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Inhibitors of IMP Dehydrogenase Stimulate the Phosphorylation of the Anti-Human Immunodeficiency Virus Nucleosides 2′,3′-Dideoxyadenosine and 2′,3′-Dideoxyinosine

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SUMMARY

2′,3′-Dideoxyadenosine (ddAdo) and its deamination product 2′,3′-dideoxyinosine (ddIno) (didanosine) inhibit the replication and infectivity of the human immunodeficiency virus (HIV) in a number of *in vitro* assay systems. Early clinical studies (phase I) have indicated a role for ddIno in the treatment of patients with severe HIV infection. In the present *in vitro* study, the formation in human T cells (MOLT-4, ATH8, and CCRF-CEM) of the pharmacologically active metabolite of ddIno and ddAdo, 2′,3′-dideoxyadenosine-5′-triphosphate (ddATP), was found to be stimulated 2–4-fold by appropriate concentrations of inosinate dehydrogenase (IMPD) inhibitors such as ribavirin, tiazofurin, and mycophenolic acid. Concomitant with this increase in ddATP formation from ddIno was an increase in anti-HIV activity of this agent when it was combined with ribavirin in the ATH8 cell assay system and with tiazofurin in the MOLT-4 assay system. No change was noted in the intracellular concentration of the corresponding physiological deoxynucleoside-5′-triphosphate, dATP; positive correlation was observed, however, between the increase in ddATP formation from ddIno and the increase in intracellular IMP occurring as a consequence of IMPD inhibition. The results support the hypothesis that the stimulation of ddATP formation seen when ddIno is combined with ribavirin or other IMPD inhibitors is a consequence of an increased concentration of IMP, the major phosphate donor for the initial phosphorylation step in the anabolism of ddIno to ddATP, i.e., ddIno → ddIMP.

In a recent study, we demonstrated that the phosphorylation of ddGuo, a purine nucleoside analogue with anti-HIV and antihepadnavirus activity, is stimulated in human T cells by as much as 20-fold by IMPD inhibitors such as ribavirin, tiazofurin, and mycophenolic acid (1). In parallel with the increase in phosphorylation of ddGuo produced by these three IMPD inhibitors, the anti-HIV activity of this agent in the H9 cell assay system was stimulated severalfold by low concentrations of ribavirin (1–10 μM), an effect that has also been described by Baba *et al.* (2) in the MT-4 assay system. These results are in accordance with the generally held view that ddGuo and related purine dideoxynucleoside analogues must undergo anabolism to their corresponding 5′-triphosphates in order to exert their anti-HIV effect (3, 4).

Although extensively studied in antiviral test systems *in vivo* and in cell culture (5, 7, 8), ddGuo has not yet been subjected to clinical trial as an antiviral agent. However, the purine dideoxynucleoside analogues ddAdo (Fig. 1) and ddIno (didanosine) have undergone extensive clinical study (9–11), and large-scale phase II/III controlled trials of the latter agent (ddIno) are currently in progress. Because ddIno is phosphorylated primarily by the same cytoplasmic phosphotransferase (5′-nucleotidase) as is ddGuo, with IMP as the preferred phosphate donor (12) (Fig. 2A), and because ddAdo, although not itself subject to phosphorylation by this enzyme, is readily and quantitatively deaminated to yield ddIno *in vivo* (13) and also in whole-cell assay systems (14) (Fig. 2B), it appeared of possible relevance to the study of the pharmacological activity of ddAdo and ddIno to ascertain the effect of ribavirin and other IMPD inhibitors on the phosphorylation and the antiviral activity of these two anti-HIV agents.

Materials and Methods

Chemicals. [^{3}H]ddAdo (30 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). The percentage of label in the 2′- and 3′-

ABBREVIATIONS: ddGuo, 2′,3′-dideoxyguanosine; ddAdo, 2′,3′-dideoxyadenosine; ddIno, 2′,3′-dideoxyinosine; HIV, human immunodeficiency virus; ddATP, 2′,3′-dideoxyadenosine-5′-triphosphate; IMPD, inosinate dehydrogenase; ddIMP, 2′,3′-dideoxyinosine-5′-monophosphate; AZT, 3′-azido-2′,3′-dideoxythymidine; ddCyd, 2′,3′-dideoxycytidine; $TCID_{50}$, 50% tissue culture infective dose; ddADP, 2′,3′-dideoxyadenosine-5′-diphosphate; ddGDP, 2′,3′-dideoxyguanosine-5′-diphosphate; ddGTP, 2′,3′-dideoxyguanosine-5′-triphosphate; HPLC, high performance liquid chromatography; ddAMP, 2′,3′-dideoxyadenosine-5′-monophosphate; p24 gag, major capsid protein of HIV-1.

positions of the dideoxyribose moiety averaged 97%, with the remaining tritium being associated with the 8-position of the purine base. [^{3}H] ddIno was prepared by means of enzymatic deamination of [^{3}H]ddAdo, utilizing calf intestinal adenosine deaminase (Sigma Chemical Co., St. Louis, MO). Ribavirin (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide; NSC-163039), tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide; NSC-286193), mycophenolic acid (NSC-129185), ddAdo (NSC-98700), and ddIno (NSC-612049) were supplied by Dr. Karl Flora, Developmental Therapeutics Program, National Cancer Institute, National Institutes of Health. ddATP was purchased from Pharmacia (Piscataway, NJ); all other nucleoside/nucleotide standards were purchased from Sigma Chemical Co.

Cells. ATH8 and CCRF-CEM cells were grown as previously described (5, 6). MOLT-4 cells were grown at 37° in RPMI 1640 medium supplemented with 10% heat-inactivated (56° for 30 min) fetal bovine serum, 44 μg/ml gentamycin, and 4 mM L-glutamine, in a humidified atmosphere of 95% air/5% CO_2. Cells were verified to be in logarithmic growth at the time of use.

Metabolism studies. Metabolism studies with ddAdo or ddIno were carried out in exponentially growing ATH8, MOLT-4, or CEM cells, in the presence or absence of IMPD inhibitors. Ten-milliliter aliquots of cell suspensions (about 10^6 cells/ml) were incubated with IMPD inhibitors (ribavirin, tiazofurin, or mycophenolic acid) for 30 min. After this preincubation period, cells were exposed to a 5 μM concentration of the radiolabeled dideoxynucleoside (5 μCi/ml). After 5 hr of incubation, cells were centrifuged, and the cell pellets were washed with 1 ml of cold normal saline and extracted with 0.4 ml of 60% methanol. Extracts were heated for 1 min at 95° and, after centrifugation, 200 μl of the supernatant were subjected to chromatography on an ion exchange Partisil 10-SAX column (15). One-minute fractions were collected, and radioactivity was determined.

Determination of IMP concentration. Methanolic cell extracts were subjected to chromatography on an ion exchange Partisil 10-SAX column that had been prestandardized with known amounts of IMP. Under the HPLC conditions used (15), IMP eluted at 7.0 ± 0.1 min. Basal intracellular levels of IMP in control cells (not treated with IMPD inhibitors) ranged from 0.02 to 0.05 mM.

Assay for anti-HIV activity. ATH8 cells, which are sensitive to the cytopathic effect of HIV, were exposed to 3.16×10^3 $TCID_{50}$ of HIV-1/III_B (1000 virus particles/cell) for 1 hr. Cell suspensions (2 ml) were then exposed to ribavirin or other IMPD inhibitor for 30 min before the addition of various concentrations of ddIno or ddAdo. On day 7, total viable cells were counted for quantitation of cytopathic effect. p24 gag protein concentration in the supernatant fraction was quantitated by radioimmunoassay (Dupont, NEN Research Products, Boston, MA). MOLT-4 cells (2×10^4 cells/5 ml of culture medium) were exposed to HIV-1/III_B for 40 min but were otherwise treated similarly. On day 7 in culture, supernatants were collected and the amount of p24 gag protein was quantitated by radioimmunoassay.

Determination of deoxyribonucleoside triphosphate concentrations. Deoxyribonucleoside-5′-triphosphate concentrations in MOLT-4 cell extracts were determined by the method of Garrett and Santi (16), with the following modifications. The trichloroacetic acid extraction method of Khym (17) was used instead of the perchloric acid method. Cyclohexyl ammonium chloride (5 M) and 2 M glycerol were substituted for the methylamine and rhamnose, respectively, as suggested by Dr. W. Plunkett, University of Texas M. D. Anderson Hospital and Tumor Institute. The chromatographic system used a Whatman Partisil 10-SAX 25- × 0.4-cm column, with gradient elution from 0.02 M ammonium phosphate, pH 3.5, to 0.7 M ammonium phosphate, pH 3.5, over 40 min, at 2 ml/min and 45°. Detection was by UV absorption at 254 and 270 nm.

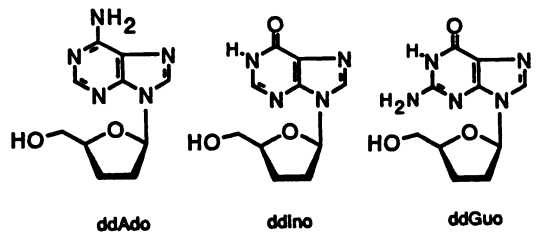

Fig. 1. Structures of ddAdo, ddIno, and ddGuo.

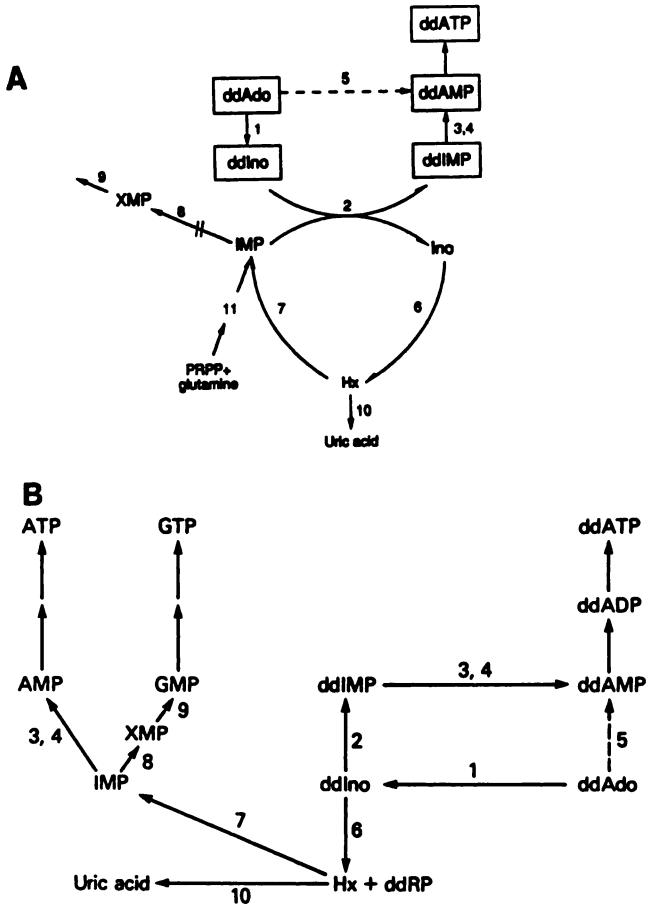

Fig. 2. A, Role of IMP in ddIno phosphorylation (11, 17, 18). *1*, Adenosine deaminase; *2*, high-K_m 5′-nucleotidase (phosphotransferase); *3*, adenylosuccinate synthetase; *4*, adenylosuccinate lyase; *5*, 2′-deoxycytidine kinase; *6*, purine nucleoside phosphorylase; *7*, hypoxanthine-guanine phosphoribosyl transferase; *8*, IMPD; *9*, GMP synthetase; *10*, xanthine oxidase; *11*, pathway for purine biosynthesis *de novo*. PRPP, 5-phosphoribosyl-1-pyrophosphate; Hx, hypoxanthine. B, Pathways of ddAdo and ddIno metabolism. *1–10*, See A. ddAdo and ddIno are also susceptible to nonenzymic hydrolytic cleavage at acid pH. ddRP, 2,3-dideoxyribose-5-phosphate.

Results

Chromatographic separation of nucleotides arising from ddIno and ddAdo. We initially determined the effect of ribavirin on the formation of the metabolic products arising from radiolabeled ddIno (5 μM) upon incubation with MOLT-4 cells. As indicated in Fig. 3 (*upper*), the detectable radiolabeled products were ddADP and ddATP and the purine ribonucleotides GDP, GTP, ADP, and ATP, the four latter compounds arising, as we have previously demonstrated (18, 19), from the enzymic cleavage of ddIno and the reutilization, through purine salvage pathways, of the labeled hypoxanthine thus liberated. Pretreatment with ribavirin (10 μM) resulted in a 3–4-fold increase in both ddADP and ddATP formation (Fig. 3, *lower*). A significant decrease, to undetectable levels, of radiolabeled GTP arising from ddIno was also observed on

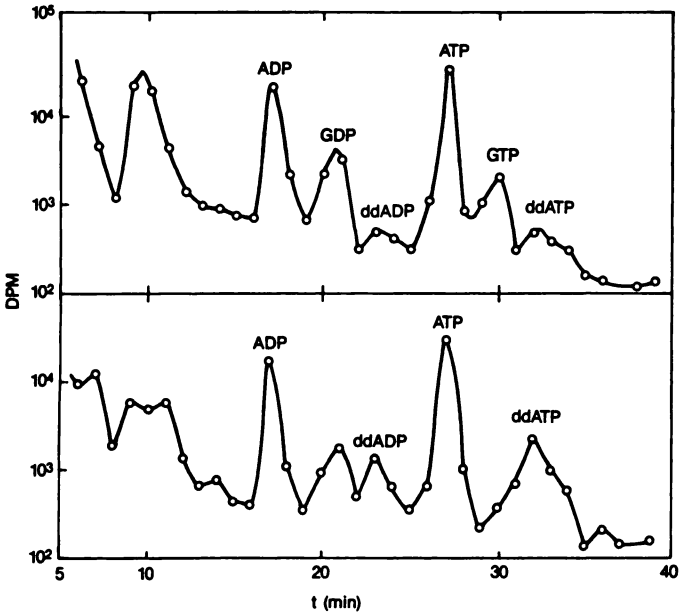

Fig. 3. Separation of ^{3}H-labeled metabolites arising from [^{3}H]ddIno. MOLT-4 cells (10^6 cells/ml) were incubated with ^{3}H-labeled ddIno (5 μM; 5 μCi/ml) for 5 hr, as described in Materials and Methods. Methanolic extracts of 5×10^6 cells were subjected to ion exchange HPLC (Partisil 10-SAX), using an elution program previously described (15). *Upper*, control. *Lower*, plus ribavirin (10 μM). Note logarithmic scale (*ordinate*).

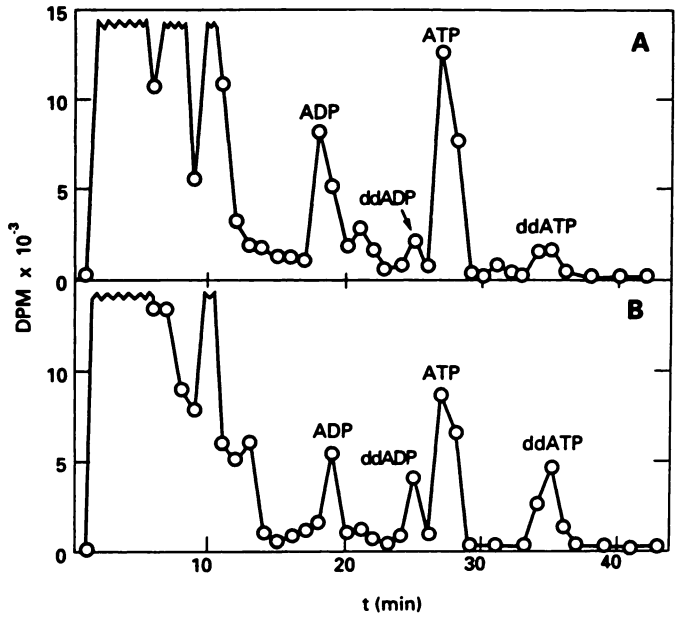

Fig. 4. Separation of ^{3}H-labeled metabolites arising from [^{3}H]ddAdo. MOLT-4 cells (10^6 cells/ml) were incubated with ^{3}H-labeled ddAdo (5 μM; 5 μCi/ml) for 5 hr, as described in Materials and Methods. Elution program was as described for Fig. 2. A, Control; B, plus ribavirin (10 μM).

ribavirin treatment. When radiolabeled ddAdo rather than ddIno was utilized as precursor, a similar chromatographic pattern and an identical stimulation by ribavirin of ddADP and ddATP formation were observed (Fig. 4), a result to be expected in view of the rapid deamination of ddAdo to ddIno in whole-cell assay systems (14). ddIMP/ddAMP eluted at 13–14 min but could not be accurately quantitated by this method. A similar stimulation of ddAdo nucleotide formation from ddIno was seen with tiazofurin (10 μM) and mycophenolic acid (1 μM) (data not shown).

TABLE 1

Stimulation by ribavirin of ddAdo phosphorylation and IMP pool size in ATH8 cells

ATH8 cells growing in logarithmic phase (10^7 cells/10 ml at $t = 0$) were incubated with 5 μM ddAdo (5 μCi/ml) in the presence or absence of ribavirin (25 μM). Cells were incubated with ribavirin for 30 min before the addition of radiolabeled ddAdo. After 5 hr of incubation, cells were extracted with 60% cold methanol, and extracts were analyzed by ion exchange HPLC, as described in Materials and Methods. Each value represents the mean of duplicate samples, with the individual values obtained varying by <10%. ddIMP/ddAMP eluted at 13–14 min but could not be accurately quantitated by this method.

Addition	Concentration		Increase		IMP
	ddADP	ddATP	ddADP	ddATP	
	pmol/10^6 cells		*fold*		*nmol/10^6 cells*
ddAdo (5 μM)	0.076	0.110			0.02
ddAdo (5 μM) + ribavirin (25 μM)	0.168	0.219	2.2	2.0	0.13

Phosphorylation of ddIno was also examined in two other T cell lines, CCRF-CEM and ATH8. In CEM cells, the fold stimulation of ddATP formation by ribavirin and tiazofurin was similar in magnitude to that seen in MOLT-4 cells (data not shown). In ATH8 cells, however, the effects of these two agents were considerably lesser in magnitude. At the maximal concentration of ribavirin examined (25 μM), a 6.5-fold increase in IMP was observed and the increase in ddADP and ddATP was approximately 2-fold (Table 1). With tiazofurin (25 μM), there was only a 2.4-fold increase in IMP, no significant increase in ddADP, and only a 1.6-fold increase in ddATP (Table 2).

Relative effectiveness of IMPD inhibitors. In a quantitative comparison of the three IMPD inhibitors studied, mycophenolic acid was found to be about twice as effective in stimulating ddATP formation from ddAdo or ddIno in MOLT-4 cells and in increasing IMP pools as was ribavirin or tiazofurin, when the three compounds were compared at equimolar levels (Table 3). Ribavirin was more potent than tiazofurin, with a doubling of ddATP formation at 5 hr being observed with 6.5 μM ribavirin and with 10 μM tiazofurin (Fig. 5). The greater effectiveness of ribavirin than of tiazofurin was seen at all time periods examined, up to 8 hr (Table 4). All three compounds showed similar patterns of stimulation, being much more effective in enhancing ddGDP formation from ddGuo than ddADP formation from ddAdo (Fig. 6, *left*); this differential effect was not observed with 5′-triphosphate formation, however, where enhancement of ddATP formation from ddAdo and that of ddGTP formation from ddGuo were almost identical (Fig. 6, *right*).

Enhancement of anti-HIV activity. In order to determine whether the increase in ddATP formation seen in the presence of IMPD inhibitors was accompanied by an increase in anti-HIV activity, we determined the effect of ribavirin and tiazofurin on the activity of ddIno in the ATH8 and MOLT-4 cell assay systems. Assays of anti-HIV activity were carried out over a 7-day period, in contrast to the 5-hr incubation period for the metabolism studies. In the presence of ribavirin (5 μM), the protective effect of ddIno against the cytopathic effect of HIV in ATH8 cells was increased significantly throughout the range of ddIno concentrations examined; maximal increase (92%) was seen at a ddIno concentration of 2 μM (Table 5). No reversal of the HIV cytopathic effect was noted with 5 μM

TABLE 2

Effect of tiazofurin on ddAdo phosphorylation and IMP pool size in ATH8 cells

ATH8 cells growing in logarithmic phase (10^7 cells/10 ml at $t = 0$) were incubated with 5 μM ddAdo (5 μCi/ml) in the presence of tiazofurin at the concentrations indicated. Cells were incubated with tiazofurin for 30 min before the addition of radiolabeled ddAdo. After 5 hr of incubation, cells were extracted with 60% cold methanol, and extracts were analyzed by ion exchange HPLC, as described in Materials and Methods. Each value represents the mean of duplicate samples, with the individual values obtained varying by <10%. ddIMP/ddAMP eluted at 13–14 min but could not be accurately quantitated by this method.

Addition	Concentration		Increase		IMP
	ddADP	ddATP	ddADP	ddATP	
	pmol/10^6 cells		*fold*		*nmol/10^6 cells*
ddAdo (5 μM)	0.055	0.095			0.015
ddAdo (5 μM) + tiazofurin (2.5 μM)	0.054	0.105	None	1.1	0.016
ddAdo (5 μM) + tiazofurin (5 μM)	0.054	0.115	None	1.2	0.029
ddAdo (5 μM) + tiazofurin (25 μM)	0.063	0.152	1.1	1.6	0.036

TABLE 3

Effect of IMPD inhibitors on IMP pool size and on 5′-phosphorylation of ddAdo (5 μM) in MOLT-4 cells

MOLT-4 cells growing in logarithmic phase (10^6 cells/ml at $t = 0$) were incubated with 5 μM ddAdo (5 μCi/ml) in the presence or absence of 5 μM tiazofurin, ribavirin, or mycophenolic acid. Cells were incubated with the respective IMPD inhibitors for 30 min before the addition of radiolabeled ddAdo. After 5 hr of incubation, cells were extracted with 60% cold methanol, and extracts were analyzed by ion exchange HPLC, as described in Materials and Methods. Each value represents the mean of duplicate samples, with the individual values obtained varying by <10%. ddAMP/ddIMP eluted at 13–14 min but could not be accurately quantitated by this method (see Figs. 3 and 4).

Addition	Concentration		Increase		IMP
	ddADP	ddATP	ddADP	ddATP	
	pmol/10^6 cells		*fold*		*nmol/10^6 cells*
None	0.127	0.190			0.026
Tiazofurin (5 μM)	0.213	0.311	1.7	1.6	0.608
Ribavirin (5 μM)	0.235	0.352	1.9	1.9	0.653
Mycophenolic acid (5 μM)	0.383	0.551	3.0	3.0	1.052

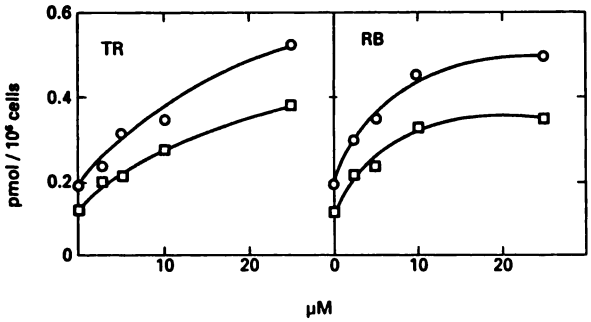

Fig. 5. Effect of tiazofurin and ribavirin on ddADP and ddATP formation from ddIno. MOLT-4 cells growing in logarithmic phase (10^6 cells/ml at $t = 0$) were incubated with 5 μM ddIno (5 μCi/ml) and varying concentrations (0–25 μM) of tiazofurin (*TR*) or ribavirin (*RB*) for 5 hr. IMPD inhibitors were added 30 min before radiolabeled ddIno. Methanolic cell extracts were analyzed as described in Materials and Methods. *Ordinate*, intracellular ddAdo nucleotide concentration (pmol/10^6 cells). □, ddADP; ○, ddATP.

TABLE 4

Effect of inosine on IMP pool size and on ddAdo phosphorylation in MOLT-4 cells

MOLT-4 cells growing in logarithmic phase (10^6 cells/ml at $t = 0$) were incubated with 5 μM ddAdo (5 μCi/ml) in the presence or absence of inosine (100 μM). After 5 hr of incubation, cells were extracted with 60% cold methanol, and extracts were analyzed by ion exchange HPLC, as described in Materials and Methods. Each value represents the mean of duplicate samples, with the individual values obtained varying by <10%.

Addition	IMP	ddATP
	nmol/10^6 cells	*pmol/10^6 cells*
None	0.040	0.068
Inosine (100 μM)	0.077	0.079

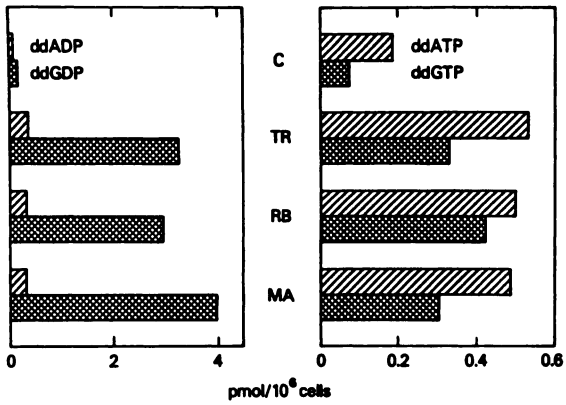

Fig. 6. Effect of IMPD inhibitors on dideoxynucleoside diphosphate and triphosphate formation from ddIno and ddGuo. MOLT-4 cells growing in logarithmic phase (10^6 cells/ml at $t = 0$) were incubated with 5 μM ddIno or ddGuo (5 μCi/ml), in the presence or absence of 25 μM tiazofurin (*TR*), 25 μM ribavirin (*RB*), or 1 μM mycophenolic acid (*MA*). *C*, Control cells (no inhibitor). Cells were incubated with the respective IMPD inhibitors for 30 min before the addition of the radiolabeled nucleosides. Methanolic cell extracts were analyzed as described in Materials and Methods. *Left*, ddADP versus ddGDP formation (pmol/10^6 cells); *right*, ddATP versus ddGTP formation (pmol/10^6 cells).

ribavirin alone. Potentiation of the ddIno protective effect in ATH8 cells by ribavirin was also seen utilizing the p24 gag protein assay system (data not shown). Similar results have been reported by Baba *et al.* (2) for ddAdo plus ribavirin in the ATH8 and MT-4 cell assay systems and by Balzarini *et al.* (20) for ddIno plus ribavirin in the MT-4 and the Moloney murine sarcoma retrovirus assay systems. Tiazofurin (2.5 μM) was inactive in potentiating ddIno activity in the ATH8 system; at this concentration, tiazofurin was also inactive in stimulating ddIno phosphorylation and only marginally active in increasing IMP levels (Table 2). Higher concentrations of this agent were not explored in the ATH8 antiviral assay system.

In MOLT-4 cells, significant potentiation of ddIno activity was seen with the addition of tiazofurin (2.5 μM) (Fig. 7). Mycophenolic acid and ribavirin were not explored in this assay system.

IMP pool size. It has previously been shown that, in human

TABLE 5

Potentiation by ribavirin of the protection by ddIno from the cytopathic effect of HIV-1/III_B in ATH8 cells

ATH8 cells (1×10^5), which are sensitive to the cytopathic effect of HIV, were exposed to a high multiplicity of infectious HIV-1/III_B (3.16×10^3 $TCID_{50}$ of HIV-1/III_B; 1000 virus particles/cell). Cell suspensions (2 ml) were then exposed to ribavirin for 30 min before the addition of various concentrations of ddIno. Uninfected cells were treated identically with ddIno, with or without ribavirin, but were not exposed to the virus; no inhibition of cell growth was noted at these drug concentrations in the latter group. On day 7, total viable cells were counted. Control cell counts at 7 days were as follows: untreated infected cells, 0.4×10^5 cells/2 ml; untreated uninfected cells, 2.9×10^5 cells/2 ml. No reversal of the HIV cytopathic effect was noted with 5 μM ribavirin alone.

ddIno concentration	Reversal by ddIno of HIV cytopathic effect		Increase in protection
	Without ribavirin	With ribavirin (5 μM)	
μM	%		%
1	34	56	65
2	48	92	92
5	60	88	47

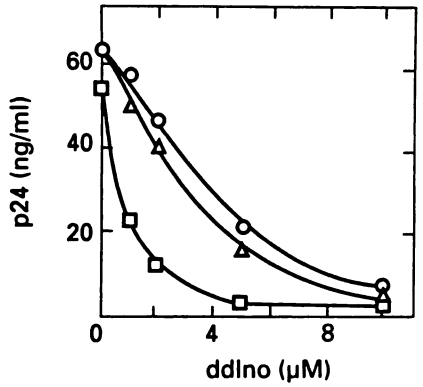

Fig. 7. Effect of increasing concentrations of tiazofurin on anti-HIV activity of ddIno in MOLT-4 cells. MOLT-4 cells (2×10^4) were exposed to HIV-1/III_B for 40 min and cultured with the indicated concentrations of drugs in 5 ml of culture medium. On day 7 in culture, supernatants were collected and the amount of p24 gag protein was quantitated. ○, Tiazofurin omitted; △, tiazofurin at 0.5 μM; □, tiazofurin at 2.5 μM.

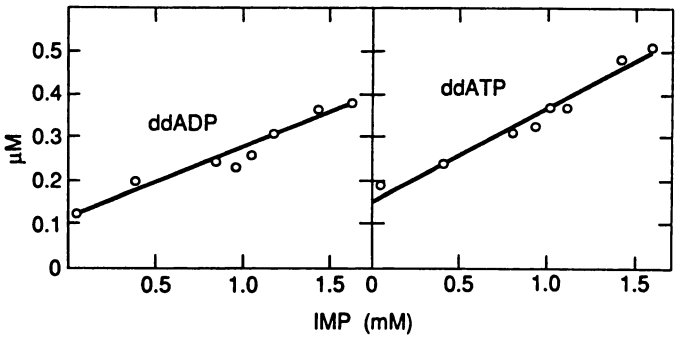

Fig. 8. Relationship between ddAdo nucleotide formation and intracellular IMP concentration. MOLT-4 cells growing in logarithmic phase were incubated for 5 hr with 5 μM ddAdo (5 μCi/ml) and varying concentrations of ribavirin (0–25 μM). Intracellular IMP, [^{3}H]ddADP, and [^{3}H]ddATP concentrations were determined as described in Materials and Methods. Correlation coefficients: *left*, $r = 0.97$; *right*, $r = 0.98$.

lymphoid cells, a cytosolic 5′-nucleotidase, acting in the anabolic direction as a phosphotransferase, plays a major role in the initial 5′-phosphorylation of ddIno (12). The preferred phosphate donor in this reaction is IMP (12), and it, thus, appears likely that inhibitors of IMPD, such as those studied

TABLE 6

Time dependence of enhancement by tiazofurin and ribavirin of ddATP formation from ddIno

MOLT-4 cells growing in logarithmic phase (10^6 cells/ml at $t = 0$) were incubated with 5 μM ddIno (5 μCi/ml) and tiazofurin or ribavirin, at the concentrations indicated, for varying time periods (1–8 hr). IMPD inhibitors were added 30 min before radiolabeled ddIno. Methanolic cell extracts were analyzed as described in Materials and Methods. Each value represents the mean of duplicate samples, with the individual values obtained varying by <10%.

Addition	ddATP			
	1 hr	2 hr	4 hr	8 hr
	pmol/10^6 cells			
None	0.104	0.145	0.184	0.238
Tiazofurine (2.5 μM)	0.173	0.264	0.344	0.387
Ribavirin (2.5 μM)	0.227	0.326	0.373	0.464
Tiazofurin (5 μM)	0.182	0.285	0.366	0.468
Ribavirin (5 μM)	0.287	0.336	0.469	0.522

TABLE 7

Effect of ribavirin on deoxyribonucleotide concentrations in MOLT-4 cells

MOLT-4 cells were exposed to the indicated agents for 6 hr. Cells were extracted, and deoxyribonucleoside-5′-triphosphate levels were determined as described in Materials and Methods. Values shown are means ± standard errors from three separate determinations.

Addition	Concentration			
	dCTP	dTTP	dATP	dGTP
	pmol/10^6 cells			
None	18 ± 2	54 ± 5	54 ± 8	25 ± 3
ddAdo (5 μM)	18 ± 3	50 ± 7	52 ± 5	24 ± 1
Ribavirin (10 μM)	49 ± 7	135 ± 18	58 ± 6	25 ± 2
ddAdo (5 μM) + ribavirin (10 μM)	48 ± 10	124 ± 8	54 ± 8	26 ± 1

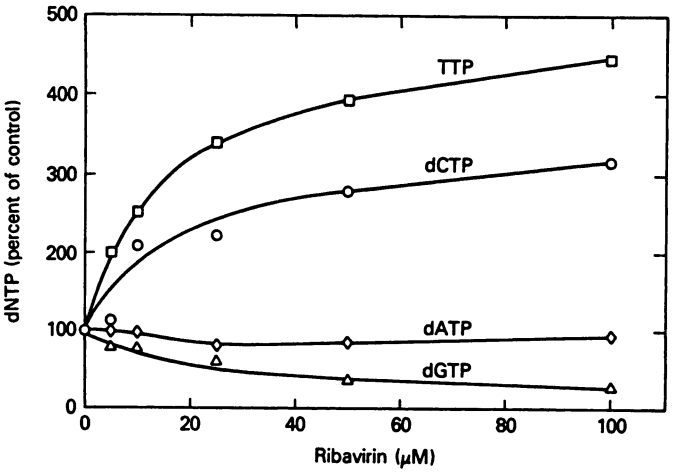

Fig. 9. Effect of ribavirin on deoxyribonucleoside-5′-triphosphate concentrations in MOLT-4 cells. MOLT-4 cells (2.2×10^7 cells at a density of 4.4×10^5 cells/ml) were incubated at 37° for 5 hr with varying concentrations of ribavirin. Deoxyribonucleoside concentrations were determined as described in Materials and Methods. Each *point* represents the mean of duplicate determinations. Control values (no ribavirin) were as follows: dCTP, 19.1 pmol/10^6 cells; TTP, 64.5 pmol/10^6 cells; dATP, 53.8 pmol/10^6 cells; and dGTP, 40.7 pmol/10^6 cells.

here, exert their effect on ddIno phosphorylation by blocking the utilization of IMP for xanthylate and guanylate synthesis, thus increasing the intracellular IMP concentration. Because the affinity of IMP for the 5′-nucleotidase is relatively low (K_m = 2.1 mM), the latter enzyme would be undersaturated under basal conditions, and an increase in IMP concentration beyond

its usual intracellular level (approximately 0.03 mM) could result in enhanced ddIno phosphorylation. The relationship between ddIno phosphorylation and intracellular IMP concentration was, thus, determined in MOLT-4 cells over a wide range of ribavirin concentrations (0–25 μM). As shown in Fig. 8, there was a positive correlation between intracellular IMP and the formation of both ddADP ($r = 0.97$) and ddATP ($r = 0.98$) from ddIno.

Effect of inosine on IMP pool size and on ddIno phosphorylation. In view of the marked enhancement of IMP pool size seen with ribavirin and other IMPD inhibitors, we felt it to be of interest to determine whether high concentrations of inosine could increase IMP pools through the purine salvage pathway and, thus, increase ddIno phosphorylation. In contrast to the effects seen with IMPD inhibitors, however, inosine, even at concentrations as high as 100 μM, was found to be relatively inefficient in increasing IMP pool size (Table 6). Similarly low activity was seen with 100 μM hypoxanthine (data not shown). It is likely, therefore, that in MOLT-4 cells the *de novo* pathway, rather than the purine salvage pathway, plays the major role in IMP biosynthesis.

Effect on deoxynucleotide pool sizes. It is generally accepted that 2′,3′-dideoxynucleosides such as ddIno, ddGuo, and AZT act at the 5′-triphosphate level as inhibitors of retroviral reverse transcriptase, in competition with the corresponding physiological deoxyribonucleoside-5′-triphosphates (3, 4). As a consequence, the activity of such agents depends not only on the concentrations of their 5′-triphosphate anabolites but also on the concentrations of the corresponding endogenous deoxyribonucleoside triphosphates. It, thus, seemed possible that enhanced anti-HIV activity of ddIno or ddAdo could be attributable to a decrease in dATP formation as well as to an increase in ddATP. We, therefore, determined the effect of ribavirin on the levels of dATP and other deoxyribonucleotides in MOLT-4 cells under the conditions employed here. As shown in Table 7 and Fig. 9, however, no change was observed in dATP level after treatment with ribavirin, even at levels as high as 100 μM. Significant increases in TTP and dCTP were seen; these changes would be without effect on the anti-HIV activity of ddAdo or ddIno but could adversely influence the activity of the antiviral agents AZT and ddCyd, respectively. dGTP levels were less affected by ribavirin (10 μM) than were TTP or dCTP levels (Table 7); at higher ribavirin concentrations, however, dGTP levels were significantly decreased (Fig. 9), an effect that could potentiate the activity of ddGuo and ddGuo analogues such as 2,6-diaminopurine-2′,3′-dideoxyriboside (20), 3′-azido-2,6-diaminopurine-2′,3′-dideoxyriboside (21), and carbovir (carbocyclic 2′,3′-didehydro-2′,3′-dideoxyguanosine) (22).

Discussion

These studies, together with our previous observations with ddGuo (1), provide a possible explanation for the empirical observation that ribavirin enhances by severalfold the anti-HIV activity of the purine dideoxynucleosides ddAdo and ddGuo, i.e., that ribavirin, as a result of its ability to act as an IMPD inhibitor, blocks the utilization of IMP for guanine nucleotide biosynthesis, thus yielding a higher level of IMP to act as a phosphate donor for the reaction dideoxynucleoside → dideoxynucleoside monophosphate, the first step in the activation of purine dideoxynucleosides to their corresponding 5′-triphosphates. Supporting this interpretation is our observation that the enhancement of ddAdo and ddGuo phosphorylation is seen also with other agents that increase IMP levels by inhibiting IMPD, such as tiazofurin and mycophenolic acid. Of the three compounds examined, ribavirin, although less active than mycophenolic acid as an IMPD inhibitor, would appear to have the greatest potential for combination therapy with ddIno or ddGuo, because of the low cytotoxicity seen with this agent and the consistent reproducibility of the stimulation effects observed. Incubation of MOLT-4 cells with high levels (100 μM) of inosine or hypoxanthine resulted in only a slight increase in IMP; however, other strategies for increasing intracellular IMP concentration and, thus, purine dideoxynucleoside-5′-triphosphate concentration are possible and remain to be explored.

The results with ddIno may have particular relevance to therapeutic application, in view of the extensive phase II/III clinical trials of this compound that are ongoing at the present time. Although additional studies would be required to ascertain the effect of IMPD inhibitors on ddIno anti-HIV activity and toxicity *in vivo*, it is of interest that Baba *et al.* (2) reported that, in an *in vitro* assay system, the combination of ddGuo or ddAdo with ribavirin resulted not only in increased antiviral activity of these compounds but also in an increase in therapeutic index, i.e., the increase in anti-HIV activity was greater than the increase in cytotoxicity. In addition, Balzarini *et al.* (20), utilizing the Moloney murine sarcoma retrovirus assay system, found that ribavirin enhances both the potency and the therapeutic index of ddIno *in vivo*. If this relationship is also seen with HIV *in vivo*, the increased therapeutic ratio could be of considerable clinical relevance.

It should be noted that these effects would only be anticipated for purine dideoxynucleosides whose initial phosphorylation is catalyzed, in whole or in part, by cytoplasmic phosphotransferase (5′-nucleotidase). This situation would not obtain with the other major class of dideoxynucleosides, i.e., agents that are analogues of pyrimidine rather than purine nucleosides; prominent among these are AZT and ddCyd, analogues of thymidine and 2′-deoxycytidine, respectively. In the case of AZT, a significant antagonism of its anti-HIV activity by ribavirin was reported by Vogt *et al.* (23) and also by Baba *et al.* (2); this decrease in activity appears to be due to a substantial decrease in the formation of AZT-triphosphate in the presence of ribavirin. Because AZT is phosphorylated by thymidine kinase, the decrease in AZT-triphosphate formation could well be due to the increase in TTP levels seen with ribavirin (Table 7) (24) and the consequent feedback inhibition of this enzyme. A similar antagonism by ribavirin of the antiviral effects of the pyrimidine nucleoside analogues 2′,3′-dideoxythymidine-2′,3′-ene and ddCyd has also been reported by Baba *et al.* (2).

As in other studies involving combinations of chemotherapeutic agents, the question arises of whether the effects described here could be due in part to direct antiviral activity of the IMPD inhibitors studied, rather than solely to an effect of the latter on the activity of purine dideoxynucleosides. Of the IMPD inhibitors studied here, ribavirin has been reported to have direct anti-HIV activity in some *in vitro* assay systems, although only at concentrations considerably higher than those studied here (25). In clinical studies, ribavirin may produce slowing in progression to acquired immunodeficiency syndrome of HIV-infected patients with lymphadenopathy, although no improvement was noted for this group of patients in immunological competence or other surrogate markers (26). *In vitro*,

ribavirin is without anti-HIV activity in the MT-4 and ATH8 assay systems at 10 μM, a concentration at which it strongly enhances the antiviral activity of ddAdo and ddGuo (2). In the case of tiazofurin, *in vitro* anti-HIV activity has been detected, but only at concentrations at which this agent also shows direct cytotoxicity toward host cells (27). Because the enhancement of phosphorylation and the antiviral effect of ddIno and ddGuo described in this and the preceding study (1) are seen with low ribavirin concentrations, at which the latter agent is without detectable cytotoxic or anti-HIV effects in the assay systems utilized (ATH8, MOLT-4, and CEM), the preponderance of evidence would support the contention that, in the present studies, ribavirin and the other IMPD inhibitors studied are acting through the intermediation of their effect on dideoxypurine nucleoside anabolism.

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