deoxynucleosides (or dideoxynucleosides) and antimetabolites exert varied effects on dNTP levels and some of these changes influence HIV replication (3,4). Here we have investigated the effects of HU, a ribonucleotide reductase inhibitor which depletes dNTP pools, and 4-aminoimidazole carboxamide riboside (AICA riboside), a natural precursor of purine nucleotides, on HIV replication, either alone or in combination with antiviral dideoxynucleosides (ddNs).

MATERIALS AND METHODS

Reagents and Chemicals. The T lymphoid cell lines CEM-SS, CEMx174, MT-2, H9, as well as the wild-type HIV isolates IIIB & pNL4, and the ddC-resistant pJ4-A were all obtained from the NIH/NIAID AIDS Research and Reference Reagent Repository (Ogden BioServices, Rockville, MD). The B-lymphocytic WIL-2 cells were obtained from ATCC. A ddI-resistant HIV isolate, MB-48 (5) was obtained from Dr. Marty St.Clair (Burroughs-Wellcome, Research Triangle Park, NC). ddG, ddA, and ddI were obtained from Dr. Karl Flora, Developmental Therapeutics Program, NCI, NIH. AICA riboside was purchased from Sigma Chemical Co. (St. Louis, MO).

Antiviral assays. Inhibition of HIV-induced cytopatholgy in MT-2 cells was monitored by XTT-assay (6), while virus yield reduction from HIV-infected cell lines was monitored by reverse transcriptase assay (7).

RESULTS AND DISCUSSION

Effects of HU and AICA riboside on cell proliferation and HIV replication in T-lymphocytic cell lines. Uninfected or HIV-infecetd (IIIB, moi ~1 RTcpm/cell) CEM cells were incubated in the absence or presence of HU or AICA riboside,

[¶] This work was supported in parts by NIH grants RO1 AI 27652, AI 31145, Cancer Support (CORE) Grant 5 P30 CA 21765 and by the American Lebanese Syrian Associated Charities.

and the virus production was determined by RT assays after 5 days of culture. Parallell uninfected cultures were also incubated with these compounds to determine the drug effects on cell proliferation. Both virus production and cell proliferation was inhibited in HU and AICA riboside treated cells possiibly due to altered nucleotide pools.

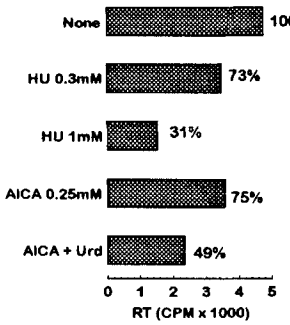

Figure I. Effect of HU and AICA riboside on HIV replication

In order to reduce the drug cytotoxic effect, cells were pretreated with HU or AICA riboside for 4 h prior to infection, and washed free of the drug 12 h post-infection. No change in cell growth was observed in uninfected cultures exposed to 1 mM HU or 0.25 mM AICA riboside for16 h. However, the virus replication was significantly reduced by 1 mM HU or 0.25 mM AICA riboside (Fig. 1). These results support the notion that a transient perturbation of cellular nucleotide pools can limit virus proliferation without affecting cell proliferation. AICA riboside causes a build-up of purine nucleotide, primarily ATP, within the cells and it can also inhibit pyrimidine nucleotide which is thought to result in growth inhibition (6,8). Consistent with an earlier report, we found that the growth-inhibitory effects of AICA riboside was more active against T-lymphocytic CEM-SS or MT-2 cells (IC_{50} ~500 μM) than to B-lymphocytic WIL-2 cells (IC_{50} ~ 1200 μM), and T & B hybrid CEMx174 (IC_{50} ~800 μM). The growth-inhibitory effects of AICA riboside was readily reversed by uridine or cytidine indicating that the antiproliferative effect is mediated by pyrimidine depletion, an effect thought to occur only *in vitro*, but not *in vivo* (8,9). As shown in Fig. 1, 0.25 mM AICA riboside partially inhibited HIV replication; interestingly, this inhibition was enhanced by the addition of 25 μM uridine. These results suggest that a transient perturbation of cellular nucleotide pools can restrict virus replication without directly affecting cell growth. The effect of AICA riboside-uridine combination on HIV replication is unclear, but it may be related to an imbalance in dNTP pools caused by a build-up of pyrimidine nucleotides in the cells. In a recent report, deoxythymidine was shown to decrease intracellular dCTP levels and inhibit HIV replication, whereas deoxycytidine elevated dCTP levels and enhanced HIV replication (3). Together, these results suggest that an optimum ratio of the different dNTPs, in addition to the absolute amounts of the different dNTPs is required for efficient reverse transcription and HIVreplication.

Effect of hydroxyurea and AICA riboside on the antiviral efficacy of dideoxynucleosides. The purine and pyrimidine dideoxynucleosides (ddNs) are potent inhibitors of HIV reverse transcription, and mediate their effects via their triphosphates (ddNTPs) which compete with their dNTP counterparts for binding to reverse transcriptase, and/or cause chain termination. The efficacy of ddNs vary directly with intracellular ddNTP levels, and inversely with the levels of competing dNTPs. Thus, agents which perturb dNTP pools are likely to modulate the antiviral efficacy of the various ddNs as well. We therefore investigated the effects of HU on the antiviral efficacy of AZT and ddI, and the results are summarized in Fig. II. A much greater inhibiton of virus replication was seen with a combination of HU & ddN, than either compound alone for both AZT and ddI. Our findings contrast with those of Gao et al. (Molecular Pharmacology, in press) who have recently shown that HU enhances the activity of ddI, but not AZT, in PBMCs. This differential effect of HU on ddN anti-HIV activity was explained by a depletion of dATP (compared to TTP) in HU-treated cells. HU has recently been shown to enhance the phosphorylation of AZT in CEM cells (10) which may account for the AZT potentiation seen in our experiments. These

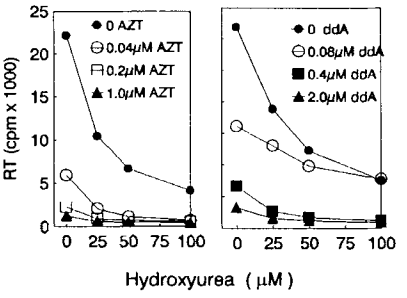

Figure. II Effect of Hydroxyurea on antiviral activity of ddNs. CEM-SS cells were infected with HIV-1 IIIB at a multiplicity of 1 RT cpm/cell and maintained in media containing indicated concentrations of HU and AZT or ddA. Virus production was monitored by RT assay on 5th day post-infection.

results highlight cell-type specific differences in the effects of modulatory agents on ddN, and the mechanisms of ddN potentiation by HU may involve an increase in the import and/or anabolic phosphorylation of the ddNs, in addition to alterations in ddNTP/dNTP ratios.

We have recently shown that AICA riboside at subtoxic concentrations potentiates the antiviral efficacy of ddI (6). We have extended these initial observations to include other ddNs, and more importantly studied the ability of such combinations to suppress ddN-resistant HIV (Tables I & II). Based on the previous results which indicated that AICA riboside and uridne combination was more effective than AICA riboside alone, AICA riboside-uridine combination was used in these studies. As shown in Table I, 0.25 mM AICA riboside (with 25 μM uridine) enhanced the antiviral activities of all purine ddNs tested (ddI, ddG, CBV and F-β-ddA) against IIIB. AICA riboside also potentiated drug toxicity, *albeit* to a lesser extent, thus resulting in enhanced drug selectivities. The ddI-resistant isolate MB-48 showed reduced susceptibility to ddI, CBV and ddG, while J4A was ~4-fold less sensitive to CBV, compared to wild-type viruses. AICA riboside enhanced the antiviral efficacy of ddI, ddG and CBV against MB-48 (and CBV against J4A) to a degree comparable to, or greater than that seen with IIIB. Furthermore, the ED_{50} values of these purine nucleosides against MB-48 or JF4A were now comparable to the ED_{50} values obtained against IIIB and other sensitive isolates (Table II). AICA riboside, unlike HU, neither potentiated nor antagonized the pyrimidine nucleosides AZT and ddC. AZT and ddI are synergistic, and several trials are currently underway to evaluate the clinical efficacy of AZT-ddI combination.

Table I. Effect of AICA riboside on ddN efficacy

Treatment	IC50 (μM)	ED50 (mM)	T.I.
ddI	1090	6.5	165
ddI+AICA	484 (2)	1 (6)	480 (3)
FβddA	650	25	26
FβddA+AICA	450 (1)	4 (6)	112 (4)
ddG	830	4.1	200
ddG+AICA	847 (1)	1.4 (3)	600 (3)
CBV	175	2.3	75
CBV+AICA	66 (3)	0.6 (4)	110 (1)

Table II. Efficacy of ddN-AICA riboside against resistant isolates.

HIV	ddN	ED50 (μM)	
		- AICA	+ AICA
MB48	ddI	20	2.1 (9)
(ddI^R)	CBV	4.1	0.8 (5)
	ddG	6.5	0.8 (8)
J4A	ddC	8.0	8.3
(ddC^R)	CBV	1.2	0.5 (2)
pNL4	ddC	3.2	3.9
(WT)	CBV	0.3	0.3
IIIB	ddC	4.3	5.2
(WT)	AZT	0.2	0.1

The toxicity (IC50) and antiviral activities (ED50) were determined by XTT assay. The experiments were carried out in the absence or presence of 0.25 mM AICA riboside and 25 μM uridine. Each figure represents a mean of three experiments and the parenthesis show fold-increase over control.

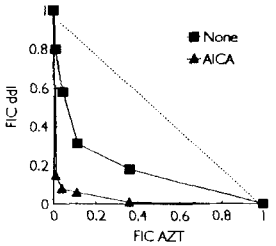

Figure III. Effect of AICA riboside on AZT-ddI synergy

We therefore determined the effect of AICA riboside on the antiviral efficacy of the AZT-ddI combination. The fractional inhibitory concentrations (FIC_{90}) of AZT and ddI against IIIB were determined at different ratios and plotted as an isobologram. The results show that the FIC_{90} of ddI is lower at all concentration of AZT in presence of 0.25 mM AICA riboside supporting the idea that AICA riboside enhances AZT-ddI synergy.

The mechanisms of purine ddN potentiation by AICA riboside appears to be distict from HU, and do not involve a depletion of the competing dNTPs. Unlike HU which depletes purine nucleotide pools, AICA riboside actually enhances the flux of purine

nucelotide biosynthesis. Many purine ddNs, including ddI, are phosphorylated initially by the cytosolic 5'-nucleotidase using IMP, rather than ATP, a phosphate donor (10-12). We and others have previously shown that an expansion of IMP pools by ribavirin or AICA riboside can increase the rate of phosphorylation, and concomitant antiviral efficacy of ddI (6,13-15). Consistent with this idea, the intracellular pools of both IMP and ddNTP were markedly elevated in cells incubated with CBV, ddG, or ddI in presence of AICA riboside (not shown). Thus, increased phosphorylation of the purine ddNs are likely to be mainly responsible for the enhanced antiviral activities.

The clinical significance of our findings are presently unclear, but suggest that agents which modulate nucleotide metabolism, in addition to restricting HIV replication, can enhance the efficacy of various ddNs. Such combinations may be useful in the management of HIV infections, particularly for resistant viruses, or for drug dosage reductions for patients unable to tolerate the current regimens. It is of interest to note that at least two clinical trials are curently evaluating the combinations of ribavirin & ddI (ACTG 231) and HU & ddI (16) in HIV-infected patients.

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