0026-895X/93/030519-05$03.00/0

MOLECULAR PHARMACOLOGY, 44:519–523

ACCELERATED COMMUNICATION

Enhanced Stimulation by Ribavirin of the 5′-Phosphorylation and Anti-Human Immunodeficiency Virus Activity of Purine 2′-β-Fluoro-2′,3′-dideoxynucleosides

DAVID G. JOHNS, GURPREET S. AHLUWALIA, DAVID A. COONEY, HIROAKI MITSUYA, and JOHN S. DRISCOLL

Laboratory of Medicinal Chemistry, Developmental Therapeutics Program (D.G.J., G.S.A., D.A.C., J.S.D.), and Experimental Retrovirology Section, Medicine Branch, Clinical Oncology Program (H.M.), Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Received April 12, 1993; Accepted June 1, 1993

SUMMARY

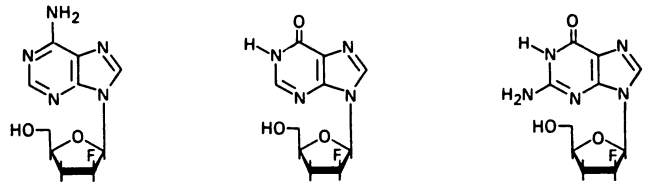
The purine dideoxynucleosides 2′-β-fluoro-2′,3′-dideoxyadenosine (2′-β-F-ddAdo), 2′-β-fluoro-2′,3′-dideoxyinosine, and 2′-β-fluoro-2′,3′-dideoxyguanosine (2′-β-F-ddGuo) are active inhibitors of the replication of the human immunodeficiency virus (HIV) in the ATH8 assay system, with 2′-β-F-ddAdo and 2′-β-fluoro-2′,3′-dideoxyinosine showing activity and potency equivalent to those of their respective parent compounds, 2′,3′-dideoxyadenosine (ddAdo) and 2′,3′-dideoxyinosine. Because inhibitors of IMP dehydrogenase such as ribavirin and tiazofurin stimulate the 5′-phosphorylation and consequently the anti-HIV activity of the three nonfluorinated parent compounds (ddAdo, 2′,3′-dideoxyinosine, and 2′,3′-dideoxyguanosine), we have undertaken a study in MOLT-4 cells to determine whether a similar stimulatory effect is observed with their 2′-β-fluorinated analogs. The 5′-phosphorylation of all the fluoro compounds was found to be greatly enhanced by low levels (10 μM) of either ribavirin or tiazofurin, with the greatest increase being seen with 2′-β-F-ddAdo, where stimulation of the formation of the 5′-mono-, di-, and triphosphorylated nucleotides was approximately 20-fold, 6-fold, and 5-fold, respectively. These increases were approximately 3-fold greater than the increases seen with the nonfluorinated parent compound ddAdo. In the case of 2′-β-F-ddGuo, the greatest stimulation (8-fold) was seen in the formation of the 5′-diphosphate. In parallel with the increased phosphorylation of 2′-β-F-ddAdo and 2′-β-F-ddGuo, the anti-HIV potency of these two compounds at the 5 μM level was approximately doubled in the presence of ribavirin (5 μM).

In 1987, Baba *et al.* (1) reported that ribavirin (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide) enhanced the anti-HIV effects of the purine 2′,3′-dideoxynucleosides ddAdo and ddGuo in the MT-4 test system. This observation has since been confirmed by other investigators and has been extended to other test systems and to other purine dideoxynucleosides such as 2′,3′-dideoxy-2,6-diaminopurine riboside (2) and ddIno (2, 3). In studies to elucidate the basis for this increased antiviral effect, using the dideoxynucleoside ddGuo, we noted that the intracellular concentrations of ddGuo-5′-diphosphate and ddGTP were 15-fold and 4-fold greater, respectively, than control levels in the presence of low levels of ribavirin (4). Other metabolic changes noted in these cells were major increases (up to 35-fold) in intracellular levels of IMP and significant decreases (up to 70%) in levels of GTP and dGTP.

Because these biochemical changes are characteristic of inhibition of IMPD, the enzyme that converts IMP to xanthosine-5′-monophosphate, we extended the study to other inhibitors of this enzyme; in molar terms, the most effective of these was mycophenolic acid, which produced similar increments in the phosphorylation of ddGuo at levels as low as 1 μM. A third IMPD inhibitor, tiazofurin (2-β-ribofuranosylthiazole-4-carboxamide), was equivalent to ribavirin over a concentration range of 2.5–25 μM. Stimulation by IMPD inhibitors of the 5′-phosphorylation of other purine dideoxynucleosides (ddIno and ddAdo) was also seen (3); in these cases, however, the magnitude of the effect was smaller by half than that seen with ddGuo.

Because IMP is the major phosphate donor for the initial phosphorylation of ddGuo to ddGuo-5′-monophosphate and of ddIno to ddIno-5′-monophosphate, reactions catalyzed by a cytosolic 5′-nucleotidase (5), it appeared likely that the ob-

ABBREVIATIONS: HIV, human immunodeficiency virus; 2′-β-F-ddAdo, 2′-β-fluoro-2′,3′-dideoxyadenosine; 2′-β-F-ddIno, 2′-β-fluoro-2′,3′-dideoxyinosine; 2′-β-F-ddGuo, 2′-β-fluoro-2′,3′-dideoxyguanosine; ddAdo, 2′,3′-dideoxyadenosine; ddIno, 2′,3′-dideoxyinosine; ddGuo, 2′,3′-dideoxyguanosine; IMPD, inosine monophosphate dehydrogenase; ddATP, 2′,3′-dideoxyadenosine-5′-triphosphate; ddGTP, 2′,3′-dideoxyguanosine-5′-triphosphate; 2′-β-F-ddATP, 2′-β-fluoro-2′,3′-dideoxyadenosine-5′-triphosphate; 2′-β-F-ddGTP, 2′-β-fluoro-2′,3′-dideoxyguanosine-5′-triphosphate.



2′-β-F-ddAdo 2′-β-F-ddIno 2′-β-F-ddGuo

Fig. 1. Structures of 2′-β-F-ddAdo, 2′-β-F-ddIno, and 2′-β-F-ddGuo.

served increases in phosphorylation were a consequence of the increased level of IMP arising from inhibition of IMPD. Furthermore, intracellular concentrations of IMP tended to remain elevated over their normal levels because this nucleotide, as a result of the fall in GTP, was no longer able to utilize its alternate anabolic pathway, i.e., conversion to AMP, a reaction for which GTP is an essential co-substrate (6, 7).

In view of the clinical interest in this class of compounds, we have now extended these studies to the 2′-β-fluoro analogs of ddAdo, ddIno, and ddGuo (Fig. 1). These fluorinated analogs possess the potential advantage of acid stability (8), whereas the parent compounds, because of the extreme acid lability of their glycosylic bonds (8), require administration with antacids or in an enteric-coated formulation to be orally available (9). In addition, the fluorinated analogs are not susceptible to cleavage by purine nucleoside phosphorylase, a major route of disposition of ddIno (10).

Materials and Methods

Chemicals. The purine 2′,3′-dideoxynucleosides ddAdo, ddGuo, and ddIno and the IMPD inhibitors ribavirin, tiazofurin, and mycophenolic acid were supplied by Dr. Karl Flora, Pharmaceutical Resources Branch, Developmental Therapeutics Program, National Cancer Institute. 2′-β-F-ddAdo and 2′-β-F-ddIno were synthesized within the Laboratory of Medicinal Chemistry by methods previously described (8), and 2′-β-F-ddGuo was synthesized by a method presently being prepared for publication.[1] 2′-β-F-ddATP was synthesized by Mrs. Pamela Russ of the Laboratory of Medicinal Chemistry, using the general method of Kovács and Ötvös (11); an additional supply was prepared by Sierra Bioresearch (Tucson, AZ), using 2′-β-F-ddAdo furnished by this laboratory. 2′-β-F-ddAdo-5′-monophosphate was synthesized by the general method of Yoshikawa and Takenishi (12). ddATP and ddGTP were purchased from Pharmacia (Piscataway, NJ). 2′-β-F-[5′-^{3}H]ddAdo (10 Ci/mmol), [2′,3′-^{3}H]ddAdo (30 Ci/mmol), [2′,3′-^{3}H]ddGuo (44 Ci/mmol), and 2′-β-F-[8-^{3}H]ddGuo (1.5 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). 2′-β-F-[5′-^{3}H]ddIno and [2′,3′-^{3}H]ddIno were prepared by means of enzymatic deamination of 2′-β-F-[5′-^{3}H]ddAdo and [2′,3′-^{3}H]ddAdo, respectively, using calf intestinal deaminase (Sigma Chemical Co., St. Louis, MO); the enzyme was removed by heat denaturation (95° for 1 min), followed by centrifugation to remove the precipitated protein. In a few early experiments, 2′-β-F-[2,8-^{3}H]ddAdo (25 Ci/mmol) was used (American Radiolabeled Chemicals Inc., St. Louis, MO). Venom phosphodiesterase (1.5 units/mg) was obtained from Boehringer-Mannheim (Indianapolis, IN).

Cells. MOLT-4 cells were grown in RPMI 1640 tissue culture medium, supplemented with 10% heat-inactivated (56° for 30 min) fetal bovine serum, 45 μg/ml gentamycin, and 4 mM L-glutamine, at 37° in a humidified atmosphere of 95% air/5% CO_2. Cells were verified to be in logarithmic growth at the time of use. Typically, a cell concentration of $<1 \times 10^6$ cells/ml was used in metabolism studies.

[1] Ford, H., Jr., J. S. Driscoll, M. Siddiqui, J. A. Kelley, H. Mitsuya, T. Shirasaka, D. G. Johns and V. E. Marquez. Chemistry and anti-HIV activity of 2′-β-fluoro-2′,3′-dideosyguanosine. Submitted for publication.

Metabolism studies. Metabolism of the dideoxynucleosides ddAdo, ddIno, ddGuo, 2′-β-F-ddAdo, 2′-β-F-ddIno, and 2′-β-F-ddGuo was determined in exponentially growing MOLT-4 cells in the presence or absence of varying concentrations of IMPD inhibitors. Ten-milliliter aliquots of cell suspensions (approximately 1×10^6 cells/ml) were incubated with IMPD inhibitors (ribavirin, tiazofurin, or mycophenolic acid) for the indicated time periods (30 or 45 min). Cells were then exposed to a 5 μM concentration of the radiolabeled dideoxynucleoside (5 μCi/ml). After 5 hr of incubation, cells were collected by centrifugation and the cell pellets were washed with 1 ml of cold normal saline and then extracted with 0.4 ml of 60% methanol. The methanolic 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, as described previously (10). One-minute fractions were collected, and radioactivity was determined by liquid scintillation counting. Identity of radiolabeled phosphorylated anabolites was established by co-chromatography with known standards, where available.

Assay for anti-HIV activity. The assay method used was that described by Mitsuya and Broder (13). ATH8 cells (2×10^5), which are sensitive to the cytopathic effect of HIV, were exposed to a high multiplicity of infectious HIV-1/LAI (3.16×10^3 times the 50% tissue culture infectious dose of HIV-1/LAI). Cell suspensions (2 ml) were then exposed to ribavirin (5 μM) for 30 min before the addition of various concentrations of 2′-β-F-ddAdo or 2′-β-F-ddGuo. Uninfected cells were treated identically but were not exposed to the virus. On day 7, total viable cells were counted for quantitation of cytopathic effects.

Results

Chromatographic separation of nucleotides arising from 2′-β-F-ddAdo and 2′-β-F-ddGuo. We initially determined the effect of a low level of ribavirin (10 μM) on the 5′-phosphorylation of 2′-β-F-ddAdo and 2′-β-F-ddGuo under the conditions previously described for analysis of the metabolism of the parent compounds ddAdo (3) and ddGuo (4). As illustrated in Fig. 2A, with 2′-β-F-ddAdo ribavirin provoked a striking increase in the formation of all four nucleotides, i.e., 2′-β-F-ddAdo-5′-monophosphate (20-fold), 2′-β-F-ddIno-5′-monophosphate (9-fold), 2′-β-F-ddAdo-5′-diphosphate (6-fold), and 2′-β-F-ddATP (5-fold). In the case of 2′-β-F-ddGuo, however (Fig. 2B), the ribavirin stimulation effect was most marked at the 5′-diphosphate level (7-fold), with lesser increases of 2′-β-F-ddGuo-5′-monophosphate (3-fold) and 2′-β-F-ddGTP (2-fold). A similar but lesser accumulation at the 5′-diphosphate level has also been noted with the nonfluorinated parent compound (4). The differential stimulation seen with 2′-β-F-ddAdo and 2′-β-F-ddGuo is summarized in Table 1.

Comparison with nonfluorinated parent compounds. The effect of the IMPD inhibitors ribavirin and tiazofurin on the 5′-phosphorylation of 2′-β-F-ddAdo was compared with the effect of these two agents on the nonfluorinated purine dideoxynucleoside ddIno over a wide range of ribavirin and tiazofurin concentrations. As illustrated in Fig. 3, the efficiency of phosphorylation was approximately 10-fold greater in the case of the fluorinated nucleoside. The two IMPD inhibitors were almost identical in their ability to stimulate phosphorylation when compared over the range of 2.5–25 μM, with ribavirin being slightly more effective than tiazofurin in stimulating the formation of 2′-β-F-ddAdo-5′-diphosphate and maximal stimulation of the latter being achieved at a ribavirin concentration as low as 5 μM (Fig. 3, *lower*). No differences in direct cytotoxicity of ribavirin and tiazofurin were detectable over these short periods of incubation (5 hr); however, when the cell growth-

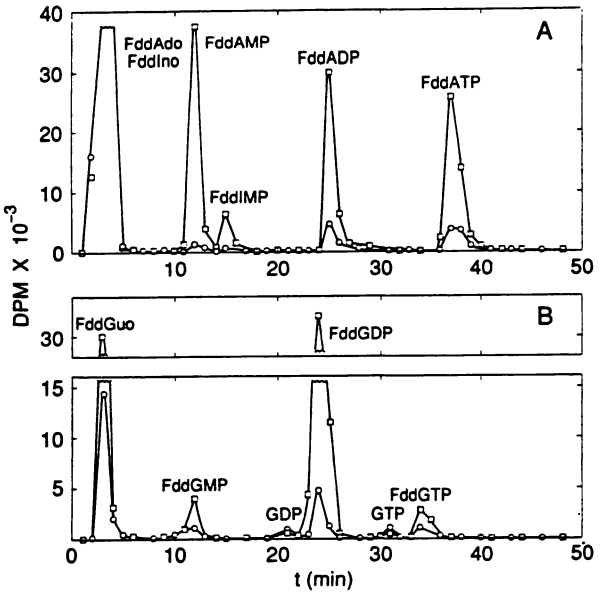


Fig. 2. Chromatographic separation of ^{3}H-labeled metabolites arising from 2′-β-F-[^{3}H]ddAdo (A) or 2′-β-F-[^{3}H]ddGuo (B). MOLT-4 cells (approximately 10^6 cells/ml at time 0) were incubated with either ^{3}H-labeled 2′-β-F-ddAdo or ^{3}H-labeled 2′-β-F-ddGuo (5 μM; 5 μCi/ml) for 5 hr in the presence or absence of ribavirin (10 μM). Cells were preincubated with ribavirin for 45 min before addition of the radiolabeled drug. Methanolic extracts of an equivalent of 5 × 10^6 cells were subjected to ion exchange high performance liquid chromatography (Partisil 10-SAX), using an elution program previously described (10). Representative chromatograms are shown (three or more experiments). A, 2′-β-F-ddAdo metabolites with (□) or without (○) ribavirin; B, 2′-β-F-ddGuo metabolites with (□) or without (○) ribavirin.

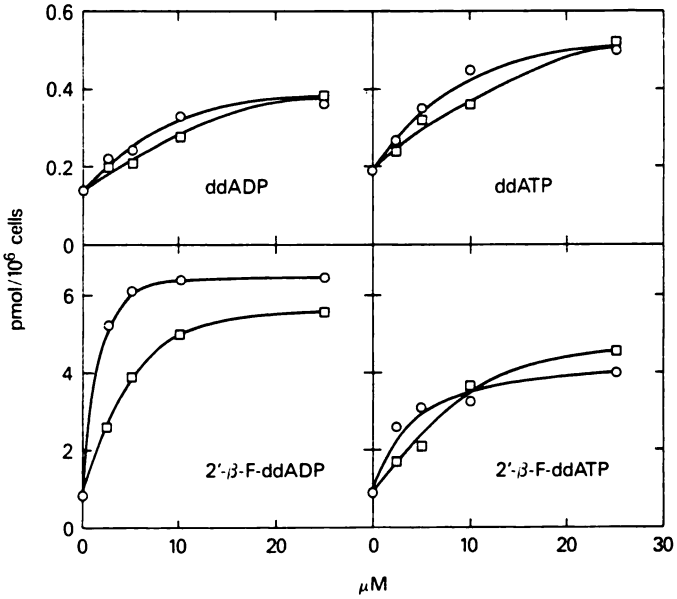


Fig. 3. Effect of ribavirin and tiazofurin on 5′-phosphorylation of ddIno and 2′-β-F-ddAdo in human T lymphoblasts. MOLT-4 cells in logarithmic growth (10^6 cells/ml at time 0) were incubated with either ^{3}H-labeled 2′,3′-ddIno (*upper*) or ^{3}H-labeled 2′-β-F-ddAdo (5 μM; 5 μCi/ml) (*lower*) for 5 hr in the presence of varying concentrations (0–25 μM) of tiazofurin or ribavirin. The IMPD inhibitors were added 30–45 min before addition of the labeled drug. Methanolic extracts equivalent to 5 × 10^6 cells were subjected to ion exchange high performance liquid chromatography as described for Fig. 2. Each value represents the average of duplicate samples, with the individual values obtained varying by <10%. ○, Plus ribavirin; □, plus tiazofurin.

TABLE 1

Stimulation by ribavirin of 5′-phosphorylation of purine 2′-β-fluoro-2′,3′-dideoxynucleosides

MOLT-4 cells (approximately 10^6 cells/ml at time 0) were incubated with ^{3}H-labeled 2′-β-F-ddAdo or ^{3}H-labeled 2′-β-F-ddGuo (5 μM; 5 μCi/ml of cell suspension) for 5 hr in the presence or absence of ribavirin (10 μM). Cells were preincubated with ribavirin for 45 min before addition of the radiolabeled drug. Methanolic extracts equivalent to 5 × 10^6 cells were subjected to ion exchange high performance liquid chromatography as described in the legend to Fig. 2. Values represent the average of duplicate experiments, with the individual values varying by <10%. Numbers in parentheses, fold increase after ribavirin treatment.

Addition	Dideoxynucleoside 5′-phosphate			
	F-ddNMP*	F-ddIMP	F-ddNDP	F-ddNTP
	pmol/10⁶ cells			
2′-β-F-ddAdo (5 μM)	0.15	0.06	0.55	0.54
2′-β-F-ddAdo (5 μM) + ribavirin (10 μM)	3.02 (20)	0.57 (9)	3.21 (6)	2.69 (5)
2′-β-F-ddGuo (5 μM)	0.14		0.52	0.19
2′-β-F-ddGuo (5 μM) + ribavirin (10 μM)	0.39 (3)		3.86 (7)	0.40 (2)

* F-ddNMP, 2′-β-fluoro-2′,3′-dideoxynucleoside-5′-monophosphate; F-ddIMP, 2′-β-fluoro-ddIno-5′-monophosphate; F-ddNDP, 2′-β-fluoro-2′,3′-dideoxynucleoside-5′-diphosphate, F-ddNTP, 2′-β-fluoro-2′,3′-dideoxynucleoside-5′-triphosphate.

inhibitory activities of the two compounds were compared over 24 hr, tiazofurin was found to be about twice as cytostatic as ribavirin in MOLT-4 cells (IC_{50} for tiazofurin, 21 μM; IC_{50} for ribavirin, 43 μM).

Effect of deamination of 2′-β-F-ddAdo on its conversion to 5′-di- and triphosphate anabolites. We previously noted that the conversion of both ddAdo and its deamination product ddIno to ddATP is stimulated by ribavirin; however, the efficiency of conversion of ddIno to ddATP is less than that of its parent compound, an apparent consequence of the lower octanol/water partition coefficient of ddIno. This lower lipophilicity results in less efficient entry of ddIno into the intracellular space (14). Because a similar relationship of octanol/water partition coefficients pertains with the fluorinated analogs (log*P* for 2′-β-F-ddIno, −1.21; log*P* for 2′-β-F-ddAdo, −0.18) (15), we assessed the relative efficiency of 5′-phosphorylation of 2′-β-F-ddIno and 2′-β-F-ddAdo in the presence and absence of pharmacologically induced inhibition of IMPD. As shown in Fig. 4, the ribavirin stimulation of 5′-diphosphorylation was approximately 8-fold less efficient and that of 5′-triphosphorylation was approximately 5-fold less efficient when 2′-β-F-ddIno rather than 2′-β-F-ddAdo was used as the precursor.

With 2′-β-F-ddGuo, the ribavirin stimulation effect was readily demonstrable at the 5′-diphosphate level (approximately 8-fold) (Fig. 5). As was the case with the nonfluorinated parent compound, however (4), the further conversion to the 5′-triphosphate was inefficient (Figs. 2 and 5) and there was little absolute difference between the net overall stimulation of 5′-triphosphorylation of 2′-β-F-ddGuo and its nonfluorinated parent compound (Fig. 5).

Effect of ribavirin on the anti-HIV effectiveness of 2′-β-F-ddAdo and 2′-β-F-ddGuo. It is generally accepted that anabolism to their 5′-triphosphate nucleotides is essential for the anti-HIV effectiveness of 2′,3′-dideoxynucleosides as inhibitors of HIV reverse transcriptase and chain terminators of viral DNA synthesis. In an attempt to determine whether the increased levels of 2′-β-F-ddATP and 2′-β-F-ddGTP after ribavirin treatment would coincide with increased antiviral potency, we assessed the anti-HIV-1 activities of 2′-β-F-ddAdo

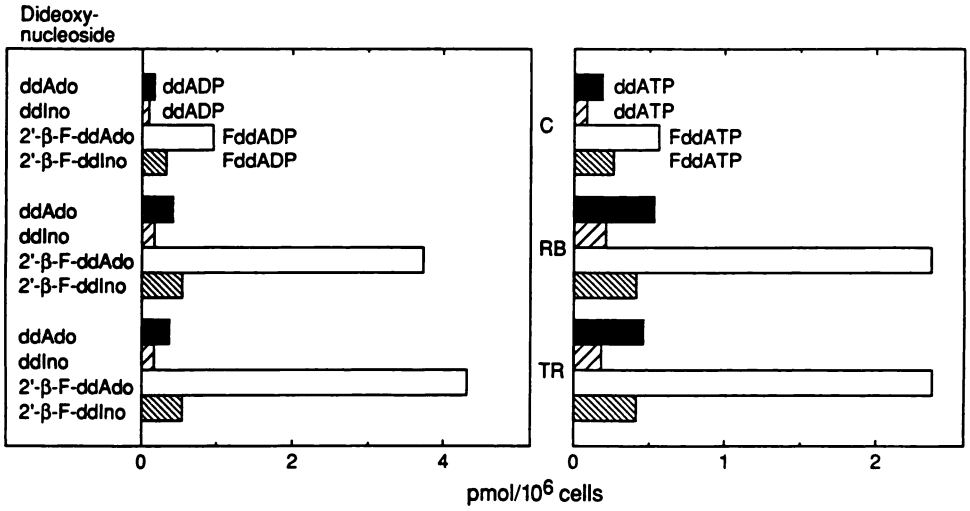


Fig. 4. Effect of IMPD inhibitors on 5′-phosphorylation of ddAdo, ddIno, 2′-β-F-ddAdo, and 2′-β-F-ddIno. MOLT-4 cells in logarithmic growth (10^6 cells/ml at time 0) were incubated with ^{3}H-labeled ddAdo, ddIno, 2′-β-F-ddAdo, or 2′-β-F-ddIno, at a drug concentration of 5 μM (5 μCi/ml of cell suspension), for 5 hr in the presence or absence of 10 μM tiazofurin or ribavirin. The IMPD inhibitors were added 45 min before addition of the labeled drug. Extracts equivalent to 5×10^6 cells were subjected to ion exchange high performance liquid chromatography as described in the legend to Fig. 2. *Left*, dideoxynucleoside-5′-diphosphate; *right*, dideoxynucleoside-5′-triphosphate. *C*, Control; *RB*, plus ribavirin; *TR*, plus tiazofurin.

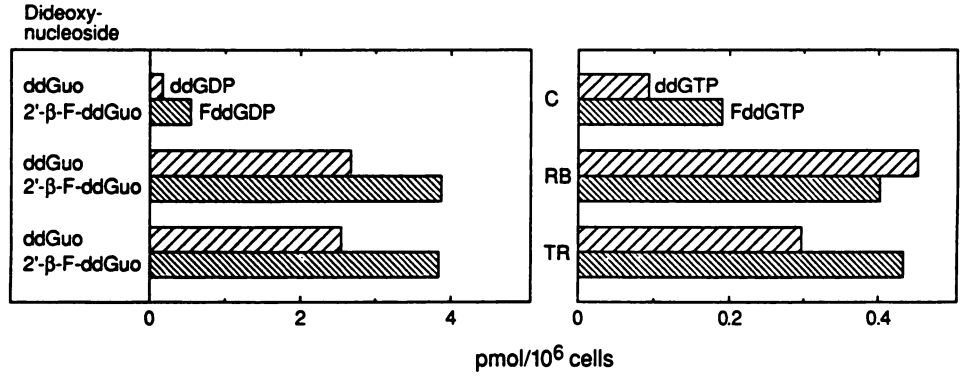


Fig. 5. Effect of IMPD inhibitors on 5′-phosphorylation of ddGuo and 2′-β-F-ddGuo. MOLT-4 cells in logarithmic growth (10^6 cells/ml at time 0) were incubated with either ^{3}H-labeled ddGuo or ^{3}H-labeled 2′-β-F-ddGuo (5 μM; 5 μCi/ml of cell suspension) for 5 hr in the presence or absence of tiazofurin or ribavirin (10 μM). The IMPD inhibitors were added 45 min before addition of the radiolabeled drug. Extracts equivalent to 5×10^6 cells were subjected to ion exchange high performance liquid chromatography as described in the legend to Fig. 2. *Left*, dideoxynucleoside-5′-diphosphate; *right*, dideoxynucleoside-5′-triphosphate. *C*, Control; *RB*, plus ribavirin; *TR*, plus tiazofurin.

TABLE 2

Potentiation by ribavirin of the protection by 2′-β-F-ddAdo and 2′-β-F-ddGuo from the cytopathic effect of HIV-1/LAR in ATH8 cells

ATH8 cells (2×10^5), which are sensitive to the cytopathic effect of HIV, were exposed to a high multiplicity of infectious HIV-1/LAR (3.16×10^3 times the 50% tissue culture infectious dose of HIV-1/LAR). Cell suspensions (2 ml) were then exposed to ribavirin for 30 min before the addition of various concentrations of 2′-β-F-ddAdo or 2′-β-F-ddGuo. Uninfected cells were treated identically but were not exposed to the virus. On day 7, total viable cells were counted. Control cell counts at 7 days were as follows: untreated uninfected cells, 3.5×10^5/2 ml; untreated infected cells, 0.3×10^5/2 ml. Ribavirin alone (5 μM) did not show any reversal of the HIV cytopathic effect but exhibited slight cytotoxicity toward uninfected cells [uninfected cells plus ribavirin (5 μM), 2.7×10^5 cells/2 ml]. No cytotoxicity was observed with 2′-β-F-ddAdo or 2′-β-F-ddGuo at these concentrations. Results shown are the average of duplicate values.

Drug treatment	Protection against HIV-1 cytopathic effect		Increased protection with ribavirin
	Without ribavirin	With ribavirin	
	%		%
None		<1	<1
2′-β-F-ddAdo			
5 μM	29	69	138
10 μM	44	64	45
2′-β-F-ddGuo			
5 μM	23	46	100
10 μM	30	62	106

and 2′-β-F-ddGuo in the ATH8 assay system (13) in the presence and absence of a low level of ribavirin (5 μM). The two fluorinated dideoxynucleosides were tested at suboptimal activity levels for this assay (5 and 10 μM), so that any potentiating effects would be demonstrable. As shown in Table 2, there was marked potentiation at all levels examined, with the greatest increase in protection against the HIV cytopathic effect (138%) being observed with the combination of 5 μM 2′-β-F-ddAdo and 5 μM ribavirin. Ribavirin alone showed slight cytotoxicity but no anti-HIV activity in this assay system.

Although the drop in dGTP accompanying ribavirin pretreatment (4) might be expected to further enhance the antiviral activity of 2′-β-F-ddGuo, the accumulation of this compound at the 5′-diphosphate level (see above), rather than at the biologically active 5′-triphosphate level, as seen with 2′-β-F-ddAdo, would appear to account for the antiviral potentiation by ribavirin of 2′-β-F-ddGuo being less than might be anticipated.

Discussion

As we and other investigators have observed, 2′-β-F-ddATP is active but less potent as an inhibitor of HIV-1 reverse transcriptase than is its parent compound ddATP (10), with the disparity being to some degree template dependent (16). Masood *et al.* (10), using the template-primer poly(dA-dT), found the K_i values for ddATP and 2′-β-F-ddATP to be 0.1 μM and 1.0 μM, respectively (a ratio of 1:10), whereas Hitchcock *et al.* (16), using the template-primer poly(rU)-oligo(dA), found a ratio of 1:19 for ddATP versus 2′-β-F-ddATP. In general, therefore, the fluorinated compound appears to be at least 1 log unit less active as an inhibitor of reverse transcriptase. In

assays using the ATH8 system, however, 2′-β-F-ddAdo and ddAdo are approximately equally potent and active (8, 17), an observation that would appear to correlate with greater intracellular concentration of 2′-β-F-ddATP, consistent with the more efficient conversion of the 2′-β-fluoro analog to its 5′-triphosphate form (10). Thus, there would appear to be a potential practical significance to our observation that exposure to IMPD inhibitors such as ribavirin and tiazofurin can stimulate the generation of 2′-β-F-ddATP to a greater degree than is seen in the analogous stimulation of ddIno or ddAdo conversion to ddATP. This is especially so inasmuch as the stimulation noted is of considerable magnitude. For example, where 2′-β-F-ddAdo yields 4–5 times as much 5′-triphosphate as does ddAdo without ribavirin stimulation, the addition of ribavirin (10 μM) results in a 12-fold increase in 2′-β-F-ddATP (Fig. 4). With ddIno (the deaminated analog of ddAdo and the form in which ddAdo is administered clinically), the difference in the rates of 5′-triphosphate generation is even more striking; for example, in cells exposed to ddIno without ribavirin the rate is 0.07 pmol of ddATP generated/10^6 cells/5 hr, whereas for 2′-β-F-ddAdo with ribavirin the rate is 1.84 pmol of 2′-β-F-ddATP generated/10^6 cells/5 hr, a difference of approximately 26-fold (Fig. 4). With ddGuo, however, the difference is much less marked; for ddGuo without ribavirin the rate is 0.09 pmol of ddGTP/10^6 cells/5 hr, whereas for 2′-β-F-ddGuo with ribavirin it is 0.41 pmol of 2′-β-F-ddGTP/10^6 cells/5 hr, a difference of 4.5-fold (Fig. 5). It would appear, therefore, that ribavirin or tiazofurin enhancement of 5′-triphosphate formation would be more likely in the case of 2′-β-F-ddAdo, rather than 2′-β-F-ddGuo, to compensate for the decrease in affinity for HIV reverse transcriptase resulting from 2′-β-fluorination.

When 2′-β-F-ddAdo and its deamination product 2′-β-F-ddIno were compared directly, the ribavirin stimulation of 5′-triphosphorylation was approximately 5-fold less efficient with 2′-β-F-ddIno (Fig. 4). This observation could be of greater importance with the 2′-β-fluoro analogs than with their nonfluorinated parent compounds, because the rate of adenosine deaminase-catalyzed deamination of 2′-β-F-ddAdo is approximately 10–15-fold slower than that of ddAdo (8, 10), with the result that 2′-β-F-ddAdo persists unchanged in plasma (and likely within cells as well) for a much longer period than does ddAdo (8). *In vivo*, the latter compound is deaminated to ddIno virtually instantaneously (9).

The reason for the enhancement of 5′-phosphorylation and the resulting increase in anti-HIV potency of purine dideoxynucleosides in combination with ribavirin and other IMPD inhibitors has not been fully elucidated. As indicated above, IMPD inhibitors cause both an increase in intracellular IMP levels and a decrease in GTP and dGTP. Both of these factors can contribute to greater 5′-phosphorylation, because IMP can act as a phosphate donor for purine dideoxynucleosides and the decrease in GTP can contribute to IMP accumulation by blocking the utilization of this nucleotide in the adenylosuccinate synthetase reaction, for which GTP is an obligatory source of energy (6, 7). These considerations do not explain, however, the greatly enhanced 5′-phosphorylation of 2′-β-F-ddAdo, compared with ddAdo, even in the absence of IMPD inhibitors (10). It would appear from the present results either that 2′-β-fluorination must result in more active substrate activity of the purine dideoxynucleoside for one of the initial enzyme steps necessary for 5′-phosphate formation or that the 2′-β-fluorodideoxynucleosides can utilize a pathway that is unavailable or only poorly available to the nonfluorinated parent dideoxynucleosides.

Acknowledgments

The authors would like to thank Mrs. Sandra Taubenkibel for excellent secretarial assistance.

References

1. Baba, M., R. Pauwels, J. Balzarini, P. Herdewijn, E. DeClercq, and J. Desmyter. Ribavirin antagonizes inhibitory effects of pyrimidine 2′,3′-dideoxynucleosides but enhances inhibitory effects of purine 2′,3′-dideoxynucleosides on replication of human immunodeficiency virus *in vitro*. *Antimicrob. Agents Chemother.* **31**:1613–1617 (1987).
2. Balzarini, J., L. Naesens, M. J. Robins, and E. DeClercq. Potentiating effect of ribavirin on the *in vitro* and *in vivo* antiretrovirus activities of 2′,3′-dideoxyinosine and 2,6-diaminopurine riboside. *J. Acquired Immune Deficiency Syndrome* **8**:1140–1147 (1990).
3. Hartman, N. R., G. S. Ahluwalia, D. A. Cooney, H. Mitsuya, S. Kageyama, A. Fridland, S. Broder, and D. G. Johns. Inhibitors of IMP dehydrogenase stimulate the phosphorylation of the anti-human immunodeficiency virus nucleosides 2′,3′-dideoxyadenosine and 2′,3′-dideoxyinosine. *Mol. Pharmacol.* **40**:118–124 (1991).
4. Ahluwalia, G., D. A. Cooney, L. L. Bondoc, Jr., M. J. Currens, H. Ford, D. G. Johns, H. Mitsuya, and A. Fridland. Inhibitors of IMP dehydrogenase stimulate the phosphorylation of the antiviral nucleoside 2′,3′-dideoxyguanosine. *Biochem. Biophys. Res. Commun.* **171**:1297–1303 (1990).
5. Johnson, M. A., and A. Fridland. Phosphorylation of 2′,3′-dideoxyinosine by cytosolic 5′-nucleotidase of human lymphoid cells. *Mol. Pharmacol.* **36**:291–295 (1989).
6. Muirhead, K. M., and S. H. Bishop. Purification of adenylosuccinate synthetase from rabbit skeletal muscle. *J. Biol. Chem.* **249**:459–464 (1974).
7. Balzarini, J., C.-K. Lee, P. Herdewijn, and E. De Clercq. Mechanism of the potentiating effect of ribavirin on the activity of 2′,3′-dideoxyinosine against human immunodeficiency virus. *J. Biol. Chem.* **266**:21509–21514 (1991).
8. Marquez, V. E., C. K.-H. Tseng, H. Mitsuya, S. Aoki, J. A. Kelley, H. Ford, Jr., J. S. Roth, S. Broder, D. G. Johns, and J. S. Driscoll. Acid-stable 2′-fluoro purine dideoxynucleosides as active agents against HIV. *J. Med. Chem.* **33**:978–985 (1990).
9. Hartman, N. R., R. Yarchoan, J. M. Pluda, R. V. Thomas, K. S. Marczyk, S. Broder, and D. G. Johns. Pharmacokinetics of 2′,3′-dideoxyadenosine and 2′,3′-dideoxyinosine in patients with severe human immunodeficiency virus infection. *Clin. Pharmacol. Ther.* **47**:647–654 (1990).
10. Masood, R., G. S. Ahluwalia, D. A. Cooney, A. Fridland, V. E. Marquez, J. S. Driscoll, Z. Hao, H. Mitsuya, C.-F. Perno, S. Broder, and D. G. Johns. 2′-Fluoro-2′,3′-dideoxyarabinosyladenine: a metabolically stable analogue of the antiretroviral agent 2′,3′-dideoxyadenosine. *Mol. Pharmacol.* **37**:590–596 (1990).
11. Kovács, T., and L. Ötvös. Simple synthesis of 5-vinyl- and 5-ethynyl-2′-deoxyuridine-5′-triphosphates. *Tetrahedron Lett.* **29**:4525–4528 (1988).
12. Yoshikawa, T. K., and T. Takenishi. Studies of phosphorylation. III. Selective phosphorylation of unprotected nucleosides. *Bull. Chem. Soc. Jpn.* **42**:3505–3508 (1969).
13. Mitsuya, H., and S. Broder. Inhibition of the *in vitro* and cytopathic effect of human T-lymphotropic virus, type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. *Proc. Natl. Acad. Sci. USA* **83**:1911–1915 (1986).
14. Ahluwalia, G., D. A. Cooney, H. Mitsuya, A. Fridland, K. P. Flora, Z. Hao, M. Dalal, S. Broder, and D. G. Johns. Initial studies on the cellular pharmacology of 2′,3′-dideoxyinosine, an inhibitor of HIV infectivity. *Biochem. Pharmacol.* **36**:3797–3801 (1987).
15. Ford, H., Jr., C. L. Merski, and J. A. Kelley. A rapid microscale method for the determination of partition coefficients by HPLC. *J. Liquid Chromatogr.* **14**:3365–3386 (1991).
16. Hitchcock, M. J. M., K. Woods, H. De Boeck, and H.-T. Ho. Biochemical pharmacology of 2′-fluoro-2′,3′-dideoxyarabinosyladenine, an inhibitor of HIV with improved metabolic and chemical stability over 2′,3′-dideoxyadenosine. *Antiviral Chem. Chemother.* **1**:319–327 (1990).
17. Marquez, V. E., C. K.-H. Tseng, J. A. Kelley, H. Mitsuya, S. Broder, J. S. Roth, and J. S. Driscoll. 2′,3′-Dideoxy-2′-fluoro-ara-A: an acid-stable purine nucleoside active against human immunodeficiency virus (HIV). *Biochem. Pharmacol.* **36**:2719–2722 (1987).

Send reprint requests to: David G. Johns, Building 37, Room 5B22, National Cancer Institute, NIH, Bethesda, MD 20892.