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RAPID COMMUNICATION

Sensitivity of L-(-)2′,3′-Dideoxythiacytidine Resistant Hepatitis B Virus to Other Antiviral Nucleoside Analogues

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ABSTRACT. L-(-)2′,3′-Dideoxythiacytidine (L(-)SddC, Lamivudine) resistant hepatitis B virus (HBV) develops in patients after prolonged treatment. Point mutations detected in the viral genome from these patients have been shown to be responsible for L(-)SddC resistance. Therefore, new drugs active against L(-)SddC resistant HBV are needed. Using a transient transfection system, we studied the sensitivity of L(-)SddC resistant HBV to other anti-HBV nucleoside analogues. It was found that the L526M mutation alone caused greater resistance to penciclovir (PCV) than did the V553I mutation alone. Both mutations also caused the virus to be less sensitive to L(-)SddC and 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil (L-FMAU), although the degree of resistance was much less than that to PCV. The A546V mutation had no impact on the sensitivity to L(-)SddC, L-FMAU, and PCV. When these single mutations were coupled with the M550V/I mutation, all the double mutants were resistant to those drugs. Although 2′,3′-dideoxy-2′,3′-didehydro-β-L(-)-5-fluorocytidine (L(-)Fd4C) was also less active, the IC_{50} of L(-)Fd4C against the L(-)SddC resistant mutant was at least fifty times lower than that against cell growth in culture. DNA polymerase associated with L(-)SddC resistant virions was also found to be less sensitive than that with wild-type HBV to those L-nucleoside triphosphates. All the L(-)SddC resistant mutants were still sensitive to 9-(2-phosphonylmethoxyethyl)-adenine (PMEA). These results suggest that different mutations in the HBV genome have a different impact on its sensitivity to those compounds, and L(-)SddC resistant HBV may also be resistant to PCV, L-FMAU, and L(-)Fd4C. A nucleoside analogue less toxic than PMEA could be developed against L(-)SddC resistant HBV. BIOCHEM PHARMACOL **57**;12:1351–1359, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. hepatitis B virus (HBV); mutation; nucleoside analogues; L(-)SddC (3TC) resistance; cross-resistance

HBV† infection is a worldwide public health problem. Chronic HBV infection in patients increases the risk of developing hepatocellular carcinoma about 150-fold [1, 2]. Vaccination was used for the prevention of viral infection [3]. Interferon could be beneficial to some patients with chronic infection, but it has some undesirable side-effects [4–6]. Several nucleoside analogues were found to have potent anti-HBV activity. L(-)SddC, which was discovered independently in our laboratory [7] and the laboratory of others [8], is the first L-nucleoside analogue to have potent biological activity. In the clinic, L(-)SddC can rapidly reduce viral load in patient serum to undetectable levels, although viral replication may return to former levels when treatment is withdrawn, which could be due to the presence of HBV cccDNA in the infected hepatocytes during the treatment [7, 9, 10]. Therefore, long-term treatment is required for chronically infected HBV patients. Unfortunately, drug resistance emerges during long-term treatment [11]. The resistant phenotype was found to be associated with the change of methionine (M550) to valine or isoleucine at the YMDD motif (encoding for tyrosine, methionine, aspartate, aspartate) of HBV DNA polymerase. Our previous study indicated that this mutation alone could render viruses to be less replication efficient than the wild-type virus. Additional mutations of DNA polymerase may be necessary to compensate for the effect on the viral replication, as well as to have an impact on the sensitivity of mutated viruses to L(-)SddC [12].

The emergence of viral resistance during monotherapy suggests that new drugs and drug combinations for the control of HBV infection and viral resistance are needed. Results of clinical trials of anti-HIV therapy and *in vitro* drug combination studies have shown that the combination of different types of inhibitors improves the antiviral efficacy, prolongs the development of the resistant mutants, reduces drug toxicity, and has potential for antiviral synergy [13–15]. For HBV treatment, drug combinations may also have some benefit. Therefore, the study of drug cross-

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† *Abbreviations*: HBV, hepatitis B virus; L(-)SddC (3TC, Lamivudine), L-(-)2′,3′-dideoxythiacytidine; L-FMAU, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil; L(-)Fd4C, 2′,3′-dideoxy-2′,3′-didehydro-β-L(-)-5-fluorocytidine; PMEA, 9-(2-phosphonylmethoxyethyl)-adenine; and PCV, penciclovir.

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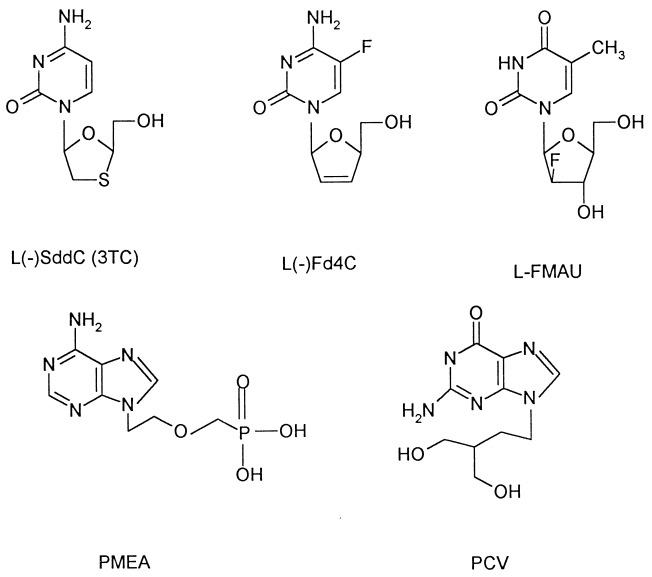

FIG. 1. Structures of the compounds in this study.

resistant profiles *in vitro* will be important for the determination of drug combination or treatment protocols in the clinic.

Currently, nucleoside analogs PMEA, PCV, and lobucavir, which were discovered to be active against HBV [16–19], are also in clinical trials. It was reported that some patients developed resistance during PCV treatment, and point mutations were detected in the virion genome isolated from patients who had viral rebound [20, 21]. It was noticed that some of the mutation sites in the L(-)SddC resistant viruses and PCV resistant viruses are the same [22–24].

In our laboratory, other L-nucleoside analogues with different structures were found to be active against HBV in cell culture. These compounds include L(-)Fd4C [25], which is a deoxycytidine analogue and ten times more potent than L(-)SddC, and L-FMAU [26], which is a thymidine analogue and has a different mode of action against HBV in woodchuck from L(-)SddC. At this point it is not clear what their activity is against L(-)SddC resistant HBV. In this study, we employed an HBV transient transfection system to study the action of L(-)Fd4C, L-FMAU, PMEA, and PCV against the L(-)SddC resistant mutants.

MATERIALS AND METHODS

Chemicals

L(-)SddC (3TC, Lamivudine) and L-FMAU were provided by Dr. Chung K. Chu from the University of Georgia [27]. L(-)Fd4C was synthesized in the laboratory of the late Tai-Shun Lin at Yale University [25]. PMEA was provided by Gilead Sciences Inc. PCV was purchased from Moravek. The structures of these analogues are shown in Fig. 1.

HBV Genomes

The wild-type HBV genomic clone pHBV-adr used in this study was a gift from Professor Yuan Wang, Shanghai Institute of Biochemistry, Academia Sinica. The three sets of HBV mutants (set I, pL526M and pL526MM550V; set II, pV553I and pM550IV553I; and set III, pA546V and pA546VM550I) were generated by site-directed mutagenesis. The genomic construction, mutation generation, and characterization of the viral replication and sensitivity to L(-)SddC were described in our previous publications [12, 28, 29].

Drug Sensitivity of HBV in Transiently Transfected Cells

The effects of the compounds on HBV DNA replication were assessed as described previously [12]. HBV wild-type or mutated genomes were transiently transfected into human hepatoma cells (HepG2, from the American Type Culture Collection) by calcium phosphate precipitation. Plasmid (pSEAP) encoding for alkaline phosphatase was co-transfected with the HBV genomes as an internal control to normalize the variation of the transfection efficiency among individual samples. The compounds were added to the culture when the medium was replaced 1 day after transfection. After 9 days of treatment, the cells were harvested, and the intracellular progeny DNA replication was detected by Southern blot analysis.

Inhibition of HBV DNA Polymerase by Triphosphate of L-Nucleosides

The triphosphate of L-FMAU was synthesized in our laboratory. The triphosphates of L(-)Fd4C and L(-)SddC were provided by Vion Pharmaceuticals and Dr. R. Schinazi of Emory University, respectively. The activity assay for virion-associated HBV DNA polymerase was performed according to the methodology described previously [30]. Briefly, HBV virion particles were accumulated from culture medium of the HBV-producing cell lines (HepG2-WT10, HepG2-SM1, and HepG2-DM2) by utilizing a polyethylene glycol precipitation method. These cell lines produce HBV viral particles, wild-type, L526M mutant, and L526MM550V mutant, respectively. The generation time and characterization of these cell lines have been determined.* The viral particles were added to the polymerase reaction mixture that contained 42 mM Tris–HCl (pH 7.5), 34 mM $MgCl_2$, 340 mM KCl, 22 mM β-mercaptoethanol, and 0.4% Nonidet P-40. The reaction mixture for deoxycytosine analogues contained dATP, dTTP, and dGTP (each at 70 μM), 0.175 μM dCTP, 10 μCi of [α-^{32}P]dCTP (3000 Ci/mmol, Amersham Corp.), and different concentrations of the triphosphates of L(-)SddC or L(-)Fd4C. For the thymidine analogue, the reaction mix-

* Fu L, Liu SH and Cheng YC, unpublished data.

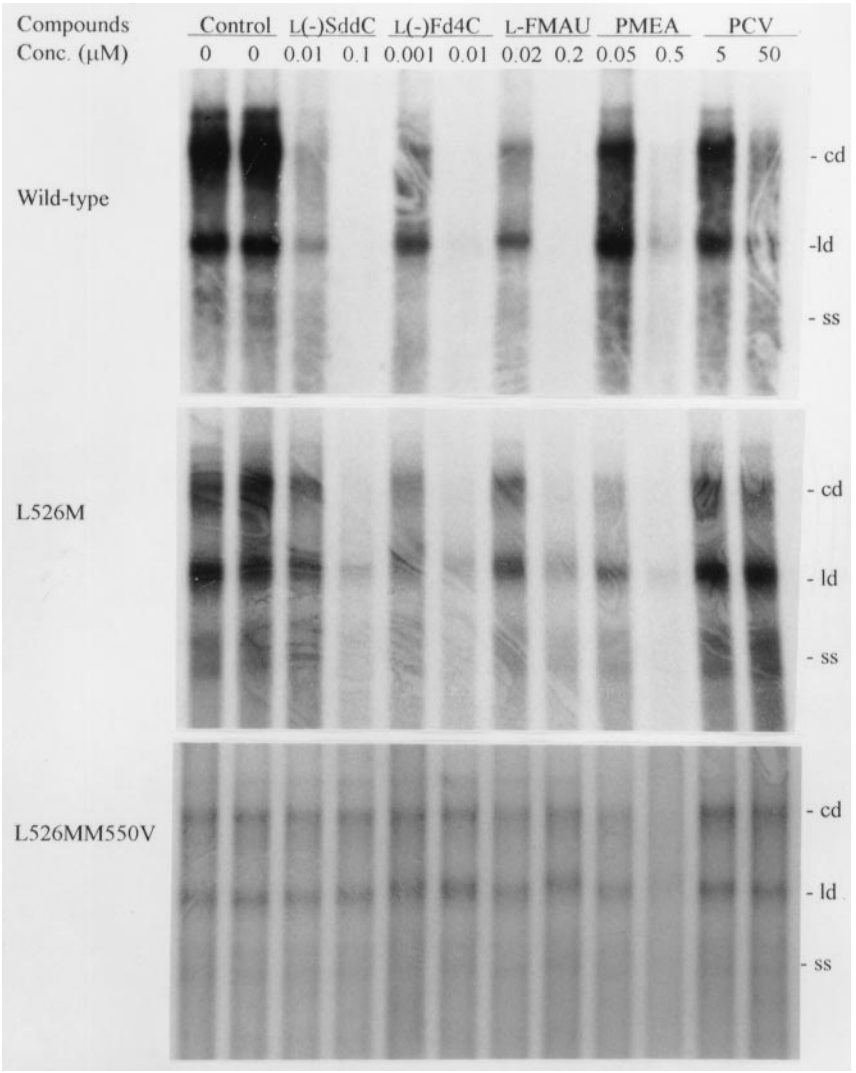

FIG. 2. Sensitivity of HBV wild-type and mutants to antiviral nucleoside analogues L(-)SddC, L(-)Fd4C, L-FMAU, PMEA, and PCV. HepG2 cells transiently transfected with HBV genome were treated with these drugs at two different concentrations, as indicated. Abbreviations: cd, circular partial double-stranded HBV DNA; ld, linear partial double-stranded HBV DNA; and ss, single-stranded HBV DNA. This is a representative experiment out of three.

ture contained dATP, dCTP, and dGTP (each at 70 μM), 0.175 μM dTTP, 10 μCi of [α-^{32}P]dTTP (3000 Ci/mmol, Amersham Corp.), and different concentrations of the triphosphate of L-FMAU. After a 2-hr incubation at 37°, the reaction was stopped by proteinase K digestion (100 mg/mL of proteinase K and 1% SDS) at 50° for 30 min. The newly synthesized ^{32}P-labeled viral DNA was analyzed by agarose gel electrophoresis and autoradiography.

RESULTS

Sensitivity of L(-)SddC Resistant HBV Mutants to L(-)Fd4C, L-FMAU, PMEA, and PCV

In our previous study, three sets of HBV mutants (set I, pL526M and pL526MM550V; set II, pV553I and pM550IV553I; and set III, pA546V and pA546VM550I) were generated, and their viral replication and sensitivity to L(-)SddC were characterized [12]. To compare the impact of the mutations on the sensitivity of HBV to other antiviral nucleoside analogues, we tested the anti-HBV replication activity of L(-)Fd4C, L-FMAU, PMEA, and PCV in HepG2 cells that were transiently transfected by HBV mutants. Two concentrations with a 10-fold difference were used for each compound. Figure 2 illustrates a typical result out of three independent experiments. It was found that the L526M mutant, which has been demonstrated to be 6-fold resistant to L(-)SddC [12], showed decreased sensitivity to L(-)Fd4C and L-FMAU compared with wild-type and resistance to PCV, but maintained sensitivity to PMEA. The double mutant L526MM550V, which was more resistant to L(-)SddC, was also resistant to L(-)Fd4C, L-FMAU, and PCV, but was still sensitive to PMEA.

Using the same methodology, the sensitivity of other sets of mutants was also examined, and similar results were observed on the set II mutants. The V553I mutant, which

TABLE 1. Sensitivity of HBV mutants to antiviral nucleoside analogues

Compounds	Concentration (μM)	Wild-type	L526M		V553I		A546V	
			−*	+†	−*	+†	−*	+†
			(% of Control)					
L(−)SddC	0.01	35 ± 4	70 ± 9	100 ± 9	66 ± 2	105 ± 6	32 ± 7	94 ± 6
	0.1	6 ± 1	16 ± 2	100 ± 7	6 ± 1	100 ± 10	4 ± 1	84 ± 5
L(−)Fd4C	0.001	42 ± 6	58 ± 2	100 ± 9	80 ± 2	103 ± 5	33 ± 11	91 ± 4
	0.01	7 ± 2	23 ± 2	92 ± 7	8 ± 7	97 ± 2	4 ± 1	76 ± 13
L-FMAU	0.02	46 ± 5	64 ± 8	100 ± 6	70 ± 7	105 ± 9	33 ± 7	89 ± 8
	0.2	6 ± 1	28 ± 2	89 ± 5	16 ± 2	96 ± 7	3 ± 1	78 ± 5
PMEA	0.05	51 ± 8	48 ± 5	60 ± 5	50 ± 7	53 ± 4	49 ± 19	44 ± 3
	0.5	15 ± 3	12 ± 4	37 ± 3	10 ± 4	14 ± 3	12 ± 4.6	20 ± 1
PCV	5	59 ± 3	100 ± 9	100 ± 5	88 ± 2	110 ± 8	63 ± 6	90 ± 5
	50	16 ± 2	100 ± 8	100 ± 7	53 ± 4	104 ± 6	14 ± 3	76 ± 4

The relative amount of viral progeny DNA in the drug-treated cells transfected with the viral genome was calculated as a percentage of the control with the same viral genome in the absence of drugs. The quantitative densitometry was generated from the autoradiograph. Values are means ± SD, N = 3.

*HBV mutants without YMDD mutation M550V/I.

†HBV mutants with YMDD mutation M550V/I.

was 3-fold resistant to L(-)SddC [12], also showed a decreased sensitivity to L(-)Fd4C, L-FMAU, and PCV, but was still sensitive to PMEA. The double mutant M550IV553I was more resistant to all the nucleoside analogues examined in this test except for PMEA. In set III, it was found that the mutant with an A546V single mutation was sensitive to all five compounds used in this study. However, the mutant with the A546V and M550I double mutation became resistant to L(-)SddC, L(-)Fd4C, L-FMAU, and PCV. Table 1 summarizes these observations.

Concentration-Dependent Relationship of HBV Wild-Type, L526M Mutant, and L526MM550V Double Mutant to L-FMAU and L(-)Fd4C

In the above study, we showed that there was some degree of cross-resistance between L(-)SddC and L(-)Fd4C or L-FMAU. However, it was not known whether the replication of L(-)SddC resistant mutants could be suppressed by using an increased concentration of L(-)Fd4C and L-FMAU. L526M and L526MM550V mutants, which were the most common mutations observed in virions isolated from L(-)SddC resistant patients, were selected as a model to determine their sensitivity to L-FMAU and L(-)Fd4C. HepG2 cells transiently transfected with the HBV genomes of wild-type and the two mutants were treated with concentrations of the compounds as indicated. Panel a of Fig. 3 shows a portion of the results of the Southern blot analysis of the progeny HBV DNA replication, and based on the quantitative densitometric data generated from autoradiographs, the concentration–response curves are shown in panels b and c. As indicated, the wild-type HBV was highly sensitive to L-FMAU and L(-)Fd4C, with IC_{50} values of 0.02 and 0.001 μM, respectively. The L526M mutant was less sensitive to these compounds than the wild-type. The L526MM550V mutant was resistant to L-FMAU, with an IC_{50} higher than 2.5 μM. However, the IC_{50} of L(-)Fd4C against the L526MM550V HBV mutant was around 0.2 μM, which is much lower than that against cell growth (about 10 μM) [25].

Sensitivity of HBV Virion-Associated DNA Polymerase to the Triphosphates of L(-)SddC, L(-)Fd4C, and L-FMAU

In culture, the resistance of HBV L526M and L526MM550V mutants to L(-)SddC, L(-)Fd4C, and L-FMAU was observed. Whether the mutated viral polymerase was also resistant to the triphosphate metabolites of these compounds was examined. The concentration-dependent inhibition of these triphosphate nucleotide analogues on viral DNA polymerase activity is shown in Fig. 4. HBV mutant-associated DNA polymerase was resistant to these compounds. This correlates with the sensitivity of viral growth to these compounds in culture.

DISCUSSION

In this study, three sets of L(-)SddC mutants, which were generated according to the mutation sites reported in the clinical L(-)SddC resistance cases [22, 23, 31], were used as models for testing the cross-resistance patterns of HBV mutants between L(-)SddC and other nucleoside analogues, L(-)Fd4C, L-FMAU, PMEA, and PCV. Due to the defective replication of M550V and M550I mutants, it was difficult to detect the viral progeny DNA [12]. In addition, most clinical cases showed that these two mutations at the YMDD motif were coupled with other mutations in the same viral variants [20–24, 32]. Therefore, in this study, we examined the sensitivity of the double mutants containing both M550V/I mutation and an additional mutation beyond the YMDD motif instead of the mutants with M550V/I mutation alone.

L(-)Fd4C and L-FMAU were discovered in our laboratory and shown to have potent anti-HBV activity [25, 26].

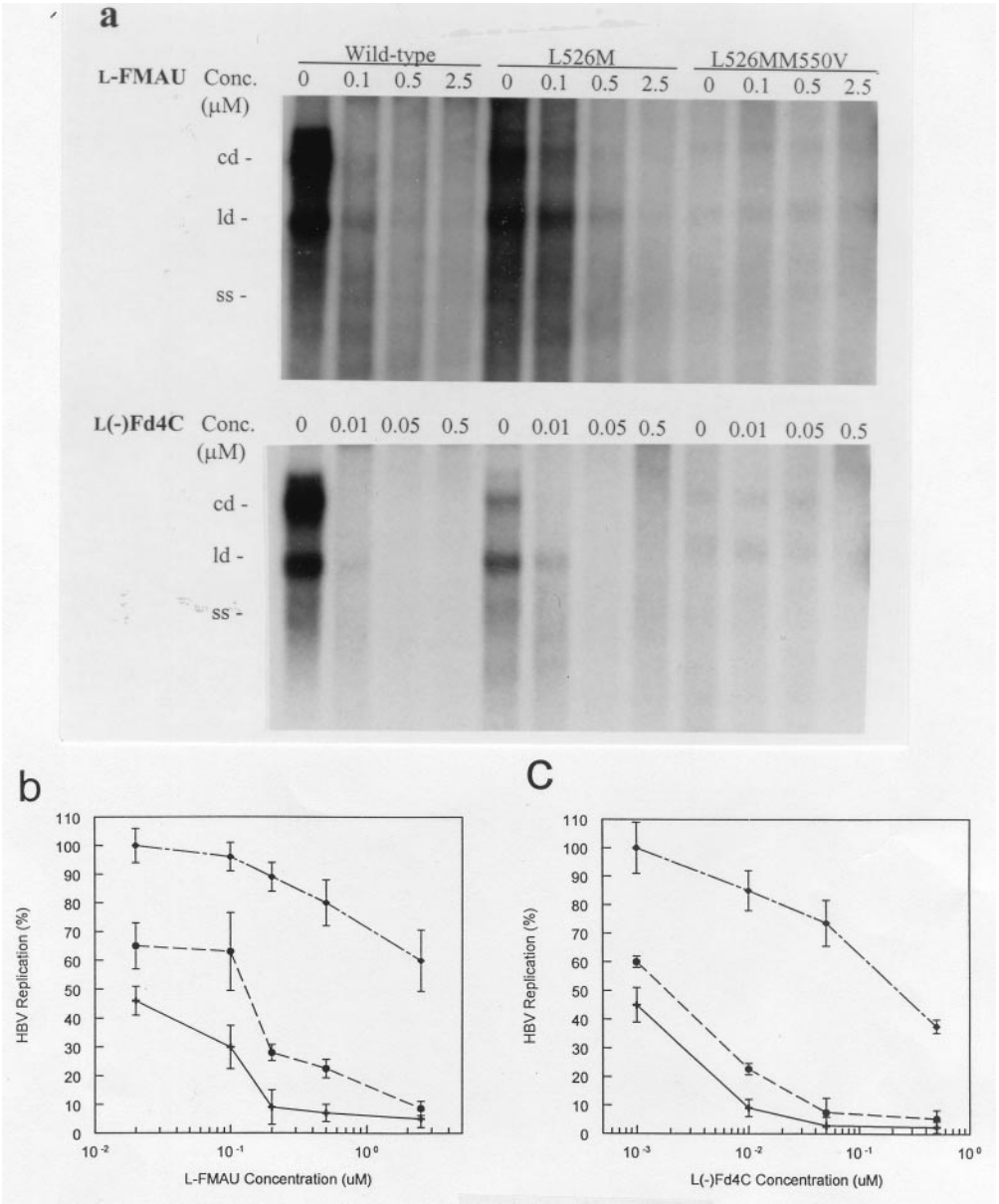

FIG. 3. Drug sensitivity of HBV to L-FMAU and L(-)Fd4C. HepG2 cells transiently transfected with wild-type genome and L(-)SddC resistant mutants were treated with L-FMAU or L(-)Fd4C for 9 days. (a) Southern hybridization of intracellular progeny DNA. (b) and (c) Concentration-dependent relationship of L-FMAU and L(-)Fd4C. Panel (a) is an experiment representative of two. Abbreviations: cd, circular partial double-stranded HBV DNA; ld, linear partial double-stranded HBV DNA; and ss, single-stranded HBV DNA. Values of (b) and (c) are means ± SD of three separate experiments, except for the 0.5 and 2.5 μM concentrations of L-FMAU where the means ± range of two experiments are shown. Key: (+) wild-type, (●) L526M, and (♦) L526MM550V.

Using a woodchuck model, L-FMAU, an L-thymidine analogue that has a unique metabolism [33], could suppress HBV replication without HBV rebounding once the L-FMAU treatment was stopped [34], whereas HBV rebound always occurred once L(-)SddC treatment was stopped.* Unfortunately, it was found in this study that all the L(-)SddC resistant mutants were also cross-resistant to L-FMAU. This suggested that the administration of L-FMAU to L(-)SddC resistant patients with the same mutation genotypes of these three sets might have no effect. L(-)Fd4C, a deoxycytidine analogue that is ten times more potent than L(-)SddC, could dramatically inhibit viral DNA synthesis and decrease the persistence of viral replication intermediates and cccDNA in the duck model.† Although, to some extent, cross-resistance was observed between L(-)SddC and L(-)Fd4C in our transient transfection model, the concentration of L(-)Fd4C required to inhibit 50% of viral progeny DNA replication of L(-)SddC

* Zoulim F, Aguesse S, Trepo C, Chevalier M and Cheng YC, unpublished data.

† Le Guerhier F, Pichoud C, Chevallier M, Germon S, Liu SH, Chen SH, Li XY, Trepo C, Cheng YC and Zoulim F, Antiviral activity of a novel L-nucleoside analog, β-L-Fd4C, in the duck HBV infection model. *Molecular Biology of Hepatitis B Viruses*, p. 152, Aug. 30–Sep. 3, UCSD, 1998.

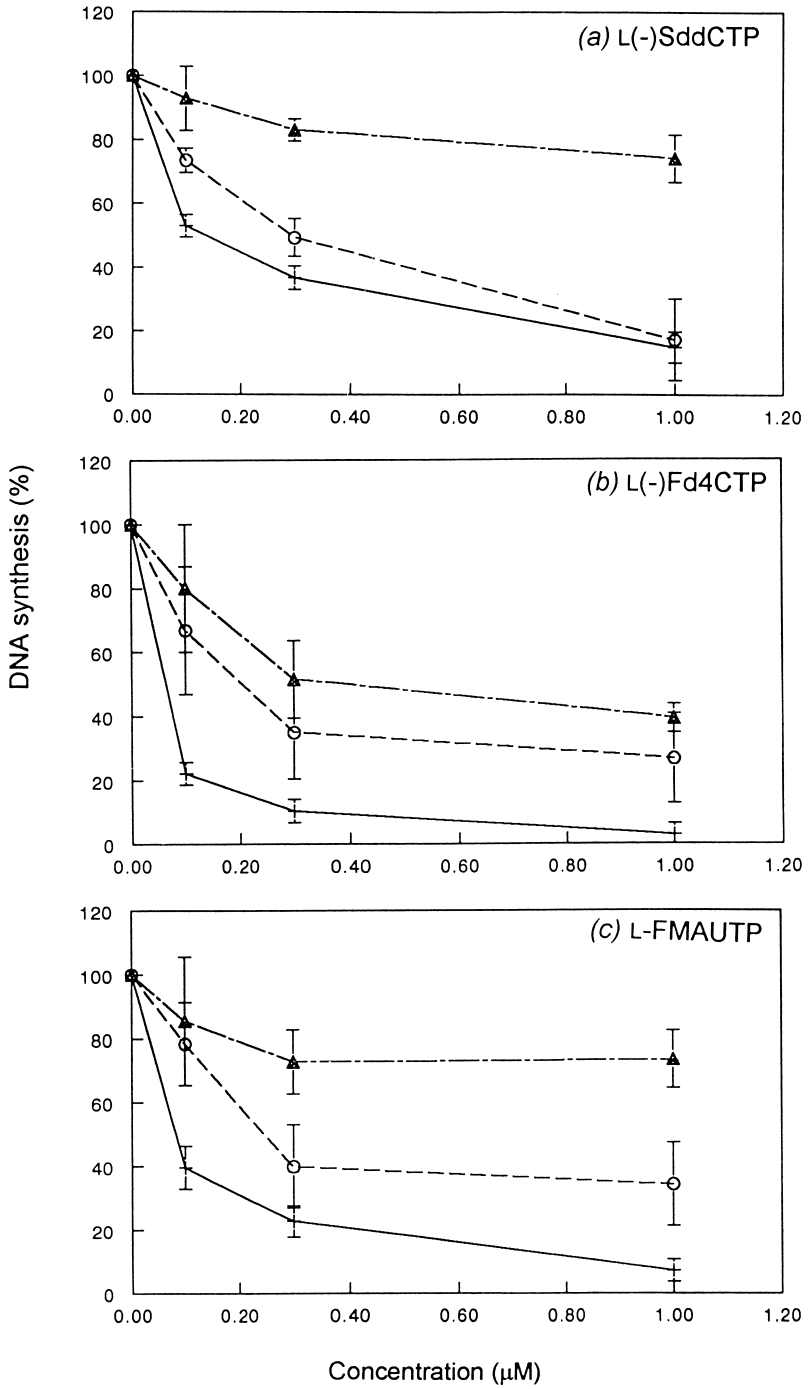

FIG. 4. Inhibition of HBV DNA polymerase by L-nucleoside triphosphates of L(-)SddC (a), L(-)Fd4C (b) and L-FMAU (c). The virion-associated HBV DNA polymerase assay was performed as described in Materials and Methods. HBV DNA synthesis levels quantitated by densitometry were calculated as percentages of the control. Values are means ± SD, N = 3. Key: (+) wild-type, (○) L526M, and (▲) L526MM550V.

resistant mutant L526MM550V was about 0.2 μM. This concentration is much lower than that against cell growth by L(-)Fd4C, which is about 10 μM [25]. Therefore, it is still possible to use a high concentration of L(-)Fd4C to overcome L(-)SddC resistant mutants in the clinic.

When the sensitivity of virion-associated DNA polymerase to the active metabolite, nucleoside triphosphate of L(-)SddC, L(-)Fd4C, and L-FMAU, was examined, there was a correlation of the sensitivity of virus to these compounds. The behavior of DNA polymerase to the active metabolite suggested that the loss of sensitivity of HBV mutants to the compounds studied could be due, in part, to the decrease of potency of their active metabolite in inhibiting HBV DNA polymerase. Detailed kinetic studies

involving the interaction of the active metabolites of these compounds and HBV DNA polymerase are under current investigation.

L526M mutation is common in clinical L(-)SddC resistant cases and most consistently linked to the M550V mutation [22–24, 31]. This mutation was also detected in the clinical famciclovir (the oral prodrug of PCV) resistant cases together with other mutations [20]. We found that the L526M mutation alone could render the HBV mutant resistant to PCV treatment, which is consistent with the observation of others [35]. It indicated that the HBV in L(-)SddC resistant patients containing the L526M mutation may be resistant to PCV treatment, and the PCV resistant patients may have a greater chance to develop L(-)SddC resistance if L(-)SddC is used following the development of PCV resistance. This could explain in part why some L(-)SddC resistant patients do not respond to PCV treatment [36]. It could also explain why some patients develop L(-)SddC resistance early on during treatment or do not respond to L(-)SddC treatment at all if they have been treated previously with PCV [37, *]. The occurrence of the L526M mutation was consistent in these cases.

V553I is a mutation that has been reported in both L(-)SddC and PCV resistant cases [23, 38]. In our system (adr substrain), it was found that this mutation alone only allowed the mutant to be less than 10-fold resistant to L(-)SddC and PCV, and this mutation did not change the viral replication efficiency [12]. When this mutation occurred together with the M550I mutation, the double mutant turned out to be much more resistant to L(-)SddC and PCV. However, other groups reported that in their system (ayw substrain), the V553I mutation (in their ayw genome, it was numbered as V542I) alone could dramatically decrease the viral replication and render the mutant much more resistant to PCV [38]. These different observations suggested that the same mutation in the HBV DNA polymerase of different viral substrains or variants may have different effects on drug sensitivity and the ability of the virus to replicate. The variation in the HBV DNA polymerase sequence among HBV variants, which makes subtle changes in the structure and function of the enzyme, could have significant effects on the behavior of the same mutation in HBV DNA polymerase.

It was interesting to find that all three sets of mutants did not substantially alter the sensitivity to PMEA. This is consistent with the observations by other groups [39, 40]. Clinical effects of adefovir (the prodrug form of PMEA) showed a marked reduction of HBV DNA in patient serum [41]. The potential use of PMEA for the treatment of L(-)SddC or PCV resistant chronic HBV in patients should be investigated further if the toxicity can be avoided.

In summary, among the anti-HBV nucleoside analogues examined, L(-)SddC resistant HBV was found to be resistant to L-FMAU. The clinical study of the effectiveness of L-FMAU should be targeted on L(-)SddC naive patients. There is also cross-resistance between L(-)SddC and L(-)Fd4C. However, L(-)Fd4C may still be useful in L(-)SddC resistant HBV patients at non-toxic concentrations since L(-)Fd4C is almost ten times more potent than L(-)SddC. PCV resistant HBV could predispose HBV to develop L(-)SddC resistance due to the alteration of amino acids, which will facilitate the replication of the M550V mutant. The lack of cross-resistance to PMEA suggests the possibility to develop new nucleoside analogues that have antiviral effects with less toxicity than PMEA [42] for the treatment of L(-)SddC and PCV resistance.

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References

1. Beasley RP, Hwang LY, Lin CC and Chien CS, Hepatocellular carcinoma and hepatitis B virus: A prospective study of 22,707 men in Taiwan. *Lancet* **ii:** 1129–1136, 1981.
2. Gitlin N, Hepatitis B: Diagnosis, prevention, and treatment. *Clin Chem* **43:** 1500–1506, 1997.
3. Xu ZY, Duan SC, Margolis HS, Purcell RH, Ou-Yang PY, Coleman PJ, Zhuang YL, Xu HF, Qian SG, Zhu QR, Wan CY, Liu CB, Gun ZL and the United States-People's Republic of China Study Group on Hepatitis B, Long-term efficacy of active postexposure immunization of infants for prevention of hepatitis B virus infection. *J Infect Dis* **171:** 54–60, 1995.
4. Wong DKH, Cheung AM, O'Rourke K, Naylor CD, Detsky AS and Heathcote J, Effect of alpha-interferon treatment in patients with hepatitis B e antigen positive chronic hepatitis B: A meta-analysis. *Ann Intern Med* **119:** 312–323, 1993.
5. Perrillo RP, Schiff ER, Davis GL, Bodenheimer HC Jr, Lindsay K, Payne J, Dienstag JL, O'Brien C, Tamburro C, Jacobson IM, Sampliner R, Feit D, Lefkowitch J, Kuhns M, Meschievitz C, Sanghvi B, Albrecht J, Gibas A and the Hepatitis Interventional Therapy Group, A randomized, controlled trial of interferon alpha-2b alone and after prednisone withdrawal for the treatment of chronic hepatitis B. *N Engl J Med* **323:** 295–301, 1990.
6. Perrillo RP, Treatment of chronic hepatitis B with interferon: Experience in western countries. *Semin Liver Dis* **9:** 240–248, 1989.
7. Doong SL, Tsai CH, Schinazi RF, Liotta DC and Cheng YC, Inhibition of the replication of hepatitis B virus *in vitro* by 2′,3′-dideoxy-3′-thiacytidine and related analogues. *Proc Natl Acad Sci USA* **88:** 8495–8499, 1991.
8. Tyrrell DLJ, Fischer K, Savani K, Tan W and Jewell L, Treatment of chimpanzees and ducks with lamivudine, 2′,3′ dideoxy 3′ thiacytidine, results in a rapid suppression of

* Petit MA, Buffello D, Roche B, Dussaix E, Eberle F, Thiaux C, Bismuth H, Samuel D and Feray C, Characterization of new hepatitis B virus (HBV) variants emerging from patients sequentially treated with antiviral therapies and lamivudine (3TC) for recurrent hepatitis B after liver transplantation. *Molecular Biology of Hepatitis B Viruses*, p. 132, Aug. 30–Sep. 3, UCSD, 1998.

hepaviral DNA in sera. *Clin Invest Med* **16** (Suppl 4): B77, 1993.
9. Honkoop P, de Man RA, Zondervan PE and Schalm SW, Histological improvement in patients with chronic hepatitis B virus infection treated with lamivudine. *Liver* **17:** 103–106, 1997.
10. Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C and Rubin M, A preliminary trial of lamivudine for chronic viral hepatitis B infection. *N Engl J Med* **333:** 1657–1661, 1995.
11. Hoofnagle JH, Therapy of viral hepatitis. *Digestion* **59:** 563–578, 1998.
12. Fu L and Cheng YC, Role of additional mutations outside the YMDD motif of hepatitis B virus polymerase in L(-)SddC (3TC) resistance. *Biochem Pharmacol* **55:** 1567–1572, 1998.
13. Hammer SM, Katzenstein DA, Hughes MD, Gundacker H, Schooley RT, Haubrich RH, Henry WK, Lederman MM, Phair JP, Niu M, Hirsch MS and Merigan TC for the AIDS Clinical Trials Group Study 175 Study Team, A trial comparing nucleoside monotherapy with combination therapy in HIV-infected adults with CD4 cell counts from 200 to 500 per cubic millimeter. *N Engl J Med* **335:** 1081–1090, 1996.
14. Johnson VA, Merrill DP, Videler JA, Chou TC, Byington RE, Eron JJ, D'Aquila RT and Hirsch MS, Two-drug combinations of zidovudine, didanosine, and recombinant interferon-α A inhibit replication of zidovudine-resistant human immunodeficiency virus type I synergistically *in vitro*. *J Infect Dis* **164:** 646–655, 1991.
15. St. Clair M, Pennington KN, Rooney J and Barry DW, Rapid screening of antiretroviral combinations. *J Acquir Immune Defic Syndr* **10** (Suppl 1): S24–S27, 1995.
16. Heijtink RA, De Wilde GA, Kruining J, Berk L, Balzarini J, De Clercq E, Holy A and Schalm SW, Inhibitory effect of 9-(2-phosphonylmethoxyethyl)-adenine (PMEA) on human and duck hepatitis B virus infection. *Antiviral Res* **21:** 141–153, 1993.
17. Korba B and Boyd M, Penciclovir is a selective inhibitor of hepatitis B virus replication in cultured human hepatoblastoma cells. *Antimicrob Agents Chemother* **40:** 1282–1284, 1996.
18. Shaw T, Mok SS and Locarnini SA, Inhibition of hepatitis B virus DNA polymerase by enantiomers of penciclovir triphosphate and metabolic basis for selective inhibition of HBV replication by penciclovir. *Hepatology* **24:** 996–1002, 1996.
19. Hung LF, Brumbaugh AE, Bhatia G, Marion PL, Hung PP, Norbeck DW, Plattner JJ and Robinson WS, Effects of purine nucleoside analogues with a cyclobutane ring and erythromycin A oxime derivatives on duck hepatitis B virus replication *in vivo* and in cell culture and HIV-1 in cell culture. *J Med Virol* **35:** 180–186, 1991.
20. Aye TT, Bartholomeusz A, Shaw T, Bowden S, Breschkin A, McMillan J, Angus P and Locarnini S, Hepatitis B virus polymerase mutations during antiviral therapy in a patient following liver transplantation. *J Hepatol* **26:** 1148–1153, 1997.
21. Zoulim F, Pichoud C, Wang Z, Aguesse-Germon S and Trepo C, Hepatitis B virus genome variability during famciclovir therapy. *Hepatology* **26** (No 4, Pt 2)**:** 428A, 1997.
22. Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM and Harrison TJ, Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology* **24:** 711–713, 1996.
23. Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM and Tyrrell DLJ, Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine *in vivo*. *Hepatology* **24:** 714–717, 1996.
24. Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DLJ, Brown N for the Lamivudine Clinical Investigation Group and Condreay LD, Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. *Hepatology* **27:** 1670–1677, 1998.
25. Lin TS, Luo MZ, Liu MC, Zhu YL, Gullen E, Dutschman GE and Cheng YC, Design and synthesis of 2′,3′-dideoxy-2′,3′-didehydro-β-L-cytidine (β-L(-)d4C) and 2′,3′-dideoxy-2′,3′-didehydro-β-L-5-fluorocytidine (β-L(-)Fd4C), two exceptionally potent inhibitors of human hepatitis B virus (HBV) and potent inhibitors of human immunodeficiency virus (HIV) *in vitro*. *J Med Chem* **39:** 1757–1759, 1996.
26. Pai SB, Liu SH, Zhu YL, Chu CK and Cheng YC, Inhibition of hepatitis B virus by a novel L-nucleoside, 2′-fluoro-5-methyl-β-L-arabinofuranosyl uracil. *Antimicrob Agents Chemother* **40:** 380–386, 1996.
27. Chu CK, Ma T, Shanmuganathan K, Wang C, Xiang Y, Pai SB, Yao GQ, Sommadossi JP and Cheng YC, Use of 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil as a novel antiviral agent for hepatitis B virus and Epstein-Barr virus. *Antimicrob Agents Chemother* **39:** 979–981, 1995.
28. Fu L, Wu X, Kong YY and Wang Y, Regulation of hepatitis B virus gene expression by its enhancers. *Acta Biochim Biophys Sin* **28:** 590–599, 1996.
29. Fu L, Wu X, Kong YY and Wang Y, Regulation of hepatitis B virus gene expression by core promoter and its upstream sequences. *Chin J Virol* **13:** 215–223, 1997.
30. Chang CN, Doong SL, Zhou JH, Beach JW, Jeong LS, Chu CK, Tsai CH and Cheng YC, Deoxycytidine deaminase-resistant stereoisomer is the active form of (±)-2′,3′-dideoxy-3′-thiacytidine in the inhibition of hepatitis B virus replication. *J Biol Chem* **267:** 13938–13942, 1992.
31. Bartholomew MM, Jansen RW, Jeffers LJ, Reddy KR, Johnson LC, Bunzendahl H, Condreay LD, Tzakis AG, Schiff ER and Brown NA, Hepatitis-B-virus resistance to lamivudine given for recurrent infection after orthotopic liver transplantation. *Lancet* **349:** 20–22, 1997.
32. Chayama K, Suzuki Y, Kobayashi M, Kobayashi M, Tsubota A, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Murashima N, Ikeda K and Kumada H, Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild-type after cessation of therapy. *Hepatology* **27:** 1711–1716, 1998.
33. Liu SH, Grove KL and Cheng YC, Unique metabolism of a novel antiviral L-nucleoside analog, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil: A substrate for both thymidine kinase and deoxycytidine kinase. *Antimicrob Agents Chemother* **42:** 833–839, 1998.
34. Barry DW, New therapies in development for HIV/AIDS and HBV infection. Clinical Horizons: New approaches to the pharmacotherapy of HIV and HBV disease (Symposium Syllabus). July 1998, Geneva, Switzerland.
35. Locarnini S, The challenges of treating HBV disease. Clinical Horizons: New approaches to the pharmacotherapy of HIV and HBV disease (Symposium Syllabus). July 1998, Geneva, Switzerland.
36. Shields PL, Ling L, Harrison T, Boxall E, Elias E and Mutimer DJ, Management and outcome of lamivudine (LAM)-resistant hepatitis B virus (HBV) infection after liver transplantation (LT). *Hepatology* **26** (No 4, Pt 2)**:** 260A, 1997.
37. Tillmann HL, Trautwein C, Bock T, Glowenka M, Kruger M, Boker K, Jackel E, Pichlmayr R, Condreay L, Bruns I, Deslaurias M, Gauthier J and Manns MP, Response and mutations in patients sequentially treated with lamivudine and famciclovir for recurrent hepatitis B after liver transplantation. *Hepatology* **26** (No 4, Pt 2)**:** 429A, 1997.
38. Pichoud C, Seigneres B, Wang Z, Trepo C and Zoulim F, Transient selection of a hepatitis B virus polymerase gene

mutant associated with a decreased replication capacity and famciclovir resistance. *Hepatology* **29:** 230–237, 1999.
39. Gaillard RK, Allen MI, Miller WH, Barnard J and Condreay LD, *In vitro* evaluation of potential add-on therapeutics for the treatment of lamivudine treated patients infected with YMDD mutant HBV. *Hepatology* **27** (No 4, Pt 2)**:** 318A, 1998.
40. Xiong X, Yang H, Westland CE, Toole JJ and Gibbs CS, Human hepatitis B virus DNA polymerases which contain mutations arising during famciclovir treatment remain sensitive to adefovir. *Hepatology* **27** (No 4, Pt 2)**:** 491A, 1998.
41. Gilson RJC, Murray-Lyon IM, Nelson MR, Rice SJ, Tedder RS, Murray A, Jaffe HS and Weller IVD, Extended treatment with adefovir dipivoxil in patients with chronic hepatitis B virus infection. *Hepatology* **27** (No 4, Pt 2)**:** 491A, 1998.
42. Barditch-Crovo P, Toole J, Hendrix CW, Cundy KC, Ebeling D, Jaffe HS and Lietman PS, Anti-human immunodeficiency virus (HIV) activity, safety, and pharmacokinetics of adefovir dipivoxil (9-[2-(bis-pivaloyloxymethyl)-phosphonylmethoxyethyl]adenine) in HIV-infected patients. *J Infect Dis* **176:** 406–413, 1997.