Pergamon

Biochemical Pharmacology, Vol. 48, No. 7, pp. 1477–1481, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00283-5

EFFECT OF ANTI-HIV 2′-β-FLUORO-2′,3′-DIDEOXYNUCLEOSIDE ANALOGS ON THE CELLULAR CONTENT OF MITOCHONDRIAL DNA AND ON LACTATE PRODUCTION

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(*Received* 20 *January* 1994; *accepted* 12 *May* 1994)

Abstract—Many dideoxynucleosides that are effective against human immunodeficiency virus (HIV) also are potent inhibitors of mitochondrial DNA (mtDNA) synthesis, and the resulting mtDNA decrease could be responsible for the delayed clinical toxicity sometimes observed with these drugs. The following compounds have been examined for their toxicity to human lymphoid CEM cells, and their ability to suppress mtDNA content: 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyadenosine (ddA), 2′,3′-dideoxyinosine (ddI) and 2′,3′-dideoxyguanosine (ddG); and their 2′-β-fluoro analogs; β-F-ddC, β-F-ddA, β-F-ddI and β-F-ddG. Two other fluoro analogs, 5-F-ddC and 2′-β,5-di-F-ddC were also examined. The ratio of C-IC_{50} (concentration that inhibited cell growth by 50%) to mt-IC_{50} (concentration that inhibited mtDNA synthesis by 50%) was determined for each compound. The rank-order of this ratio was ddC > 5-F-ddC ⩾ ddA > ddI > ddG > β-F-ddC > β-F-ddA > β-F-ddG with the highest ratios indicating the greatest potential for delayed toxicity. In comparison with ddC, β-F-ddC and β-F-ddA were 5,000 and 22,000 times less potent, respectively, in suppressing cellular mtDNA content, while their anti-HIV potencies were decreased only modestly relative to their unfluorinated parent compounds. β-F-ddI and 2′-β,5-di-F-ddC produced neither cellular toxicity nor mtDNA suppression at concentrations of 500 and 1000 μM, respectively. Lactic acid, the product of compensatory glycolysis that results from the inhibition of mitochondrial oxidative phosphorylation, was measured after cells were treated with these compounds. There appears to be a concentration-related correlation between the increase of lactic acid and the extent of mtDNA inhibition for the compounds examined.

Key words: anti HIV nucleoside; mitochondria DNA; lactate

2′,3′-Dideoxynucleoside analogs such as AZT§, ddC and ddI have been found to have activity against HIV, both in cell culture and in patients with AIDS [1–5]. Upon long-term treatment with these anti-HIV nucleosides, many patients develop delayed toxicity such as bone marrow suppression in the case of AZT, peripheral neuropathy in the case of ddC, and both pancreatitis and peripheral neuropathy in the case of ddI. These delayed toxicities frequently limit the clinical usefulness of the compounds. The mechanism(s) responsible for these delayed toxicities is not clear. In view of the vital role of mitochondrial metabolism, it was hypothesized that a decrease in mtDNA, and therefore in mitochondrial function in target organs, could be responsible [6–8]. A positive correlation between effect of compounds on mtDNA content and limiting delayed toxicity of clinically useful anti-HIV nucleoside analogs was observed [9]. It is possible that new antiviral nucleosides, which do not deplete cellular mtDNA content, may not have this delayed adverse effect in clinical use. During the past several years, a number of new ddN analogs with anti-HIV activity have been synthesized. Among them, 2′-β-fluoro-2′,3′-dideoxynucleoside analogs (β-F-ddNs) were found to be approximately equal in potency to their parental dideoxynucleoside analogs in ATH8 cells [10–15]. Since β-F-ddC was found to be much less potent than ddC in decreasing the mtDNA content of treated cells [16, 17], an examination of whether other β-F-ddNs have similar anti-mtDNA profiles is also of interest. In addition, it was noted that once mtDNA content is decreased in ddC-treated cells, the production of a glycolysis product, lactate, increases [9]. This increase could be due to the compensatory increase in glycolysis as a result of the decrease in oxidative phosphorylation. Because some nucleoside analogs or their metabolites may also alter glycolysis and oxidative phosphorylation *independent* of the effect on mtDNA

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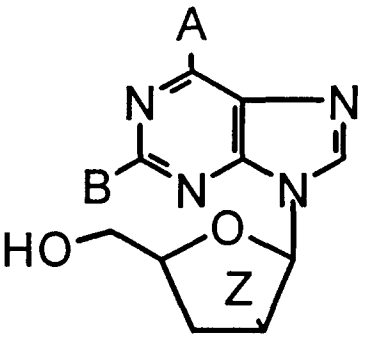
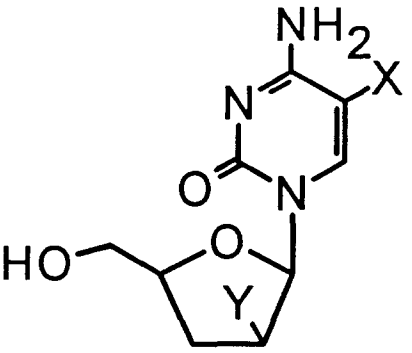
§ Abbreviations: AZT, azidothymidine; ddC, 2′,3′-dideoxycytidine; ddI, 2′,3′-dideoxyinosine; ddA, 2′,3′-dideoxyadenosine; ddG, 2′,3′-dideoxyguanosine; ddN, dideoxynucleoside; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; mtDNA, mitochondrial DNA; C-IC_{50}, concentration that inhibited cell growth by 50%; mt-IC_{50}, concentration that inhibited mtDNA synthesis by 50%.



Compound	A	B	Z
ddA	NH_2	H	H
ddI	OH	H	H
ddG	OH	NH_2	H
β-F-ddA	NH_2	H	F
β-F-ddI	OH	H	F
β-F-ddG	OH	NH_2	F



Compound	X	Y
ddC	H	H
β-F-ddC	H	F
5-F-ddC	F	H
2′-β,5-diF-ddC	F	F

Fig. 1. Nucleoside structures.

synthesis, and lactate production could be a parameter for the measurement of impairment of overall mitochondrial function, the effect of β-F-ddNs and their parent compounds on lactate production was also examined to determine (a) whether there is a correlation between the degree of decrease in mtDNA content and the amount of lactate produced for a given compound, and (b) whether different compounds having the same impact on mtDNA content would increase lactate production from cells to the same extent.

In this paper, the effects of several β-F-ddNs analogs (Fig. 1) on mtDNA content and lactic acid production are described.

MATERIALS AND METHODS

Compounds. All the fluoro-containing compounds were synthesized at the National Cancer Institute [10, 18–22]. Non-fluorinated ddNs were commercially available. All nucleoside analogs were dissolved in phosphate-buffered saline except for ddG and β-F-ddG, which were dissolved in DMSO.

In vitro *assay for cellular toxicity (C-IC_{50}).* CEM cells, a human T lymphoblastoid cell line, were suspended at a concentration of 2×10^4 cells in 5 mL of RPMI 1640 medium supplemented with 5% fetal bovine serum and 100 μg/mL of kanamycin. The cells were treated with at least four different concentrations of compounds for 4 days. At the end of this time, cells were counted using a Coulter counter. The C-IC_{50} of the compounds was determined by plotting various drug concentrations versus percentage of viable cells. The assay was done in triplicate and was performed at least twice. Cells (1×10^5) were harvested to measure their mtDNA content, and the lactic acid content of 50 μL supernatant was determined.

Measurement of mtDNA content by a slot blot method. CEM cell pellets were resuspended in 10 mM buffer (Tris–HCl, pH 7.5) and were freeze–thawed three times. The cell lysates were incubated with RNase (10 μg/mL) at 37° for 1 hr. Then the samples were treated with proteinase K (100 μg/mL) at 55° for 3 hr. An equal volume of 20× SSC (3 M NaCl and 0.3 M sodium citrate) was added to the samples, and the samples were boiled for 10 min and immobilized on Hybond paper using a slot blot apparatus (Schleicher & Schuell, Keene, NH). The mtDNA on the Hybond paper was detected with a human mtDNA probe as described previously [17].

Determination of lactic acid production from cells. Supernatants (50 μL) from compound-treated cells were assayed for lactic acid production by using a kit from Boehringer (Catalog No. 139084). Triplicate results were averaged and are given as percent lactic acid production relative to that of the control cells.

RESULTS AND DISCUSSION

It has been noted previously that CEM 4-day cell growth is not affected if only mtDNA synthesis is inhibited [16]. Those compounds that show strong cell growth inhibitory effects at concentrations equal to or lower than the concentration having an inhibitory effect on CEM mtDNA synthesis (C-IC_{50}/mt-$IC_{50} < 1$) usually have limiting toxicity against proliferating tissues. In contrast, those compounds having anti-mtDNA synthesis effects much stronger than anti-cell growth after 4 days (ratio ≥ 1) exhibit delayed organ toxicity upon long-term usage. To assess whether certain fluorine-containing analogs (Fig. 1) have the potential to cause delayed toxicity in the absence of toxicity in proliferating tissues, 2′-β-F-dideoxynucleosides as well as their parental ddNs were examined for their effectiveness in

Table 1. Effects of various dideoxynucleoside analogues on mtDNA synthesis and cell growth

Compound	C-IC_{50} (μM)	mt-IC_{50} (μM)	C-IC_{50}/mt-IC_{50}	HIV EC_{50}* (μM)
ddC	10 ± 0.5	0.022 ± 0.02	450	0.2†
5-F-ddC	110 ± 45	0.56 ± 0.32	200	0.2†
β-F-ddC	82 ± 5	124 ± 16	0.66	1‡
2′-β,5-di-F-ddC	>1000	>1000	ND§	5‡
ddA	670 ± 78	75 ± 25	8.9	6†
β-F-ddA	178 ± 22	>500	<0.36	10‖
ddG	10.5 ± 1.5	13 ± 3.0	0.81	7†
β-F-ddG	1.6 ± 0.8	20 ± 4.5	0.08	20¶
ddI	>1000	290 ± 120	>3.5	8†
β-F-ddI	>500	>500	ND**	10‖

Values are the means ± SD of at least three determinations.

* Concentration affording 50% protection against the cytopathogenic effects of HIV-1 in ATH8 cells.

† Ref. 23.

‡ Ref. 21.

§ This value could not be determined at 1000 μM.

‖ Ref. 18.

¶ Ref. 22.

** This value could not be determined at 500 μM.

inhibiting mtDNA synthesis as well as 4-day cell growth (Table 1). All the non-fluorinated dideoxynucleosides with the exception of ddG had a more pronounced effect in decreasing mtDNA content than in decreasing cell growth. ddG had similar C-IC_{50} (10.5 ± 1.5 μM) and mt-IC_{50} (13 ± 3.0 μM) values, indicating an equal potency of ddG in decreasing mtDNA content and in inhibiting cell growth. 2′-β-Fluoro substitution, however, reversed this situation. Much smaller decreases in mtDNA content were found using the 2′-β-fluoro analogues. In the case of ddC, the substitution of a 2′-β-hydrogen atom by fluorine decreased the potency for reducing mtDNA content by >5000-fold, accompanied by an 8-fold decrease in cellular toxicity (Table 1). Based on this observation, it is anticipated that β-F-ddC may have a different limiting toxicity from that of ddC, and a dose of β-F-ddC that does not show toxicity to proliferating tissue might not cause the delayed toxicity seen in patients treated with ddC, ddA and ddI [15]. When compared with ddC, β-F-ddA was >22,000 times less toxic towards mtDNA suppression with a 17-fold decrease in cellular toxicity and a 50-fold decrease in anti-HIV potency in the ATH8 system (Table 1). Having both the 2′- and 5′-positions substituted by fluorine resulted in a compound (2′-β,5-di-F-ddC) that did not affect either cell growth or mtDNA content. It should be noted that the inhibition of cell growth is not, by itself, sufficient to decrease mtDNA content. For example, arabinosyl cytosine (ara-C) at a cytotoxic dosage does not decrease mtDNA content [9].

According to our results, the C-IC_{50}/mt-IC_{50} toxicity ratios of the compounds evaluated were ranked as follows: ddC > 5-F-ddC ⩾ ddA > ddI > ddG > β-F-ddC > β-F-ddA > β-F-ddG (Table 1). The larger this ratio, the more potent mitochondrial damage (and delayed cytotoxicity) is relative to general cellular cytotoxicity. Note that the β-F-ddI and 2′-β,5-di-F-ddC did not have any effect on either cell growth or mtDNA content at the highest concentrations studied in this assay (500 and 1000 μM, respectively).

It has been demonstrated that β-F-ddCTP is less potent than ddCTP against DNA polymerase γ [14]. This enzyme is the major DNA polymerase involved in mtDNA synthesis [24, 25]. In view of the fact that β-F-ddA is less potent than ddA in inhibiting mtDNA synthesis, the active metabolites, β-F-ddATP and ddATP, were examined directly for their inhibitory activity against DNA polymerase γ. The K_i of ddATP to γ-DNA polymerase was 0.1 μM, whereas the K_i of β-F-ddATP was more than 200 μM (data not shown). This finding was consistent with their relative inhibitory activities for mtDNA synthesis (Table 1), and suggests that the different potencies of ddA and β-F-ddA on mtDNA synthesis could be related to the abilities of their respective dideoxynucleotide metabolites to inhibit γ-DNA polymerase.

Based on this observation, as well as the reports of others [5, 7, 26], it can be concluded that the substitution of the 2′-β hydrogen of a dideoxynucleoside triphosphate by a fluorine atom diminishes the ability of the compound to inhibit γ-DNA polymerase. It should be noted that the DNA content and function of mitochondria in different tissues may be variable, i.e. the effect of mtDNA inhibition may have different consequences in different tissues. Also, other factors, such as the metabolism of the dideoxynucleoside compounds, the stability of mtDNA, and the importance of mitochondria to organ function, could influence the amount of damage done to the organs by those compounds [27, 28].

The effects of these dideoxynucleoside analogs on cellular lactate production also were examined, because when mitochondrial oxidation is inhibited, a compensatory lactic acid-producing glycolysis pathway is initiated. These results are shown in Table 2. At a concentration where cell growth was not inhibited but cellular mtDNA content was

Table 2. Lactic acid production in CEM cells treated with dideoxynucleoside analogs

Compound	ddN Concentration (μM)						
	0.2	2	5	20	200	500	1000
	Lactic acid* (%)						
ddC	119 ± 5	136 ± 17	167 ± 4				
5-F-ddC	105 ± 7	125 ± 4		163 ± 16			
β-F-ddC		127 ± 2		108 ± 2	201 ± 49		
2′-β,5-di-F-ddC				90 ± 4	87 ± 10		102 ± 8
ddA					113 ± 3	155 ± 5	708 ± 38
β-F-ddA				108 ± 3	139 ± 2	321 ± 18	
ddG		117 ± 7	110 ± 3		282 ± 5		
β-F-ddG	118 ± 3	153 ± 10	285 ± 33				
ddI				112 ± 2	150 ± 5		173 ± 23
β-F-ddI		95 ± 5		105 ± 5	118 ± 9		

* Normalized against the amount of lactic acid production from non-drug-treated cells. Control lactate level (100%) was measured as 0.61 ± 0.06 mg/10^6 cells. No effect was noted for any compound at 0.05 μM. Values are the means ± SD of at least three determinations.

decreased, there was a concentration-dependent increase in lactate production. However, the concentration that caused an increase in lactate production was not the same concentration that gave a comparable effect on mtDNA content. For example, ddC at 10 times its mt-IC_{50} concentration increased lactate production to the same degree as 5-F-ddC at approximately two times its mt-IC_{50}. This is not unexpected since these analogs or their metabolites could have more than one mechanism of action interfering with the oxidative phosphorylation pathway. Many enzyme reactions involving nucleotides either as cofactors or as regulators could also be affected.

Based on the relationship of C-IC_{50}/mt-IC_{50} and lactic acid production, the compounds can be divided into three groups. The first group, with C-IC_{50}/mt-IC_{50} ratios > 100, had lower values of mt-IC_{50} with higher concentrations (compared with their mt-IC_{50}) required to induce cells to generate large amounts of lactic acid. This group, which contains ddC and 5-F-ddC, consists of compounds most likely to produce delayed toxicity. The second group of compounds possessed a ratio <10. In this group, a concentration approximating the mt-IC_{50} caused cells to produce significantly more lactic acid. ddA, ddI, ddG, β-F-ddC, β-F-ddA and β-F-ddG belong to this group. The third group of compounds (2′-β,5-di-F-ddC and β-F-ddI) did not affect either cell growth or mtDNA content, and the production of lactic acid could not be made to increase significantly. The reason lactic acid production is slow to respond to mtDNA synthesis inhibition may be because the energy compensation occurs only after most of the mtDNA has been depleted [29].

In conclusion, we examined the ratio of C-IC_{50}/mt-IC_{50} for various 2′,3′-dideoxynucleoside compounds. This ratio might be useful in predicting whether the limiting clinical toxicity of these compounds might be proliferating tissue toxicity or some type of delayed toxicity. The substitution of the 2′-β-hydrogen in a dideoxynucleoside by a fluorine atom results in a decreased potential for reducing mtDNA content. This appears to be related to a decrease in the triphosphate metabolite inhibitory activity of F-ddNs against γ-DNA polymerase. A concentration-dependent increase in lactic acid production occurred most strikingly with the compounds most effective in decreasing mtDNA synthesis. However, there was not a strictly quantitative correlation between the potencies for mtDNA inhibition and lactic acid production. A possible role for the effects of nucleoside analogs on mitochondrial functions rather than mtDNA synthesis is suggested.

Acknowledgement—This work was supported, in part, by Grant CA44358 from the U.S. Public Health Service.

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