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RAPID COMMUNICATION

STUDIES ON THE INHIBITION OF MITOCHONDRIAL DNA REPLICATION BY 3'-AZIDO-3'-DEOXYTHYMIDINE AND OTHER DIDEOXYNUCLEOSIDE ANALOGS WHICH INHIBIT HIV-1 REPLICATION

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AZT§, a 3'-azido analog of a dideoxynucleoside, is a potent inhibitor of HIV-1 replication in vitro [1] and is being used in AIDS therapy [2,3]. Many nucleoside analogs which lack a 3'-OH group inhibit the infectivity and cytopathic effect of retroviruses [4] including HIV-1 [1]. These effects occur presumably as a consequence of the incorporation of such compounds into the viral DNA; dd-nucleosides after phosphorylation can become incorporated into DNA which leads to premature termination of the elongating DNA chain [5] and inhibition of DNA replication since there is no attachment site for the formation of the next 3',5'-phosphodiester bond. Under these circumstances, it is not the enzyme but rather the elongating daughter strand template which is being inactivated; thus, the better a DNA polymerase utilizes such a substrate, the greater will be the inhibition of DNA replication. It seems clear that the effectiveness of AZT stems from the ability of its triphosphate to be utilized efficiently by the HIV reverse transcriptase [6-9] whereas, in common with three other previously studied DNA chain terminators, namely ddGTP, ddTTP, and ddCTP [10,11], this substrate is not well utilized by DNA polymerase α, making nuclear DNA replication resistant to the drug.

The therapeutic usefulness of AZT is limited by its toxic effects, particularly that of bone marrow depression [12], and this is reinforced by its toxicity to normal human hemopoietic progenitor cells in an in vitro model [13]. DNA polymerase γ, a mitochondrial enzyme initially isolated in one of our laboratories [14,15] and which is responsible for mtDNA replication [16,17], is known to avidly utilize ddNTPs [10,11], and ddCTP has in fact been shown to inhibit mtDNA replication [11]. It is therefore not an unreasonable hypothesis that the toxic effects of AZT result from its inhibition of mtDNA replication in bone marrow cells. Even a small effect of this kind could, through several cell cycles, lead to a diminishing cellular content of mitochondria until ATP or some other critical metabolite becomes limiting for cell growth or viability. The primary thrust of our initial studies, here reported, was to ask whether AZT can in fact inhibit mtDNA replication. In addition, we wished to learn whether other potential chain terminators could affect this process. For this purpose, we selected a group of eleven dd-nucleoside compounds all of which had been shown previously to possess active anti-HIV activity or to actively inhibit DNA replication in the reverse transcriptase assay system [1, 18-25, and unpublished observations].

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§Abbreviations: dd, dideoxy; AZT, 3'-azido-3'-deoxythymidine; AZTTP, 3'-azido-3'-deoxythymidine triphosphate; dNTP, deoxynucleoside triphosphate; d2C, dideoxycytidine; N_3-IUDR, 3'-azido-2',3'-dideoxy-5-iodouridine; N_3-BUdR, 3'-azido-2',3'-dideoxy-5-bromouridine; An-N_3-IUdR, 2,5'-anhydro-3'-azido-2',3'-dideoxy-5-iodouridine; An-N_3-BUdR, 2,5'-anhydro-3'-azido-2',3'-dideoxy-5-bromouridine; N_3-UdR, 3'-azido-2',3'-dideoxyuridine; d4T, 3'-deoxythymidin-2'-ene; An-N_3-TdR, 2,5'-anhydro-3'-azido-3'-deoxythymidine; 5-Me-d4C, 2',3'-dideoxy-5-methylcytidin-2'-ene; An-N_3-UdR, 2,5'-anhydro-3'-azido-2',3'-dideoxyuridine; d4C, 2',3'-dideoxycytidin-2'-ene; dThd, deoxythymidine; IdUrd, iododeoxyuridine; AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; and mtDNA, mitochondrial DNA.

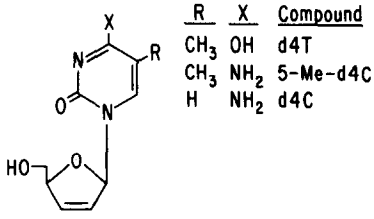
MATERIALS AND METHODS

Livers were removed aseptically [26] from 150-175 g male Sprague-Dawley rats and mitochondria were prepared as described [27] except that the 0.25 M sucrose solution contained 10 mM Tris, pH 8.0, and 1 mM EDTA. One liver provided sufficient mitochondria for nine incubation flasks. Incubation conditions for the labeled precursors, [^{3}H]thymidine [28] and [^{3}H]dATP [26], were as described except that in the latter case, unlabeled dNTP concentrations were 1 uM (20-fold less than in the [^{3}H]thymidine medium) and the concentration of [^{3}H]thymidine was 0.06 uM (20 Ci/mmol) and of [^{3}H]dATP was 0.3 uM (22 Ci/mmol). After the incubation, the contents of the flask were quantitatively transferred to a 1.5-ml Eppendorf tube which was spun at 12,000 rpm for 15 min. The supernatant fraction was removed, the walls of the tube were blotted carefully, 0.5 ml of buffer (50 mM NaCl, 5 mM Tris, pH 8.0, 0.5 mM EDTA) was added, and the pellet was resuspended with a Vortex mixer. The suspension was subjected to a freeze-thaw cycle, stirred as above, 200 ul was transferred to a second Eppendorf tube, and 200 ul of 20% trichloroacetic acid was added. The tube was stirred with the Vortex and allowed to stand in ice for 30 min. The precipitate was then filtered onto a 24 mm Millipore disc (Type HA, Cat. No. HAWP 025 00) and was washed three times with 5% trichloroacetic acid, three times with 95% ethanol and air dried. The disc was transferred to a scintillation vial so that it laid flat and 1.2 ml of 0.05 M HCl was added. The vial was closed with a cap fitted with a special Teflon-silicone liner (Tuf-Bond; Pierce Chemical Co.) and was maintained at 90° for 1 hr. Liquiscint (10 ml) was added to the clear hydrolysate and, after shaking on an orbital shaker for 30 min or until the filter disc became transparent, the vial was counted. To determine whether any label from [^{3}H]dATP had been incorporated into mtRNA, the RNA was isolated by the guanidinium isothiocyanate-CsCl step gradient procedure [29] and counted.

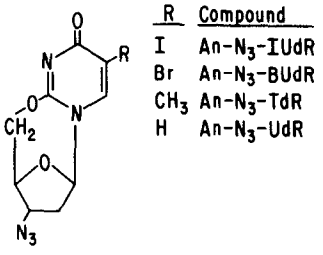
RESULTS

Labeled precursor incorporation into the DNA of isolated mitochondria [26] has been shown to reflect true DNA replication [30], and the system has been widely used. While either dNTPs or deoxynucleosides may be used as the labeled precursor, the compounds we studied were all deoxynucleosides, and would so be used *in vivo*. We therefore felt it would be a more relevant test of the *in vivo* ability of a dd-nucleoside to inhibit mtDNA replication if it did not have to compete with an added dNTP which needs no further phosphorylation. Inasmuch as [^{3}H]thymidine had been shown to be efficiently incorporated into DNA by isolated mitochondria [28], we used it as one of the labeled precursors [28]. On the other hand, since many of the compounds which were to be tested were thymidine derivatives, their competition with [^{3}H]thymidine for thymidine kinase or for other enzymes in the pathway to DNA could lead to spuriously high inhibition values. Hence, we also tested the analogs using [^{3}H]dATP, chosen because none of the compounds is a dd-adenosine derivative. Moreover, the use of the triphosphate avoids the otherwise possible effects of the analogs on the deoxyadenosine-dATP pathway. In addition, in this system we set the unlabeled dNTP concentrations very low relative to the analog concentration in order to avoid excessive substrate competition which could militate against a potential inhibitory analog exerting an effect. Finally, separation of RNA from labeled DNA after mitochondrial incubation with [^{3}H]dATP showed that the RNA was unlabeled. The structures of eleven dd-nucleoside analogs possessing anti-HIV-1 activity are shown in Fig. 1.

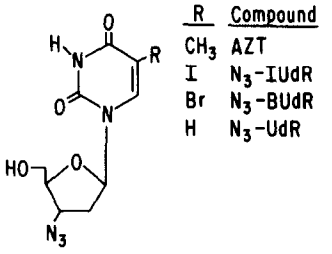
The results show (Fig. 2) that 25 uM AZT inhibits [^{3}H]thymidine incorporation about 90%, 1 uM inhibiting as much as 25-38%. When [^{3}H]dATP is used as the precursor (Table 1), the inhibition is 19% at 5 uM AZT and reaches almost 50% at 50 uM. Thus, the strong inhibitory activity of AZT irrespective of which precursor is used supports the view that AZT does in fact inhibit mtDNA replication in this system and is not acting merely as a competitive substrate for one or more of the enzymes in the pathway to dTTP. The inhibitory activity of eleven other dd-nucleosides toward [^{3}H]thymidine incorporation into mtDNA (Fig. 2) varied widely with a range at the 25 uM level from 84% to about zero, and they appeared to fall into two groups. The strong inhibitory group comprised AZT, N_3-IUdR, An-N_3-IUdR, An-N_3-BUdR, and N_3-BUdR, while the weak group comprised N_3-UdR, An-N_3-TdR, 5-Me-d4C, An-N_3-UdR, d4T, d2C and d4C, the latter two analogs having shown no inhibitory effect until the 25 uM level when inhibition by d4T rose to 17%.



R	X	Compound
CH_3	OH	d4T
CH_3	NH_2	5-Me-d4C
H	NH_2	d4C



R	Compound
I	An-N_3-IUdR
Br	An-N_3-BUdR
CH_3	An-N_3-TdR
H	An-N_3-UdR



R	Compound
CH_3	AZT
I	N_3-IUdR
Br	N_3-BUdR
H	N_3-UdR

Fig. 1. Structural formulas of dideoxynucleoside analogs tested.

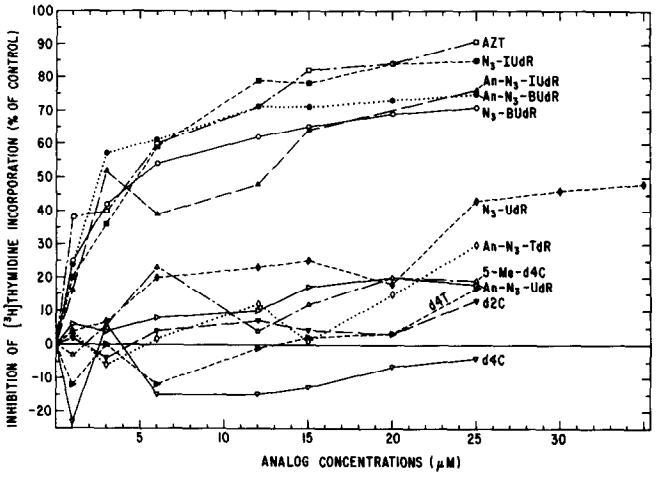


Fig. 2. Effects of nucleoside analogs on [^{3}H]thymidine incorporation into mtDNA by isolated rat mitochondria. Control values were about 6000 cpm.

Table 1. Effects of nucleoside analogs on [^{3}H]dATP incorporation into mtDNA by isolated rat liver mitochondria

	Inhibition of [^{3}H]dATP incorporation (% of control)				
	Analog Conc. (uM)				
	5	15	25	35	50
AZT	19	36	51	37	48
An-N_3-IUdR			100		
An-N_3-BUdR			90		
d4T			25		
d4C			19		
N_3IUdR			12		
d2C			9		
N_3BUdR			5		
An-N_3-TdR			5		
An-N_3-UdR			2		
5-Me-d4C			2		
N_3-UdR			-9		

Control values from experiment to experiment averaged about 2000 cpm. Results are the average of two to four experiments.

The sharp division remained when [^{3}H]dATP incorporation was examined (Table 1). AZT continued to inhibit strongly albeit less than with [^{3}H]thymidine. Inhibition by An-N_3-IUdR and An-N_3-BUdR remained very high but there was a dramatic decrease in the inhibitory effect of N_3-IUdR and N_3-BUdR as well as an appreciable decrease in the N_3-UdR inhibition. In the latter three cases, the strong inhibition of [^{3}H]thymidine incorporation may result primarily from the analog competition discussed earlier. Inhibition by d4T increased somewhat while the inhibitory effects of the remainder of the derivatives continued to be mild.

DISCUSSION

The known utilization of dd-nucleosides by DNA polymerase γ makes the dd-nucleoside analog, AZT, a candidate for inhibiting mtDNA replication. Since this could be a factor in the bone marrow suppressive effects of AZT, we have asked whether AZT can in fact inhibit mtDNA replication and we have tested this possibility on the DNA replication system in isolated rat liver mitochondria. AZT has proved to be a strong inhibitor of the incorporation into mtDNA of either [^{3}H]thymidine, or more important because the issue of substrate-thymidine analog competition does not arise, of [^{3}H]dATP. These results provide strong evidence that AZT can in fact inhibit mtDNA replication and therefore that it has potential for cytotoxicity. We emphasize, however, that our results have been obtained with isolated mitochondria and it is not yet known whether they apply to mitochondria in situ. Moreover, the highly selective toxicity of AZT against bone marrow remains to be explained. A system which may prove valuable as a first step in answering such questions is the Friend mouse leukemia cell. Preliminary experiments in our laboratory (J. Moschella et al., unpublished observations) show that the growth of uninduced Friend cells is resistant to at least a 50 uM concentration of AZT. When the cells were induced to synthesize hemoglobin, however, their growth became sensitive to AZT; 25 uM (the lowest concentration used) inhibited cell growth 58% and hemoglobin synthesis 40%.

With the exception of N_3-BUdR, N_3-IUdR and N_3-UdR, the analogs which inhibit more strongly in the [^{3}H]thymidine system do so in the [^{3}H]dATP system as well and the same is true for the more weakly inhibitory compounds. The structural basis of the variability in the extent of inhibitory activity of the different analogs, particularly toward [^{3}H]dATP incorporation which escapes the problem of possible thymidine analog-[^{3}H]thymidine competition, is a matter for further study. It is perhaps of some interest, however, that d2C, which is a weak inhibitor of mtDNA replication in our system, was found, in a phase I study, to induce peripheral neuropathy as a major side effect; unlike AZT, d2C evokes no megablastic changes which are early signs of bone marrow toxicity [31]. Also, N_3-UdR (CS-87), a non-inhibitor in the [^{3}H]dATP system and d4T, a weaker inhibitor than AZT in both the [^{3}H]thymidine and the [^{3}H]dATP systems, are less toxic than AZT in Sommadossi's human bone marrow system [13], and are expected to enter clinical trials in early 1989.

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