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Comparative Inhibition of Hepatitis B Virus DNA Polymerase and Cellular DNA Polymerases by Triphosphates of Sugar-Modified 5-Methyldeoxycytidines and of Other Nucleoside Analogs

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Of a series of 14 nucleoside 5′-triphosphates, those of 2′,3′-dideoxy-3′-fluoro-5-methylcytidine, 3′-azido-2′,3′-dideoxy-5-methylcytidine, 2′,3′-dideoxy-3′-fluoroguanosine, 2′,3′-didehydro-2′,3′-dideoxy-5-methylcytidine, 2′,3′-dideoxy-3′-fluoro-5-ethylcytidine, and 2′,3′-dideoxy-3′-fluoroadenosine emerged as the most potent inhibitors of hepatitis B virus DNA polymerase (50% inhibitory dose, 0.03 to 0.35 μM). In contrast, cellular DNA polymerases proved to be resistant to (α) or partially affected by (β) these analogs. These compounds are among the most effective and selective inhibitors of endogenous hepatitis B virus DNA polymerase recognized to date.

The group of hepatitis-inducing hepadnaviruses has been demonstrated to have a replication strategy unique to animal viruses. Like the RNA-directed DNA synthesis of retroviruses, their replication mechanism involves reverse transcription of an RNA intermediate previously synthesized by a cellular RNA polymerase (23). Therefore, it seemed reasonable to look for inhibitors of polymerase of hepatitis viruses among such compounds shown to be effective suppressors of human immunodeficiency virus reverse transcriptase (RT). Originally, 3′-azido-2′,3′-dideoxythymidine 5′-triphosphate was reported to inhibit strongly both human immunodeficiency virus RT and hepatitis B virus (HBV) DNA polymerase (1, 6, 18, 21). Meanwhile, however, 3′-azido-2′,3′-dideoxythymidine has been shown to be unable to suppress duck HBV (DHBV) replication in vitro and in vivo (9, 26) or to be effective against hepatitis B infections of AIDS patients coinfected with HBV (4). A series of compounds, such as 2′,3′-dideoxy-3′-fluorothymidine 5′-triphosphate and some closely related 3′-fluoro-modified dTTP analogs, 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-triphosphate, ddTTP, ddCTP, ddGTP, and 5-substituted dUTP derivatives, were likewise described as efficient inhibitors of human immunodeficiency virus RT (1, 8, 18, 19, 24), exhibiting promising effects on HBV DNA polymerase (20, 25) or DHBV DNA polymerase (14, 25). Of these agents, only the 2′,3′-dideoxynucleosides have been examined for their antiviral efficiency in two different HBV-producing cell systems (3, 13, 26) or in DHBV-infected animals (11, 13), with varying success. The carbocyclic analog of 2′-deoxyguanosine was found to suppress almost completely HBV replication in a cellular system (22) but, in contrast to the above-mentioned compounds, nothing is known about its effects on viral polymerases. Furthermore, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil is regarded as a promising agent which, although being less active than the corresponding 5-iodocytosine derivative against woodchuck hepatitis virus in vivo, lacks the severe toxicity of the latter (5). Several years ago, the triphosphate of 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine was found to be the most effective inhibitor of HBV DNA polymerase (K_i, 0.01 μM) (7). In the present study, we prepared triphosphates of nucleosides containing 5-methylcytosine, a 5-iodocytosine-like pyrimidine analog, and 2′,3′-dideoxyribose sugars with different modifications and tested them against endogenous HBV DNA polymerase and cellular DNA polymerases α and β. The same was done with ddCTP, ddGTP, and ddATP and their corresponding 3′-fluoro-modified counterparts (Fig. 1).

The sugar-modified nucleosides and their corresponding 5′-triphosphates were synthesized and purified by well-established procedures (10, 12), with the exception of ddCTP, ddATP, and ddGTP, which were obtained together with the substrates dTTP, dCTP, dATP, and dGTP from Boehringer (Mannheim, Germany). [^{3}H]dCTP (85 Ci/mmol), [^{3}H]dATP (88 Ci/mmol), and [^{3}H]dGTP (44 Ci/mmol) were purchased from Amersham Buchler (Braunschweig, Germany). For estimation of HBV DNA polymerase activity, virus particles were pelleted from HBV DNA-positive human sera and the endogenous DNA polymerase was assayed as described previously (20). The reaction mixture (49 μl) contained 42 mM Tris-HCl (pH 7.5), 34 mM $MgCl_2$, 340 mM KCl, 22 mM 2-mercaptoethanol, 0.4% Nonidet P-40, 70 μM concentrations each of three of the deoxynucleoside triphosphates and, on the basis of the inhibitor examined, a 0.7 μM concentration of the fourth one as a labeled triphosphate (this concentration varied between 0.22 and 1.18 μM in kinetic studies) (64 kdpm/pmol). Twenty one microliters of the virus suspension was added, and the mixture was incubated at 37°C for 180 min, during which time the incorporation had a linear course. Twenty-microliter samples were removed and processed as described previously (20). The results are given as mean values of at least three independent experiments with the same pool of serum preparations. DNA polymerases α and β were prepared and tested as described previously (16) but with activated calf thymus DNA as the template primer, 100 μM concentrations each of three of the deoxynucleoside triphosphates and, on the basis of the inhibitor tested, a 5 μM concentration of the fourth one as a labeled triphosphate (20 kdpm/pmol).

Figure 2 shows the dose-response curves for the inhibition

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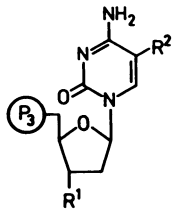

R^1	R^2	Abbreviation
-H	-H	ddCTP
-H	$-CH_3$	ddMetCTP
-F	-H	FdCTP
-F	$-CH_3$	FMetdCTP
-F	$-CH_2CH_3$	FEtdCTP
-Cl	$-CH_3$	ClMetdCTP
$-NH_2$	$-CH_3$	NH_2MetdCTP
$-N_3$	$-CH_3$	N_3MetdCTP

FIG. 1. Structural modifications of the dCTP analogs investigated. Not included were the 2',3'-unsaturated derivatives of ddCTP and ddMetCTP. P_3, triphosphate.

of endogenous HBV DNA polymerase activity by the 5'-triphosphates of 5-methyldeoxycytidine derivatives modified in the sugar moiety. Among these, 2',3'-dideoxy-3'-fluoro-5-methylcytidine triphosphate (FMetdCTP) was the most effective inhibitor, followed by 3'-azido-2',3'-dideoxy-5-methylcytidine triphosphate (N_3MetdCTP) and 2',3'-didehydro-2',3'-dideoxy-5-methylcytidine triphosphate (ddeMet CTP). At a concentration of 1.4 μM, FMetdCTP, N_3MetdCTP, and ddeMetCTP produced 95, 89, and 78% inhibition of the enzyme activity, respectively. The 50% inhibitory doses of these and all other tested compounds are presented in Table 1. The data revealed that the three mentioned derivatives are the most effective inhibitors of HBV DNA polymerase. In contrast, the sugar-modified 5-methyldeoxycytidine analogs 3'-chloro-2',3'-dideoxy-5-methylcytidine triphosphate, 2',3'-dideoxy-5-methylcytidine triphosphate (ddMetCTP), and 3'-amino-2',3'-dideoxy-5-methylcytidine triphosphate (NH_2MetdCTP) showed markedly less inhibitory activity. More effective compounds could be constructed by varying the 5-methyl group of the deoxycytidine derivatives; such has been confirmed at least for 2',3'-dideoxy-3'-fluoro-5-ethyldeoxycytidine triphosphate (50% inhibitory dose, 0.35 ± 0.1 μM).

A comparison of ddCTP and its unsaturated derivative, 2',3'-didehydro-2',3'-dideoxycytidine triphosphate, with the corresponding 3'-fluoro-substituted dCTP revealed that an

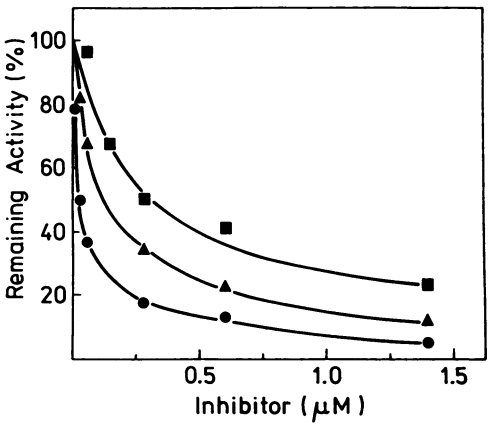

FIG. 2. Inhibition of HBV DNA polymerase activity by triphosphates of sugar-modified 5-methyldeoxycytidine analogs. The curves represent the means of four experiments. Symbols: ●, FMetdCTP; ▲, N_3MetdCTP; ■, ddeMetCTP.

TABLE 1. Comparative inhibitory activities of triphosphates of nucleoside analogs against HBV and cellular DNA polymerases

Inhibitor[a]	50% Inhibitory dose (μM) for[b]:		
	HBV DNA polymerase	Cellular DNA polymerase	
		α	β
ddCTP	3.0 ± 0.81	>200	<1
ddeCTP	2.5 ± 0.77	>200	6
FdCTP	0.5 ± 0.12	>200	1
ddMetCTP	10 ± 3.24	>200	>50
ddeMetCTP	0.28 ± 0.08	>200	8
FMetdCTP	0.03 ± 0.008	>200	1
FEtdCTP	0.35 ± 0.10	>200	3
ClMetdCTP	9 ± 2.62	>200	<100
NH_2MetdCTP	26 ± 7.96	>200	>200
N_3MetdCTP	0.12 ± 0.05	>200	>200
ddATP	1.6 ± 0.42	>100	4.0
FdATP	0.35 ± 0.09	>200	4.7
ddGTP	1.9 ± 0.63	>200	0.6
FdGTP	0.15 ± 0.04	>200	2.7

[a] ddeCTP, 2',3'-didehydro-2',3'-dideoxycytidine triphosphate; FdCTP, 2',3'-dideoxy-3'-fluorocytidine triphosphate; ddMetCTP, 2',3'-dideoxy-5-methylcytidine triphosphate; ddeMetCTP, 2',3'-didehydro-2',3'-dideoxy-5-methylcytidine triphosphate; FMetdCTP, 2',3'-dideoxy-3'-fluoro-5-methylcytidine triphosphate; FEtdCTP, 2',3'-dideoxy-3'-fluoro-5-ethylcytidine triphosphate; ClMetdCTP, 3'-chloro-2',3'-dideoxy-5-methylcytidine triphosphate; NH_2MetdCTP, 3'-amino-2',3'-dideoxy-5-methylcytidine triphosphate; N_3MetdCTP, 3'-azido-2',3'-dideoxy-5-methylcytidine triphosphate; FdATP, 2',3'-dideoxy-3'-fluoroadenosine triphosphate; FdGTP, 2',3'-dideoxy-3'-fluoroguanosine triphosphate.

[b] Means of three or four experiments; for HBV DNA polymerase, standard deviations are included.

increased sensitivity of the enzyme was achieved when hydrogen was replaced by fluorine at the C-3' position. The same was true for ddATP and ddGTP, which were both changed by the 3'-fluoro modification to yield very potent inhibitors of HBV DNA polymerase. Comparable data on the inhibition of DHBV DNA polymerase by ddCTP and ddGTP were reported recently (14), whereas an inhibitory effect of ddCTP on the DNA polymerases of both DHBV and HBV could not be confirmed (11). This lack of effect seemed to be caused by the assay conditions used, since in vivo studies in the same report demonstrated a potent antiviral activity of 2',3'-dideoxycytidine in DHBV-infected ducks (11). Kinetic studies were performed with FMetdCTP and 2',3'-dideoxy-3'-fluoroguanosine triphosphate (FdGTP), two of the most interesting compounds detected. The data in Fig. 3 indicate the competitive nature of the inhibition of HBV DNA polymerase by FMetdCTP and FdGTP with regard to the substrates dCTP and dGTP, respectively. The K_m values of dCTP and dGTP were 0.22 and 0.40 μM, respectively. The K_i values were 0.006 μM for FMetdCTP and 0.06 μM for FdGTP (means of two determinations). The resulting K_m/K_i ratios indicated ca. 37-fold (FMetdCTP) and 7-fold (FdGTP) higher affinities for the binding site (relative to the normal substrates).

In HBV-infected cells, cellular DNA polymerases as well as the viral polymerase are exposed to these inhibitors. Therefore, we tested the sensitivities of DNA polymerases α and β to the analogs. The 50% inhibitory doses in Table 1 demonstrated an almost complete insensitivity of DNA polymerase α to all of the compounds and a partial inhibition of DNA polymerase β by most of them. These properties, described earlier for the triphosphates of 2',3'-dideoxynucleosides and 2',3'-dideoxy-3'-fluoronucleosides of the four

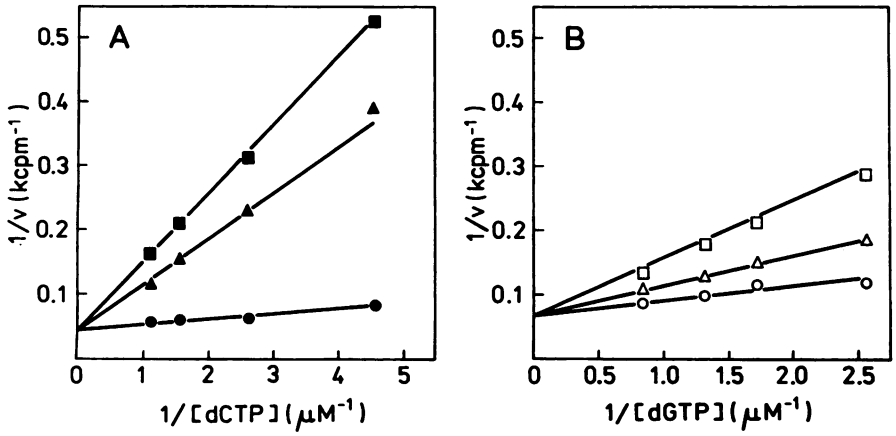

FIG. 3. Lineweaver-Burk plots for the inhibition of HBV DNA polymerase by FMetdCTP (A) and FdGTP (B). The substrates were [^{3}H]dCTP and [^{3}H]dGTP, respectively, and the reactions were performed as described in the text. Symbols: (A) ●, no FMetdCTP; ▲, 0.03 μM FMetdCTP; ■, 0.06 μM FMetdCTP; (B) ○, no FdGTP; △, 0.06 μM FdGTP; □, 0.12 μM FdGTP.

natural bases (2, 16, 18, 27), were confirmed here for a series of deoxycytidine derivatives modified in the base moiety and/or in the sugar moiety (Table 1). However, NH_2Met dCTP and N_3MetdCTP had no inhibitory effects on either cellular DNA polymerase, so N_3MetdCTP displayed the highest selectivity of all the tested compounds.

Despite the promising properties of some of the compounds described or cited here, it must be considered that the data obtained apply to the DNA-dependent polymerase activity of the serum-derived HBV particles rather than to the RNA-dependent polymerase (RT) activity detectable only in the liver-derived core particles.

Recently, it was shown (14) that the thymidine analog 5′-triphosphates ddTTP and 2′,3′-dideoxy-3′-fluorothymidine triphosphate had ca. 10-fold lower inhibitory effects and that AzdTTP had ca. 40-fold lower inhibitory effects on the DHBV RT activity than on the DNA-dependent DHBV DNA polymerase activity, whereas the two enzyme activities showed no differences in sensitivity to ddCTP and ddGTP. Therefore, it remains to be seen to what extent HBV RT can be inhibited by the described analogs.

On the basis of the findings of the present work and our earlier studies (17, 20), initial investigations on the effect of nucleoside analogs on HBV replication at the cellular level have shown that 2′,3′-dideoxy-3′-fluoro-5-methylcytidine is one of the most effective agents in suppressing HBV replication in 2.2.15 cells (15). In addition, it displays low cytotoxicity, at least in various other human cell lines and on CFU-GM of mice (data not shown), and therefore is worthy of further investigation.

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