0026-895X/94/051002-07$03.00/0

MOLECULAR PHARMACOLOGY, 46:1002–1008. 1994.

Enhancement by 2′-Deoxycoformycin of the 5′-Phosphorylation and Anti-Human Immunodeficiency Virus Activity of 2′,3′-Dideoxyadenosine and 2′-β-Fluoro-2′,3′-dideoxyadenosine

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Received April 7, 1994; Accepted August 9, 1994

SUMMARY

The anti-human immunodeficiency virus agents 2′,3′-dideoxyadenosine (ddAdo) and 2′-β-fluoro-2′,3′-dideoxyadenosine (2′-β-F-ddAdo) are rapidly converted, both *in vitro* and *in vivo*, to the corresponding inosine analogs by the widely distributed enzyme adenosine deaminase (EC 3.5.4.4). We have determined the effects of the potent adenosine deaminase inhibitor 2′-deoxycoformycin (2′-dCF) on ddAdo and 2′-β-F-ddAdo metabolism in MOLT-4 cells and on ddAdo antiviral activity in the ATH8 test system. At levels as low as 5 nM in the incubation medium, 2′-dCF effectively blocks the extracellular deamination of both agents, thus permitting their rapid cellular uptake as the unchanged parent compounds, rather than as the less lipid-soluble 2′,3′-dideoxyinosine or 2′-β-fluoro-2′,3′-dideoxyinosine. The result is a significant increase in intracellular levels of the pharmacologically active forms 2′,3′-dideoxyadenosine-5′-triphosphate and 2′-β-fluoro-2′,3′-dideoxyadenosine-5′-triphosphate. The effect becomes maximal over the range of 50–250 nM 2′-dCF and declines to control levels when extracellular 2′-dCF levels exceed 1 μM. This decrease in ddAdo and 2′-β-F-ddAdo phosphorylation with higher levels of the inhibitor appears to result from intracellular penetration of 2′-dCF and consequent inhibition of intracellular deamination, a critical step in the activation of both agents through the 5′-nucleotidase pathway. In anti-human immunodeficiency virus assays, a 2.2-fold increase in ddAdo antiviral potency was seen at 2′-dCF levels of 20 and 50 nM.

The anti-HIV agent ddAdo and its deamination product ddIno were shown to have potent anti-HIV activity in T cell assay systems by Mitsuya and Broder in 1986 (1). In early phase I studies with these agents a few patients received ddAdo, but subsequent clinical trials were carried out with ddIno after blood level studies revealed that ddAdo was converted to ddIno by plasma and erythrocyte adenosine deaminase within a few seconds after parenteral administration (2). In view of its relatively few toxic side effects, and, in particular, its lack of bone marrow toxicity (3, 4), ddIno rapidly gained clinical acceptance, particularly among patients who had become intolerant of or nonresponsive to AZT.

Cellular pharmacology studies revealed that both ddAdo and ddIno are converted ultimately to ddATP (5), with the latter nucleotide acting as an inhibitor of HIV reverse transcriptase and as a viral DNA chain terminator. Before the elucidation of the pathways of ddAdo and ddIno anabolism, however, early investigators attempted to increase the *in vitro* efficacy of ddAdo by blocking its deamination with the potent adenosine deaminase inhibitor 2′-dCF, thus rendering the compound available for 5′-phosphorylation by 2′-deoxycytidine kinase, an enzyme for which ddAdo is a slowly utilized alternate substrate (6). Unexpectedly, however, it was found that even a rather high level of 2′-dCF (10 μM) resulted in little net change either in the anti-HIV activity or in the level of ddATP attained in cells (7).

An indication that the effect of adenosine deaminase inhibitors on ddAdo might require re-examination was the recent observation by La Colla *et al.* (8) that an extremely low concentration of the adenosine deaminase inhibitor coformycin (up to 12,500 times lower than the maximum nontoxic dose) resulted in significant (up to 10-fold) potentiation of the anti-HIV activity of ddAdo, whereas no effect was seen on the anti-HIV activity of its deamination product ddIno. The present study was therefore carried out to ascertain the mechanism of this unusual effect and to determine whether 2′-dCF might be of value in potentiating the therapeutic usefulness of ddAdo. In addition, the study was extended to the ddAdo analog 2′-β-

ABBREVIATIONS: HIV, human immunodeficiency virus; ddAdo, 2′,3′-dideoxyadenosine; ddIno, 2′,3′-dideoxyinosine; 2′-dCF, 2′-deoxycoformycin; ddATP, 2′,3′-dideoxyadenosine-5′-triphosphate; ddIMP, 2′,3′-dideoxyinosine-5′-monophosphate; ddAMP, 2′,3′-dideoxyadenosine-5′-monophosphate; 2′-β-F-ddAdo, 2′-β-fluoro-2′,3′-dideoxyadenosine; EHNA, *erythro*-9-(2-hydroxyl-3-nonyl)adenine; IMPD, inosine monophosphate dehydrogenase; 2′-β-F-ddIno, 2′-β-fluoro-2′,3′-dideoxyinosine; ddADP, 2′,3′-dideoxyadenosine-5′-diphosphate; HPLC, high performance liquid chromatography; 2′-β-F-ddATP, 2′-β-fluoro-2′,3′-dideoxyadenosine-5′-triphosphate.

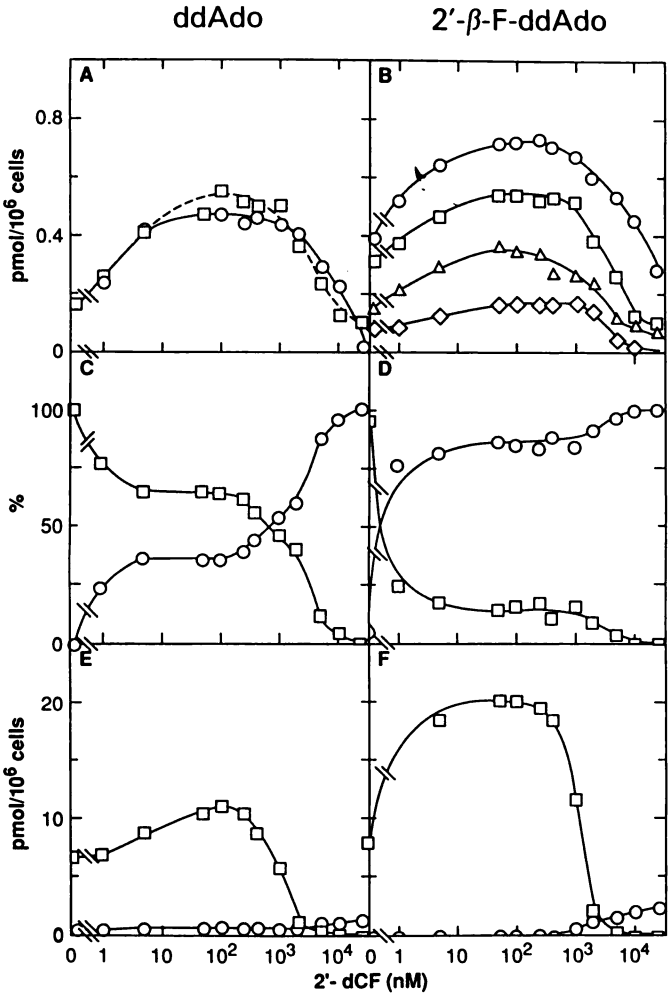

Fig. 1. Effects of 2′-dCF on the metabolism of ddAdo and 2′-β-F-ddAdo. MOLT-4 cells in logarithmic growth (10^6 cells/ml) were incubated with either ^{3}H-labeled ddAdo (*left*) or 2′-β-F-ddAdo (*right*) (5 μM, 5 μCi/ml) for 5 hr, in the presence of concentrations of 2′-dCF ranging from 1 nM to 25 μM. All incubation media contained 10% fetal bovine serum. 2′-dCF at the concentrations indicated was added to the medium 10 min before the addition of the labeled nucleosides. A and B, Intracellular levels of phosphorylated metabolites. Methanolic extracts equivalent to 5×10^6 cells were subjected to ion exchange HPLC (Partisil 10-SAX) as described in Materials and Methods. A, □, ddADP; ○, ddATP. B, ◇, 2′-β-fluoro-ddIMP; △, 2′-β-fluoro-ddAMP; □, 2′-β-fluoro-ddADP; ○, 2′-β-F-ddATP. C and D, Percentage distribution of ddAdo or 2′-β-F-ddAdo and their respective deamination products within the incubation medium. Aliquots representing 100 μl of extracellular medium were prepared as described in Materials and Methods and were subjected to reverse phase HPLC. C, ○, ddAdo; □, ddIno. D, ○, 2′-β-F-ddAdo; □, 2′-β-F-ddIno. E and F, Intracellular levels of ddAdo or 2′-β-F-ddAdo and their respective deamination products. Extracts representing 1.5×10^6 cells were prepared as described in Materials and Methods and were subjected to reverse phase HPLC. E, ○, ddAdo; □, ddIno. F, ○, 2′-β-F-ddAdo; □, 2′-β-F-ddIno. All cellular metabolism studies were repeated three times; results shown are the average of duplicate analyses from a single representative experiment.

F-ddAdo, a compound with many similarities to ddAdo but differing in having greater acid stability (9), lower substrate activity for adenosine deaminase (9), and higher affinity for direct 5′-phosphorylation by 2′-deoxycytidine kinase (10).

Materials and Methods

Chemicals. 2′-β-F-[5′-^{3}H]ddAdo (10 Ci/mmol) and [2′,3′-^{3}H] ddAdo (30 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). The percentage of the label in the 2′- and 3′-positions of the dideoxyribose moiety of the latter compound 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). ddAdo, ddIno, the IMPD inhibitor ribavirin, and the adenosine deaminase inhibitors 2′-dCF and racemic (±)-EHNA were supplied by Dr. Karl Flora, Pharmaceutical Resources Branch, Developmental Therapeutics Program, National Cancer Institute. 2′-

TABLE 1

Effect of EHNA on ddATP formation from ddAdo

MOLT-4 cells in logarithmic growth were incubated with [^{3}H]ddAdo (5 μCi/ml, 5 μM) for 6 hr, in the presence of the indicated concentrations of EHNA. Cells were collected, extracted with 60% methanol, and heated at 95° for 1.25 min, protein was removed by centrifugation, and the clarified supernatants were subjected to HPLC on a Partisil 10-SAX column, as described more fully in Materials and Methods. Each value represents the average of duplicate samples, with the individual values obtained varying by <10%. Control ddATP concentration (EHNA omitted) was 0.18 pmol/10^6 cells.

EHNA concentration	Intracellular ddATP concentration
nM	*% of control*
	100
1	100
10	135
10^2	391
10^3	391
10^4	196
10^5 (0.1 mM)	133

TABLE 2

Adenosine deaminase activity per unit volume in tissue culture medium and MOLT-4 cells

MOLT-4 cells in logarithmic growth in complete RPMI 1640 medium containing 10% heat-treated (56°, 1 hr) fetal bovine serum were collected by centrifugation, washed twice with normal saline solution, and lysed with distilled water, and the lysates were buffered to pH 7.4 with potassium phosphate. Adenosine deaminase activity per unit volume (corrected for dilution) in cells and in medium was assayed by measuring the rate of decrease in absorbance at 265 nm of the respective substrates (see Materials and Methods). Assays of cell extract enzyme were carried out in duplicate, with individual values varying by <5%. Assays of medium enzyme were carried out on two independently prepared media, with individual values varying by <10%. Intracellular enzyme activity/μl of cell volume was measured using an experimentally determined value of 1.0×10^6 MOLT-4 cells/μl of packed cells

Substrate	Enzyme activity		Cell/medium ratio
	Medium	MOLT-4 cells	
	pmol deaminated/min/μl		
Adenosine	1.46	552	378
ddAdo	1.44	508	353
2′-β-F-ddAdo	1.19	170	143

TABLE 3

Adenosine deaminase activity in MOLT-4 cells after exposure to low and high concentrations of 2′-dCF

MOLT-4 cells in logarithmic growth were incubated for 5 hr in complete RPMI 1640 medium containing either 0.2 μM or 10 μM 2′-dCF. Cells were then collected by centrifugation and washed twice with normal saline solution. The cell pellets were lysed with distilled water, and lysates were buffered with 50 mM potassium phosphate, pH 7.4, and clarified by centrifugation at 12,000 × *g* for 5 min. Adenosine deaminase activity in the cell lysates was assayed by measuring the rate of decrease in absorbance at 265 nm, with 0.1 mM adenosine as substrate (see Materials and Methods). Assays were carried out in duplicate, with individual values varying by <5%.

Addition	Intracellular adenosine deaminase activity	
		%
None	653	100
2′-dCF, 0.2 μM	517	79
2′-dCF, 10 μM	27	4

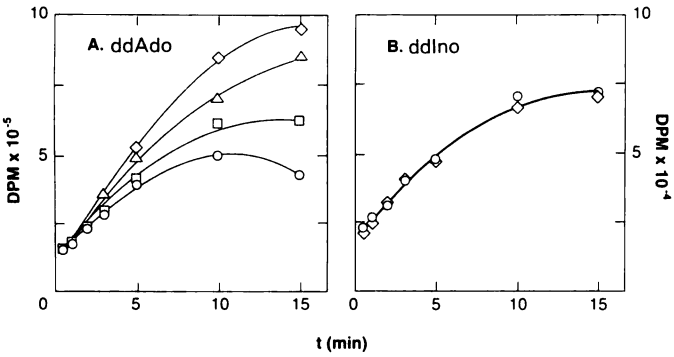

Fig. 2. Effect of 2′-dCF on the cellular uptake of ddAdo and ddIno. MOLT-4 cells in logarithmic growth were resuspended in 0.5 ml of fresh RPMI 1640 medium, containing 10% fetal bovine serum, at a concentration of 2–3 × 10^6 cells/ml (in Eppendorf tubes). After incubation for 10 min with 2′-dCF concentrations ranging from 1 to 100 nM, 5 nmol of either [^{3}H]ddAdo (A) or [^{3}H]ddIno (B) were added to each vessel (5 μCi/vessel). At the time intervals shown (1–15 min), cells were loaded onto 0.5 ml of silicone oil, separated by centrifugation, and counted as described more fully in Materials and Methods. The experiment was repeated three times, with individual values at each time point varying by <10%; results shown are the average of duplicate analyses from a single experiment. A, ○, 2′-dCF omitted; □, 1 nM 2′-dCF; △, 10 nM 2′-dCF; ◇, 100 nM 2′-dCF. B, ○, 2′-dCF omitted; ◇, 100 nM 2′-dCF. Note the 10-fold difference in ordinate scale between A (ddAdo) and B (ddIno).

β-F-ddAdo was synthesized within the Laboratory of Medicinal Chemistry by methods described previously (11). Nucleoside and nucleotide standards were purchased from Sigma or from Pharmacia (Piscataway, NJ).

Cells. MOLT-4 cells (obtained from the American Type Culture Collection, Rockville, MD) were grown at 37° in RPMI 1640 medium supplemented with 10% heat-treated (56° for 60 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. For determination of intracellular adenosine deaminase activity, MOLT-4 cells were collected by centrifugation, washed twice with normal saline solution, and lysed with distilled water (300 × 10^6 cells/ml), and the lysates were buffered to pH 7.4 with 50 mM potassium phosphate. After centrifugation at 12,000 × *g* for 3 min, the supernatants were separated and assayed as described below. For determination of adenosine deaminase activity in the medium, complete RPMI 1640 medium with 10% fetal bovine serum was assayed without dilution. To 400 μl of the medium or 25 μl of the cellular extract was added adenosine, ddAdo, or 2′-β-F-ddAdo (0.1 mM), in a total reaction volume of 1 ml. The decrease in absorbance at 265 nm was monitored at multiple time points for up to 3 hr, at room temperature. The change in extinction coefficient at the latter wavelength on complete deamination was separately determined for the three substrates. The change in absorbance per minute was calculated from the initial linear portion of the assay curves. No reaction was seen in control cuvettes to which complete reaction mixtures plus the adenosine deaminase inhibitor 2′-dCF (10 μM) had been added.

For determination of residual adenosine deaminase activity in MOLT-4 cells after exposure to low and high concentrations of 2′-dCF, MOLT-4 cells in logarithmic growth were incubated for 5 hr in complete RPMI 1640 medium containing either 0.2 μM or 10 μM 2′-dCF. Cells were then collected by centrifugation and washed twice with normal saline solution. The cell pellets were lysed with distilled water, and the lysates were buffered with 50 mM potassium phosphate, pH 7.4, and clarified by centrifugation at 12,000 × *g* for 5 min. Adenosine deaminase activity in the cell lysates was assayed by measuring the rate of decrease in absorbance at 265 nm, with 0.1 mM adenosine as substrate.

Metabolism studies. Metabolism of the dideoxynucleosides ddAdo and 2′-β-F-ddAdo was determined in exponentially growing MOLT-4 cells in the presence of varying concentrations of the adenosine deaminase inhibitors 2′-dCF and EHNA and combinations of the latter compounds with the IMPD inhibitor ribavirin. Ribavirin and other IMPD inhibitors have been reported, in previous publications from this and other laboratories, to stimulate the activation of purine dideoxynucleosides (12–16). To determine the effects of 2′-dCF on ddAdo and 2′-β-F-ddAdo metabolism, 10-ml aliquots of MOLT-4 cell suspensions (10^6 cells/ml) were preincubated for 10 min with 2′-dCF concentrations ranging from 1 nM to 25 μM or with EHNA concentrations ranging from 1 nM to 100 μM, followed by incubation with the ^{3}H-labeled dideoxynucleoside (5 μM, 5 μCi/ml). After a 5-hr incubation period, cells were centrifuged and the cell pellets were washed with 1 ml of cold normal saline solution and extracted with 0.4 ml of 60% methanol (7). The methanol extracts were heated for 1 min at 95° and, after centrifugation (2 min at 12,000 × *g*), 200 μl of the supernatant fraction were subjected to HPLC on an ion exchange Partisil 10-SAX column, utilizing an elution sequence described previously (9). One-minute fractions were collected, and radioactivity was determined by scintillation counting. For studies on the effects of the combination of ribavirin and 2′-dCF on dideoxynucleoside metabolism, cells were preincubated with ribavirin (5 μM) for 30 min before addition of 2′-dCF. All metabolism studies were repeated independently twice (occasionally three times) without significant variation being noted between different MOLT-4 cultures. Except where otherwise indicated, results shown in tables and figures represent the averages of duplicate analyses from a single representative experiment.

Uptake studies. MOLT-4 cells growing in logarithmic phase (approximately 1.0 × 10^6 cells/ml) were collected by centrifugation and the pellets were resuspended in fresh complete RPMI 1640 medium at a concentration of 2–3 × 10^6 cells/ml. Cell suspensions (0.5 ml) were added to Eppendorf polypropylene tubes containing either normal saline solution or concentrations of 2′-dCF ranging from 0.1 to 100 nM. After a 10-min incubation, 5 nmol of the ^{3}H-labeled purine dideoxynucleoside (5 μCi/vessel of [^{3}H]ddAdo or [^{3}H]ddIno) were added to the vessels. All studies were carried out at 37°. At time intervals ranging from 1 to 15 min, cells were loaded onto 0.5 ml of silicone oil (Versilube F50; GE, Waterford, NY), which had been prewarmed to 37°, in microcentrifuge tubes and were centrifuged immediately at 12,000 rpm

TABLE 4

Effect of 2′-dCF and ribavirin on formation of nucleotides of ddAdo and 2′-β-F-ddAdo

MOLT-4 cells (1 × 10^6 cells/ml) were incubated for 5 hr with 5 μM ddAdo or 2′-β-F-ddAdo (5 μCi/ml), in the presence of modulators of 5′-phosphorylation as shown. Cells were preincubated for 10 min with 2′-dCF and for 30 min with ribavirin before the addition of ddAdo or 2′-β-F-ddAdo. Methanolic cell extracts were analyzed by ion exchange HPLC, as described in Materials and Methods. Results are the average of two separate experiments, with the individual values varying by <10%.

Addition	Increase					
	ddADP	ddATP	Fluoro-ddAMP	Fluoro-ddIMP	Fluoro-ddADP	2′-β-F-ddATP
	fold					
None	1	1	1	1	1	1
2′-dCF, 0.2 μM	4.8	4.7	2.1	2.4	1.6	1.9
Ribavirin, 5 μM	1.9	1.7	8.2	4.0	2.9	3.6
2′-dCF, 0.2 μM, + ribavirin, 5 μM	11.4	10.3	13.2	6.9	5.0	5.8

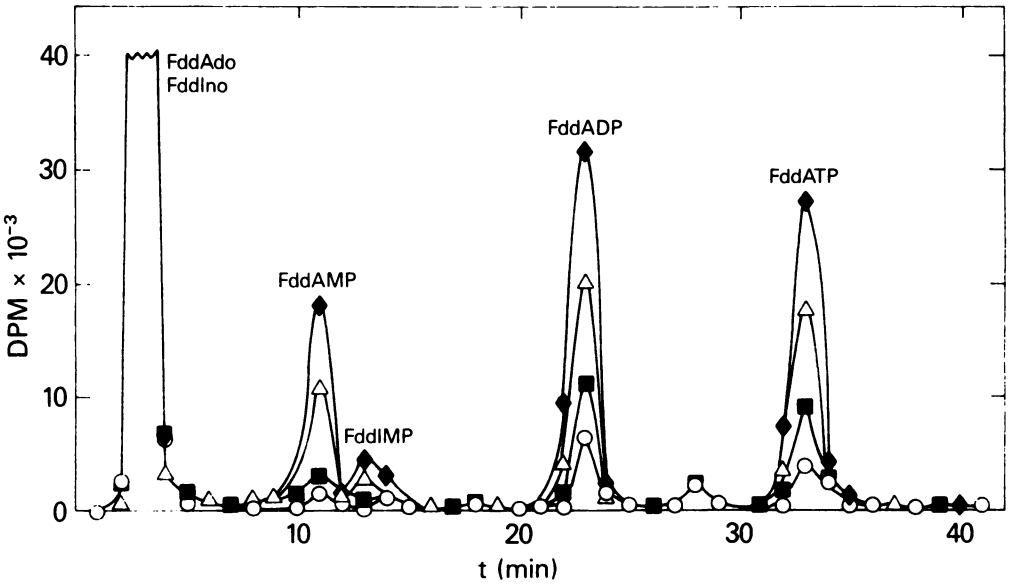

Fig. 3. Chromatographic separation of ^{3}H-labeled metabolites arising from 2′-β-F-ddAdo in the presence of 2′-dCF and/or ribavirin. MOLT-4 cells (approximately 10^6 cells/ml at time 0) were incubated with ^{3}H-labeled 2′-β-F-ddAdo (5 μM, 5 μCi/ml) for 5 hr, in the presence of 2′-dCF (200 nM) and/or ribavirin (5 μM). Cells were preincubated with 2′-dCF for 10 min and for 30 min with ribavirin before the addition of 2′-β-F-ddAdo. Methanolic extracts of an equivalent of 5×10^6 cells were subjected to ion exchange HPLC (Partisil 10-SAX), using an elution program already described (9). Representative chromatograms are shown (three or more experiments). ○, 2′-dCF and ribavirin omitted; ■, plus 200 nM 2′-dCF; △, plus 5 μM ribavirin; ♦, plus both 2′-dCF and ribavirin.

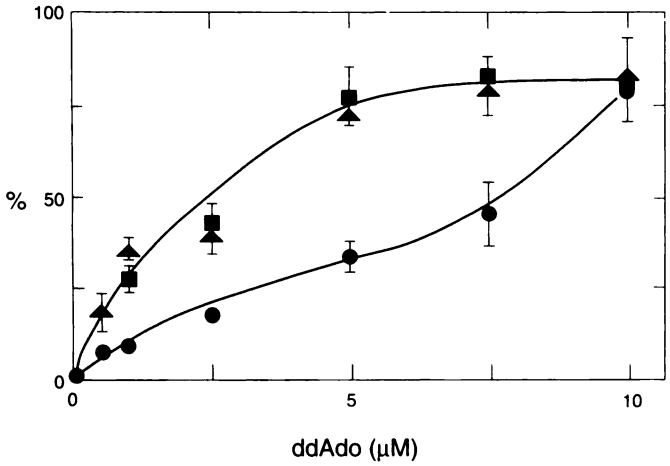

Fig. 4. Potentiation by 2′-dCF of the protection by ddAdo from the cytopathic effect of HIV-1/LAI in ATH8 cells. ATH8 cells (2×10^5), which are sensitive to the cytopathic effects of HIV, were exposed to a high multiplicity of infectious HIV-1/LAI (5.0×10^2 times the 50% tissue culture infectious dose of HIV-1/LAI). Cell suspensions were then exposed to 2′-dCF for 10 min before the addition of various concentrations of ddAdo. Uninfected cells were treated identically but were not exposed to the virus. On day 7, total viable cells were counted. *Ordinate*, percentage protection, relative to untreated uninfected cells. Control cell counts at 7 days were as follows: untreated uninfected cells (100%), 7.5×10^6/2 ml; untreated infected cells, 0.1×10^6/2 ml. ●, 2′-dCF omitted; ■, plus 20 nM 2′-dCF; ▲, plus 50 nM 2′-dCF. No cytotoxicity or anti-HIV activity was observed with 2′-dCF alone at these concentrations. Values are means ± standard deviations.

for 20 sec. Centrifuged samples were placed in powdered dry ice to freeze the upper layer of medium. The lower portion of the microcentrifuge tubes, containing the cellular pellet, was cut by means of a hot wire. Oil from above the cell pellet was drained by gentle tapping, and after vigorous agitation the pellet was digested overnight in 1 ml of 1 N NaOH; 1 ml of equimolar HCl was added to each of the digested samples and radioactivity was determined by scintillation counting.

TABLE 5

Potentiation by 2′-dCF alone and by 2′-dCF plus ribavirin of the protection by ddAdo from the cytopathic effect of HIV/LAI in ATH8 cells

ATH8 cells (2×10^5) were exposed to a high multiplicity of infectious HIV-1/LAI (5.0×10^2 times the 50% tissue culture infectious dose). Cell suspensions were then exposed to 2′-dCF and/or ribavirin for 10 min before the addition of 2.5 μM ddAdo. Uninfected cells were treated identically but were not exposed to the virus. On day 8, total viable cells were counted. Control cell counts at 8 days were as follows: uninfected control cells (100%), $5.24 \pm 0.14 \times 10^5$ cells/2 ml; untreated infected cells, 0.10×10^5 cells/2 ml. No cytotoxicity or anti-HIV activity was observed with 2′-dCF or ribavirin alone at these concentrations. Values are means ± standard deviations.

Addition	Cell number × 10^{-5}/2 ml	Protection
		% of control
ddAdo, 2.5 μM	1.41 ± 0.05	26.9
ddAdo, 2.5 μM, + 2′-dCF, 20 nM	2.28 ± 0.04	43.5
ddAdo, 2.5 μM, + ribavirin, 2.5 μM	4.15 ± 0.07	79.2
ddAdo, 2.5 μM, + ribavirin, 2.5 μM, + 2′-dCF, 20 nM	4.43 ± 0.03	84.5

Determination of purine dideoxynucleosides. The purine dideoxynucleosides ddAdo, ddIno, 2′-β-F-ddAdo, and 2′-β-F-ddIno were assayed both in the tissue culture medium and in the MOLT-4 cell extracts after treatment of cell suspensions with either ddAdo or 2′-β-F-ddAdo (5 μM, 5 μCi/ml) for 5 hr, in the presence or absence of a wide range of 2′-dCF concentrations (1 nM to 25 μM). At the termination of the 5-hr incubation period, the suspensions were centrifuged (10 min at 1500 rpm) to collect the cells. Aliquots (100 μl) of the supernatants (extracellular medium) were diluted 1/1 with methanol and heated for 1 min at 95°. After centrifugation to remove the denatured proteins, the clear supernatants were analyzed by reverse phase HPLC (see below). Cellular extracts were prepared as described above (see Metabolism studies), and 60-μl aliquots were also analyzed by reverse phase HPLC. For reverse phase HPLC analysis, a 4.6-mm × 25-cm reverse phase column was used (Microsorb-MV, C-18, 5 μm, 100 Å; Rainin Instruments, Woburn, MA). The dideoxynucleosides were eluted from the column using 0.05 M sodium acetate, adjusted to pH 5.9 with acetic acid, plus 5% acetonitrile (solvent A) and 50% acetonitrile in water

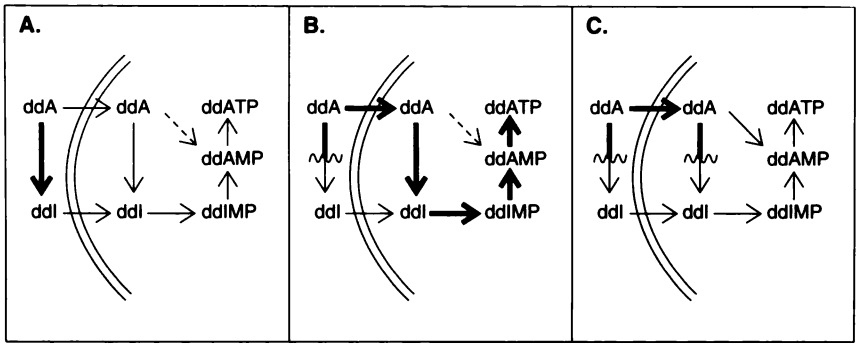

Fig. 5. Schematic diagram depicting the effects of 2′-dCF on the routes of extracellular and intracellular disposition of ddAdo. A, 2′-dCF omitted. Deamination of ddAdo is largely extracellular. B, Low 2′-dCF concentrations (<0.5 μM), producing inhibition of extracellular adenosine deaminase. Deamination of ddAdo is largely intracellular. C, High 2′-dCF concentrations (>1 μM), producing inhibition of both intracellular and extracellular deamination of ddAdo. There is increased relative significance of the direct phosphorylation route (ddAdo → ddAMP), catalyzed by 2′-deoxycytidine kinase/adenosine kinase (6, 21). *ddA*, ddAdo; *ddI*, ddIno.

(solvent B). Elution conditions were 10 min of isocratic solvent A, a linear gradient to 50% solvent A/50% solvent B over 15 min, isocratic 50% solvent A/50% solvent B for 3 min, a 2-min reverse gradient to 100% solvent A, and equilibration with 100% solvent A for 10 min before the next injection. The flow rate was 2 ml/min. Nonradiolabeled purine dideoxynucleoside standards were used to determine retention times. For extracellular medium analysis, the column eluate was monitored with an in-line Radiomatic radioactivity flow detector (Packard Instruments, Meriden, CT) to quantitate the radiolabeled peaks. For analysis of cellular extracts, 1-min fractions were collected and radioactivity was determined by scintillation counting.

Assay for anti-HIV activity. The assay method used was that described by Mitsuya and Broder (1). 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 (5.0×10^2 times the 50% tissue culture infectious dose). Cell suspensions (2 ml) were then exposed to 20 nM or 50 nM 2′-dCF for 10 min before addition of concentrations of ddAdo ranging from 0.5 to 10 μM. Uninfected cells were treated identically but were not exposed to the virus. On day 7 in culture, total viable cells were counted for quantitation of cytopathic effects.

For studies of potential additive effects of 2′-dCF and ribavirin, essentially the same protocol was followed. Suboptimal levels of ddAdo (2.5 μM), 2′-dCF (20 nM), and ribavirin (2.5 μM) were intentionally used to demonstrate possible additivity. Total viable cells were counted after 8 days of incubation.

Results

Effect of 2′-dCF on the phosphorylation of ddAdo and 2′-β-F-ddAdo. The effects of 2′-dCF on the phosphorylation of ddAdo and 2′-β-F-ddAdo were first examined. As shown in Fig. 1, A and B, low concentrations of 2′-dCF stimulated 5′-phosphate formation for both compounds, with maximum phosphorylation being reached at 50 nM 2′-dCF and with the effect remaining maximal up to 250 nM (0.25 μM). Phosphorylation then decreased steadily with increasing 2′-dCF concentrations until it fell to base-line levels at approximately 10 μM 2′-dCF, resulting in "bell-shaped" curves for phosphorylation when the 2′-dCF concentration was plotted on a logarithmic scale (Fig. 1, A and B). All of the measurable phosphorylated forms of the two drugs showed similar patterns (i.e., ddADP and ddATP for ddAdo and 2′-β-fluoro-ddIMP, 2′-β-fluoro-ddAMP, 2′-β-fluoro-ddADP, and 2′-β-F-ddATP for 2′-β-F-ddAdo). The effect was most marked with ddAdo, with ddADP and ddATP exceeding 300% of control levels over the 2′-dCF range of 50–250 nM. ddIMP and ddAMP could not be quantitated by this chromatographic method because the peaks were obscured by radiolabeled cleavage products of ddAdo (7).

Similar results were obtained with the adenosine deaminase inhibitor EHNA, with maximum enhancement of ddATP formation (341%) being observed at relatively high EHNA concentrations (0.1–1.0 μM) (Table 1), a result compatible with the somewhat lower potency of this agent as an adenosine deaminase inhibitor. EHNA also stimulated 2′-β-F-ddAdo phosphorylation (data not shown), although, as with 2′-dCF, the effect was slightly smaller with 2′-β-F-ddAdo than with ddAdo.

Extracellular and intracellular levels of purine dideoxynucleosides after exposure to increasing concentrations of 2′-dCF. To determine the relationship between 2′-dCF stimulation of ddAdo or 2′-β-F-ddAdo phosphorylation and the activity of 2′-dCF as an inhibitor of the deamination of these compounds at the nucleoside level, we next examined the levels of the parent and corresponding deaminated dideoxynucleosides in the extracellular medium and in the cytosol after 5 hr of exposure to the same range of 2′-dCF concentrations as used to study intracellular phosphorylation (1 nM to 25 μM). The heat-treated (56°, 1 hr) bovine serum present at 10% concentration in the tissue culture medium retained sufficient residual adenosine deaminase activity to deaminate both ddAdo and its 2′-fluoro analog (Table 2). In the absence of an adenosine deaminase inhibitor, as shown in Fig. 1, C and D, most of the extracellular ddAdo and 2′-β-F-ddAdo (100% and 96%, respectively) was recovered in the form of ddIno and 2′-β-F-ddIno at the termination of the 5-hr incubation period. Concentrations of 2′-dCF as low as 1 nM, however, significantly protected the parent compounds from deamination in the external medium (Fig. 1, C and D). The 2′-dCF protection of 2′-β-F-ddAdo from enzymic deamination was somewhat greater than the protection seen for ddAdo. For example, 1 nM 2′-dCF gave almost 75% protection for 2′-β-F-ddAdo but only 25% protection for ddAdo. A similar differential effectiveness was seen throughout, except at the highest 2′-dCF concentrations studied (>1 μM), which is likely a reflection of the greater activity of ddAdo as a substrate for adenosine deaminase (9).

Intracellularly, both ddAdo and 2′-β-F-ddAdo underwent rapid deamination (Fig. 1, E and F). Because 2′-dCF penetrates cells very slowly (17), intracellular adenosine deaminase did not appear to be significantly inhibited by low (nanomolar) levels of the inhibitor in the extracellular medium over this short time period (5 hr). However, at a higher (micromolar) level, a significant effect on intracellular deamination could be seen; for example, 1 μM 2′-dCF in the incubation medium was sufficient to bring about a 50% decrease in the intracellular deamination of both ddAdo and 2′-β-F-ddAdo (Fig. 1, E and F). When this point was examined experimentally, it was found that, whereas 10 μM 2′-dCF brought about 94% inhibition of intracellular adenosine deaminase after a 5-hr incubation, 200

nM 2′-dCF was sufficient only to result in 21% inhibition of the intracellular enzyme over the same time period (Table 3).

Effect of 2′-dCF on the uptake of ddAdo and ddIno by MOLT-4 cells. We have previously noted that ddIno is very slowly transported into MOLT-4 cells when its rate of entry is compared with that of its parent compound ddAdo (5). Neither compound appears to utilize the purine transport carrier (5), and the difference in rate of entry appears to depend on the greater lipid solubility of ddAdo, relative to that of ddIno (log *P* of -0.287 ± 0.005 for ddAdo versus -1.242 ± 0.028 for ddIno) (18). It therefore appeared possible that protection of ddAdo from deamination in the extracellular medium would enhance its uptake by slowing its conversion to its more slowly transported metabolite. As shown in Fig. 2A, there was a dose-dependent increase in the rate of ddAdo entry over a 2′-dCF concentration range of 1–100 nM. On the other hand, 2′-dCF had no effect on the entry of the deamination product ddIno (Fig. 2B).

Effect of the IMPD inhibitor ribavirin on the 2′-dCF-enhanced phosphorylation of ddAdo and 2′-β-F-ddAdo. In view of the previous observations by ourselves and others that agents that increase intracellular IMP levels, such as the IMPD inhibitor ribavirin, enhance the phosphorylation of ddAdo and 2-β-F-ddAdo (15, 16, 19), we next determined whether ribavirin enhancement could still be detected in cells treated with 2′-dCF, i.e., whether the ribavirin stimulation effect and the 2′-dCF stimulation effect occurred independently of each other. As shown in Table 4 and Fig. 3, treatment with an optimal level of 2′-dCF (200 nM) and a half-maximal level of ribavirin (5 μM) appeared to result in an additive stimulation both of ddATP formation from ddAdo (10.3-fold) and of 2′-β-F-ddATP formation from 2′-β-F-ddAdo (5.8-fold). Wider ranges of dose levels and additional 2′-dCF/IMPD inhibitor combinations, although of potential therapeutic interest, were not explored in the course of the present investigation.

Effect of 2′-dCF on the anti-HIV effectiveness of ddAdo. It is generally accepted that dideoxynucleosides such as ddAdo and 2′-β-F-ddAdo exert their anti-HIV activity after anabolism to their 5′-triphosphates, which act as inhibitors of HIV reverse transcriptase and chain terminators of viral DNA synthesis. To determine whether the increased levels of ddATP seen in the presence of low levels of 2′-dCF would enhance the antiviral potency of ddAdo, we assessed the anti-HIV activity of this dideoxynucleoside in the ATH8 assay system in the presence of low levels of 2′-dCF (20 and 50 nM). A wide range of ddAdo concentrations was tested, from a low suboptimal level (0.5 μM) to a level giving nearly complete protection in this assay system (10 μM). As shown in Fig. 4, the protective effect of ddAdo against the cytopathic effect of HIV-1 on ATH8 cells was increased almost equally by the two 2′-dCF concentrations tested, with potentiation being noted at all ddAdo concentrations except the highest (10 μM), where the anti-HIV effect of ddAdo was maximal even in the absence of 2′-dCF. The greatest increase in protection (2.2-fold) was noted at a 5 μM concentration of ddAdo. At the two extremely low concentrations used (20 and 50 nM), 2′-dCF alone showed no cytotoxicity or anti-HIV activity in this assay system.

As would be anticipated from the biochemical data (Table 4), the addition of a low level of ribavirin (2.5 μM) brought about a greater increase in anti-HIV activity of ddAdo than was seen with 2′-dCF alone (from 43.5% protection with 20 nM 2′-dCF alone to 84.5% protection with 2′-dCF plus ribavirin) (Table 5).

Discussion

The anti-HIV activity of ddAdo has been known since 1986 (1), whereas the ability of the compound to act as a substrate for adenosine deaminase had been reported some years earlier by Plunkett and Cohen (20). In view of the availability of potent adenosine deaminase inhibitors such as 2′-dCF, it was logical to determine whether inhibition of the deamination of ddAdo would divert more of the compound into the anabolic pathway (i.e., formation of ddATP) and thus result in greater antiviral activity. Unexpectedly, however, it was found that 2′-dCF, at the high concentration used in these early studies (10 μM), caused very little change in either the metabolism or the antiviral activity of the drug (7).

Shortly thereafter, when the metabolic pathways utilized by ddAdo became more fully understood, it was realized that the initial conversion of ddAdo to ddIno, either inside or outside the cell, was an important first step in the metabolism of the compound, with ddIno then being 5′-monophosphorylated intracellularly by one or more 5′-nucleotidases and the product being reconverted to ddAMP via the adenylosuccinate synthetase/lyase system (5, 21). It thus became understandable that 2′-dCF, by slowing the conversion of ddAdo to ddIno, would fail to enhance the activity of the former compound. On the other hand, 2′-dCF did not decrease the activity, because, given the very long duration of drug exposure in the ATH8 assay (7 days), sufficient ddIno (and thence ddATP) could still be generated from ddAdo (although more slowly) to exhibit antiviral activity, even in the presence of an adenosine deaminase inhibitor. In addition, a second, although quantitatively less important, pathway appeared to exist for direct, adenosine deaminase-independent, 5′-phosphorylation of ddAdo and 2′-β-F-ddAdo by 2′-deoxycytidine kinase and possibly adenosine kinase (see Refs. 4 and 9 for more detailed descriptions of ddAdo and 2′-β-F-ddAdo metabolic pathways).

An indication that the effects of 2′-dCF and EHNA might require re-examination was, however, the unexpected recent observation of La Colla *et al.* (8) that an extremely low concentration of the adenosine deaminase inhibitor coformycin (up to 12,500 times lower than the maximum nontoxic dose) resulted in up to 10-fold potentiation of the anti-HIV activity of ddAdo, whereas no effect was seen on the anti-HIV activity of ddIno. The present studies have served to confirm these observations and, furthermore, to offer an explanation (depicted schematically in Fig. 5) of this apparently paradoxical effect, i.e., that extremely low levels of 2′-dCF act by blocking the extracellular deamination of ddAdo to ddIno (thus permitting rapid cellular entry of the more lipid-soluble parent compound) but, because of the extremely slow cellular uptake of 2′-dCF (17), fail to inhibit intracellular adenosine deaminase in a sufficiently timely fashion to block the activation of ddAdo by the ddAdo → ddIno → ddIMP → ddAMP → ddATP pathway. Conversely, however, relatively high levels of 2′-dCF (>1 μM) result in sufficiently high intracellular concentrations to slow the intracellular reaction ddAdo → ddIno, so that the advantage gained by blocking solely extracellular deamination with low levels of 2′-dCF is lost. Experimental confirmation of this inability of low levels of 2′-dCF to block intracellular adenosine deaminase is presented in Table 3.

Although combination with low doses of 2′-dCF could result in a lowering of the clinical dose of ddAdo (by reducing the concentration of drug required to yield a maximal antiviral effect), it would not automatically follow that such a combination would be of practical benefit. Balanced against the reduction of ddAdo therapeutic dose would be the possible disadvantages of chronic administration of even low doses of 2′-dCF, a compound not without toxicity at the higher doses currently used clinically (22); no changes in the intracellular levels of dATP or other deoxynucleoside triphosphates were noted, however, after short term (5-hr) exposure of MOLT-4 cells to 2′-dCF over the concentration range of 1 nM to 1 μM, as used in the current studies (data not shown). Of greater possible interest would be the combination of 2′-β-F-ddAdo plus 2′-dCF or 2′-β-F-ddAdo plus 2′-dCF plus ribavirin, in view of the greater cost of synthesis of the 2′-β-fluoro analog and hence the potential economic advantage of a reduction in its clinical dosage. Exploration of this possibility will necessarily await information on the clinical properties of 2′-β-F-ddAdo as an anti-HIV agent.

Acknowledgments

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

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