CMCdG, a Novel Nucleoside Analog with Favorable Safety Features, Exerts Potent Activity against Wild-Type and Entecavir-Resistant Hepatitis B Virus

Nobuyo Higashi-Kuwata,^a Sanae Hayashi,^b Debananda Das,^c Satoru Kohgo,^a Shuko Murakami,^b Shin-ichiro Hattori,^a Shuhei Imoto,^d David J. Venzon,^e Kamalendra Singh,^f Stefan G. Sarafianos,^f Yasuhito Tanaka,^b Hiroaki Mitsuya^{a,c,g}

^aDepartment of Refractory Viral Infections, National Center for Global Health & Medicine Research Institute, Tokyo, Japan

^bDepartment of Virology & Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

^cExperimental Retrovirology Section, HIV and AIDS Malignancy Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

^dFaculty of Pharmaceutical Sciences, Sojo University, Kumamoto, Japan

^eBiostatistics and Data Management Section, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

^fLaboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia, USA

^gDepartment of Clinical Sciences, Kumamoto University Hospital, Kumamoto, Japan

ABSTRACT We designed, synthesized, and characterized a novel nucleoside analog, (1S,3S,5S)-3-(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl)-5-hydroxy-1-(hydroxymethyl)-2-methylene-cyclopentanecarbonitrile, or 4'-cyano-methylenecarbocyclic-2'-deoxyguanosine (CMCdG), and evaluated its anti-hepatitis B virus (anti-HBV) activity, safety, and related features. CMCdG's *in vitro* activity was determined using quantitative PCR and Southern blotting assays, and its cytotoxicity was determined with a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay, while its *in vivo* activity and safety were determined in human liver-chimeric mice infected with wild-type HBV genotype Ce (HBV_{WT}^{Ce}) and an entecavir (ETV)-resistant HBV variant containing the amino acid substitutions L180M, S202G, and M204V (HBV_{ETV-R}^{L180M/S202G/M204V}). CMCdG potentially inhibited HBV production in HepG2.2.15 cells (50% inhibitory concentration [IC₅₀], ~30 nM) and HBV_{WT}^{Ce} plasmid-transfected Huh7 cells (IC₅₀, 206 nM) and efficiently suppressed ETV-resistant HBV_{ETV-R}^{L180M/S202G/M204V} (IC₅₀, 2,657 nM), while it showed no or little cytotoxicity (50% cytotoxic concentration, >500 μM in most hepatocytic cells examined). Two-week peroral administration of CMCdG (1 mg/kg of body weight/day once a day [q.d.]) to HBV_{WT}^{Ce}-infected human liver-chimeric mice reduced the level of viremia by ~2 logs. CMCdG also reduced the level of HBV_{ETV-R}^{L180M/S202G/M204V} viremia by ~1 log in HBV_{ETV-R}^{L180M/S202G/M204V}-infected human liver-chimeric mice, while ETV (1 mg/kg/day q.d.) completely failed to reduce the viremia. None of the CMCdG-treated mice had significant drug-related changes in body weights or serum human albumin levels. Structural analyses using homology modeling, semiempirical quantum methods, and molecular dynamics revealed that although ETV triphosphate (TP) forms good van der Waals contacts with L180 and M204 of HBV_{WT}^{Ce} reverse transcriptase (RT), its contacts with the M180 substitution are totally lost in the HBV_{ETV-R}^{L180M/S202G/M204V} RT complex. However, CMCdG-TP retains good contacts with both the HBV_{WT}^{Ce} RT and HBV_{ETV-R}^{L180M/S202G/M204V} RT complexes. The present data warrant further studies toward the development of CMCdG as a potential therapeutic for patients infected with drug-resistant HBV and shed light on the further development of more potent and safer anti-HBV agents.

KEYWORDS anti-HBV drugs, drug-resistant HBV variants, human liver-chimeric mice, molecular modeling, nucleoside/nucleotide reverse transcriptase inhibitors

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Address correspondence to Hiroaki Mitsuya, hiroaki.mitsuya2@nih.gov.

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Hepatitis B virus (HBV), a double-stranded DNA virus, belongs to the family of reverse transcriptase (RT)-containing *Hepadnaviridae* and represents a human-pathogenic virus that is responsible for acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1). From 1990 to 2007, the incidence of acute hepatitis B decreased by >80% in the United States. The universal screening of pregnant women to prevent perinatal HBV transmission with the HBV vaccine has been directly responsible for this significant decline, in particular, among children and adolescents (2, 3). Nevertheless, the HBV infection prevalence among individuals older than 50 years of age persists at virtually the same level (3). Moreover, among individuals undertaking multiple sexual exposures, men who have sex with men, and illicit drug users, HBV infection remains a serious threat since those individuals have a greater risk of human immunodeficiency virus type 1 (HIV-1) coinfection than those who do not have such practices (2). Worse, it has been noted that those coinfecting with HBV and HIV-1 often undergo more rapid progression of liver fibrosis and have a greater risk of liver cirrhosis and hepatocellular carcinoma than those with HBV infection alone (4, 5).

Various strategies have been used to treat HBV infection. Nucleos(t)ide reverse transcriptase inhibitors (NRTIs) represent an important class of therapeutics for treating HBV infection, potentially reduce HBV viremia, and have changed the treatment paradigm and prognosis of HBV infection. Lamivudine (3TC or LVD), the first oral anti-HBV agent, is safe and well tolerated even in patients with decompensated liver cirrhosis (6). Other nucleosides, such as entecavir (ETV), adefovir pivoxil (ADV), telbivudine (LdT), tenofovir disoproxil fumarate (TDF), and tenofovir alafenamide (TAF), have proved efficacious in reducing HBV viremia. Among them, ETV represents the most commonly utilized therapeutic in the treatment of HBV infection due to its potent anti-HBV activity and the reduced risk of the emergence of ETV-resistant HBV variants, although 3TC-resistant HBV variants harboring amino acid substitutions in reverse transcriptase (RT), including M204V and L180M (HBV^{M204V} and HBV^{L180M/M204V}), are known to be less sensitive to ETV and significantly inclined to acquire higher levels of resistance to ETV (7). Thus, it has been recommended that ADV, TDF, or TAF be administered to individuals with HBV-resistant variants (i.e., HBV^{M204V}, HBV^{L180M/M204V}, and such) (8, 9). However, it is of note that long-term ADV or TDF treatment can cause adverse effects, such as nephrotoxicity and bone abnormalities (8, 9). TAF is a newly designed prodrug of tenofovir and has been shown to have improved bone and renal safety features compared to TDF (10); however, its long-term toxicity profiles have yet to be determined. Moreover, the cure of HBV infection or the elimination of HBV in once HBV-infected individuals is as yet elusive; therefore, novel anti-HBV therapeutics that are potent against wild-type HBV (HBV_{WT}) as well as existing drug-resistant HBV variants, that have no or less long-term adverse effects, and that do not allow the emergence of drug-resistant variants to the very drugs are urgently needed.

We previously reported on 4'-ethynyl-2'-fluoro-2'-deoxyadenosine (EFdA), which has a unique 4'-ethynyl moiety and retains the 3'-hydroxyl moiety; we demonstrated that EFdA exerts highly potent anti-HIV-1 activity *in vitro* and *in vivo* (11–16), and it is currently undergoing phase 2b clinical trials in the United States and other countries (17, 18). We have also previously reported that two EFdA congeners, 4'-C-cyano-2'-amino-2'-deoxyadenosine (CAAdA) and 4'-C-cyano-2'-deoxyguanosine (CdG; Fig. 1) exert potent activity against HBV_{WT} and drug-resistant HBV variants, such as HBV^{L180M/M204V}_{ETV-R} and HBV^{A181T/N236T}_{ADV-R}, both in cell culture and in HBV-inoculated severe combined immunodeficiency (SCID) mice that were transgenic for the urokinase-type plasminogen activator (uPA) gene (uPA/SCID mice) and in which the liver was partly replaced with human hepatocytes (human liver-chimeric mice) (19). However, both CAAdA and CdG proved to be more cytotoxic than currently available anti-HBV therapeutics and were not pursued for further development. In the present study, we designed, synthesized, and identified a novel nucleoside analog with improved properties, (1S,3S,5S)-3-(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl)-5-hydroxy-1-(hydroxymethyl)-2-methylene-cyclopentane-carbonitrile, or 4'-cyano-methylenecarbocyclic-2'-deoxyguanosine (CMCdG).

FIG 1 Structures and molecular weights (M.W.) of entecavir (ETV), 4'-C-cyano-2'-deoxyguanosine (CdG), and 4'-cyano-methylenecarbocyclic-2'-deoxyguanosine (CMCdG).

RESULTS

CMCdG potently inhibits the synthesis of HBV_{WT}^D DNA in HepG2.2.15 cells. We previously reported that CAdA and CdG highly potently suppressed the replication of a wild-type HBV genotype D (HBV_{WT}^D) in HepG2.2.15 cells. CAdA and CdG also suppressed the production of wild-type HBV genotype Ce (HBV_{WT}^{Ce}) in HBV_{WT}^{Ce} plasmid-transfected Huh7 cells and potently decreased the HBV_{WT}^{Ce} viremia level in HBV_{WT}^{Ce}-exposed human liver-chimeric mice (19). CAdA and CdG were also potent against a highly ETV-resistant HBV variant containing three critical amino acid substitutions: L180M, S202G, and M204V (HBV^{L180M/S202G/M204V}) as examined in HBV^{L180M/S202G/M204V} plasmid-transfected Huh7 cells and HBV^{L180M/S202G/M204V}-exposed human liver-chimeric mice. However, both compounds proved to be much more cytotoxic than ETV, and they were dropped from further development (19). Thus, based on the structural findings that we had obtained (19, 20), we continued the design and synthesis of approximately 220 novel nucleoside analogs containing a cyano moiety at the 4' position of the ribose and identified CMCdG. It is of note that CMCdG structurally resembles CdG, in that both compounds have a 4' cyano moiety, and it also resembles ETV, in that both CMCdG and ETV have an exocyclic double bond or methyldiene in the 4' position of the cyclopentyl moiety (Fig. 1). To determine whether CMCdG reduces the amount of intracellular HBV_{WT}^D DNA in HepG2.2.15 cells, DNA isolated from HepG2.2.15 cells, which were cultured in the presence of CMCdG over 14 days, was subjected to real-time HBV PCR as described in the Materials and Methods. Antiviral activity was scored as the percent inhibition relative to that of drug-unexposed control cells. As shown in Table 1, the activity of CMCdG against HBV_{WT}^D was significantly more potent than that of ADV and TDF and slightly more potent than that of TAF, although the activity of CMCdG was significantly less potent than that of ETV or CdG. The anti-HIV activity of CMCdG and other agents was also examined. As shown in Table 1, ETV was the least potent compound against HIV-1, as previously described (19, 21). TDF, TAF, and CdG were highly potent against HIV-1, with 50% inhibitory concentration (IC₅₀) values ranging from 0.4 to 36 nM, while ADV was found to be only moderately

TABLE 1 Anti-HBV activity, anti-HIV-1 activity, and cytotoxicity of NRTIs^a

	IC ₅₀ (nM) against:		CC ₅₀ (μM) in:					SI with:	
Drug	HBV _{WT} ^D	HIV-1 _{LAI}	HepG2.2.15 cells	Huh7 cells	HepG2 cells	PXB cells	MT-2 cells	Anti-HBV agents	Anti-HIV-1 agent
ETV	1.7 ± 0.3	2,501 ± 884.9	63 ± 11	246 ± 56	74 ± 6.5	251 ± 19.0	115 ± 6.51	37,059	46
ADV	1,031 ± 43	701 ± 118	6.5 ± 1.9	4.5 ± 2.6	19 ± 3.6	73 ± 13	5.2 ± 1.7	6.3	7.4
TDF	224 ± 27.6	36 ± 8.5	56 ± 7.6	47 ± 8.9	35 ± 9.5	31 ± 4.7	52 ± 7.5	250	1,444
TAF	54 ± 17	5.1 ± 1.2	35 ± 8.1	33 ± 4.6	20 ± 4.0	6.3 ± 1.5	20 ± 4.4	648	3,922
CdG	0.5 ± 0.1	0.4 ± 0.1	3.5 ± 0.5	0.8 ± 0.2	2.6 ± 0.5	0.32 ± 0.08	3.2 ± 0.7	7,000	8,000
CMCdG	23 ± 10	850 ± 52.8	>500	>500	89 ± 8.4	> 500	>500	>21,739	>5,882

^aThe anti-HBV activity of each compound was determined using HepG2.2.15 cells, while anti-HIV-1 activity was determined using HIV-1_{LAI} and MT-2 cells. Each assay was conducted in duplicate or triplicate. The values represent mean numbers (±1 SD) from two or three independent experiments. The selectivity index (SI) for anti-HBV activity was determined using the following formula: the CC₅₀ value of each compound in HepG2.2.15 cells/the IC₅₀ value of each compound required to suppress the synthesis of HBV DNA in HepG2.2.15 cells. The SI for anti-HIV activity was determined using the following formula: the CC₅₀ value of each compound in MT-2 cells/the IC₅₀ value of each compound required to suppress the replication of HIV-1_{LAI} in MT-2 cells.

FIG 2 Suppression of the synthesis of HBV DNA in HepG2.2.15 cells by ETV, CdG, and CMCdG. Intracellular HBV DNA levels were determined using a real-time HBV PCR and are shown as a percentage of the value for the controls without drug (control value = 100%). All data are shown as average values obtained from at least 3 independent experiments, conducted on different occasions. The broken line denotes the line for the 50% inhibitory concentration (IC_{50}). The final concentration of DMSO for the highest concentration of each agent (1 μ M) was 0.05% in culture. Thus, it is assumed that the effects of DMSO on the synthesis of HBV DNA are not significant in the data shown.

active against the wild-type strain HIV-1_{LAI}. CMCdG was also moderately active against HIV-1_{LAI}, with an IC_{50} value of 850 nM. Figure 2 illustrates representative titration curves comprised of the data from three independent assays giving IC_{50} s of 1.7, 0.5, and 23 nM to block HBV DNA synthesis in HepG2.2.15 cells for ETV, CdG, and CMCdG, respectively (Fig. 2).

CMCdG is void of significant cytotoxicity, while it potently inhibits HBV DNA synthesis. Importantly, unlike CdG, which is the 4'-cyano moiety-containing prototypic nucleoside reverse transcriptase inhibitor for CMCdG, CMCdG was almost virtually void of cytotoxicity in HepG2.2.15, Huh7, PXB, and MT-2 cells, with 50% cytotoxic concentration (CC_{50}) values of $>500 \mu$ M, and in HepG2 cells, with a CC_{50} value of 89 μ M. Thus, the selectivity index (SI) of CMCdG in relation to its activity against HBV_{WT}^D was $>21,739$, which was much greater than the SI values of ADV, TDF, TAF, and CdG. The SI values of ETV and CMCdG could not be compared since the use of concentrations greater than 500 μ M was not practically feasible for the determination of the exact CC_{50} values.

We further examined the effects of CMCdG on cellular mitochondrial DNA (mtDNA), cell growth, and cell viability using two cell lines, MOLT-4 and Daudi. After 7 days of culture in the presence of various concentrations of a test agent, the cells were harvested and subjected to determination of cell growth and cell viability and to DNA extraction (for real-time mtDNA PCR). As shown in Fig. 3A to C, in the case of ETV, a sharp decline in cell viability and growth was seen at 10 μ M in MOLT-4 cells, and the same was seen at 100 μ M in Daudi cells. There was also a sharp reduction in the amount of mtDNA at 10 and 100 μ M in MOLT-4 and Daudi cells, respectively. In contrast, there was no sharp reduction when the cells were cultivated in the presence of CMCdG in either MOLT-4 (Fig. 3B) or Daudi cells (Fig. 3D). There was only a slight reduction of cell viability, cell growth, and the amount of mtDNA at 100 μ M.

CMCdG blocks HBV_{WT}^{Ce}, HBV_{ETV-R}^{L180M/S202G/M204V}, and HBV_{ADV-R}^{A181T/N236T} replication *in vitro*. We subsequently asked whether CMCdG blocked the replication of HBV_{WT}^{Ce}, HBV_{ETV-R}^{L180M/S202G/M204V}, and HBV_{ADV-R}^{A181T/N236T}. Figure 4 shows representative results of a Southern blotting assay conducted on 2 to 4 multiple occasions. ETV effectively reduced the synthesis of HBV_{WT}^{Ce} at 1 and 10² nM concentrations, giving an IC_{50} value of 7 nM (Fig. 4a). However, as expected, ETV at 1 to 10⁵ nM concentrations failed to effectively block the DNA synthesis of HBV_{ETV-R}^{L180M/S202G/M204V}, even at the highest concentration tested (10⁵ nM), giving

FIG 3 Effects of ETV and CMCdG on cell viability, cell growth, and the amount of mitochondrial DNA (mtDNA) in MOLT-4 and Daudi cells. The cells were cultured in the presence of various concentrations of each test agent over a period of 7 days. Cell viability was determined with an MTT-based assay, and cell growth was determined by counting the number of viable cells. The cells were concurrently subjected to DNA extraction for determination of intracellular mtDNA amounts using a real-time mtDNA PCR. Each value is shown as a percentage of the value for the control without drug (control value = 100%). All data are shown as average values obtained from at least 3 independent experiments. In the case of ETV, a sharp decline in cell viability, cell growth, and the amount of mtDNA was seen between 10 and 100 μ M in MOLT-4 (A) and Daudi (C) cells. In contrast, no significant reduction was found when the cells were cultivated in the presence of CMCdG in either MOLT-4 (B) or Daudi (D) cells.

ETV an IC_{50} value of 53,090 nM, although ETV blocked the synthesis of HBV_{ADV-R}^{A181T/N236T} DNA fairly well, giving an IC_{50} value of 184 nM (Fig. 4c). ADV blocked the synthesis of HBV_{WT}^{Ce} and HBV_{ETV-R}^{L180M/S202G/M204V} DNA moderately well, giving IC_{50} values of 1,495 nM and 18,071 nM respectively, while it failed to block the synthesis of HBV_{ADV-R}^{A181T/N236T} DNA, giving an IC_{50} value of 48,905 nM (Fig. 4d to f). TDF suppressed the synthesis of HBV_{WT}^{Ce}, HBV_{ETV-R}^{L180M/S202G/M204V}, and HBV_{ADV-R}^{A181T/N236T} quite well, giving IC_{50} values of 256 nM, 521 nM, and 228 nM, respectively (Fig. 4g to i). CMCdG also effectively blocked the synthesis of HBV_{WT}^{Ce} and HBV_{ADV-R}^{A181T/N236T} DNA, with the IC_{50} values being 252 and 150 nM, respectively, and it blocked the DNA synthesis of HBV_{ETV-R}^{L180M/S202G/M204V} fairly well, giving an IC_{50} value of 3,558 nM (Fig. 4j to l). Table 2 shows the average data from the Southern blotting assay.

CMCdG blocks HBV replication in HIV_{WT}^{Ce}- and HBV_{ETV-R}^{L180M/S202G/M204V}-infected human liver-chimeric mice. We then asked whether CMCdG blocked the replication of HBV in human liver-chimeric mice. At 8 weeks following the inoculation of such mice with HBV_{WT}^{Ce}, they were orally gavaged with ETV or CMCdG (prepared at a concentration of 0.1 mg/ml in saline) using oral sondes so that the dose administered resulted in 1 mg/kg of body weight/day. Just before the administration of ETV, the HBV copy numbers in their plasma were as high as 9×10^8 copies/ml; however, the viremia levels went down significantly by day 7 of ETV administration, and a further viremia reduction to the lowest level occurred even after the termination of ETV administration (by day 21) (Fig. 5A, top left). CMCdG comparably blocked the replication of HBV_{WT}^{Ce} under the same conditions ($P = 0.10$) (Fig. 5A, top right). At 8 weeks following the inoculation of human liver-chimeric mice with HBV_{ETV-R}^{L180M/S202G/M204V}, the viremia levels had reached 7×10^7 to 2×10^8 copies/ml (Fig. 5A, bottom left). However, ETV at a dose of 1 mg/kg/day produced essentially no reduction in the HBV_{ETV-R}^{L180M/S202G/M204V} viremia levels (Fig. 5A lower left). In contrast, CMCdG at the same dose of 1 mg/kg/day brought about a significant level of viremia reduction by day 7 of administration ($P < 0.0001$, as statistically compared using the two-tailed P values corrected for multiple comparisons by the Hochberg method).

FIG 4 Reduction of HBV DNA synthesis by various NRTIs in plasmid-transfected Huh7 cells, assessed using Southern blot analysis. Huh7 cells were transfected with various HBV-harboring plasmids and cultured for 72 h in the presence of each compound, and DNA extracted from those cells was subjected to Southern blot analysis. For each compound, the relative amount of single-stranded replicative intermediate DNA (ssDNA) in the cells was determined using a chemiluminescence detection system (LAS 4000 mini-biomolecular imager; GE Healthcare). Based on the density of the signals observed, the IC_{50} values, which was the concentration at which a 50% decrease in the amount of intracellular HBV ssDNA was achieved, were determined and compared with those in untreated cells. The values were determined using the Forecast function of Microsoft Excel software. (a to l) Representative data and IC_{50} values. All cells were cultured in the presence of 0.1% DMSO, irrespective of the different agents and their concentrations.

The greatest magnitude of viremia reduction with CMCdG was $1.1 \log_{10}$ copies/ml (average, 1.2 and $1.0 \log_{10}$ copies/ml) (Fig. 5A, bottom right). Over the 14-day period of administration, the HBV_{ETV-R}^{L180M/S202G/M204V} viremia reduction in mice receiving CMCdG was significantly lower than that in mice receiving ETV ($P < 0.0001$). However, CMCdG had no impact on the levels of HBsAg in serum during the period of CMCdG administration in either HBV_{WT}^{Ce}- or HBV_{ETV-R}^{L180M/S202G/M204V}-exposed mice (Fig. 5B), suggesting that CMCdG reduced viremia by inhibiting HBV's reverse transcriptase activity but did not block the transcription of covalently closed circular DNA into mRNA or translation to produce HBV proteins.

CMCdG causes no significant changes in body weights and serum h-albumin levels in mice. Human albumin (h-albumin) levels in serum and the body weights of the treated mice were also monitored during treatment (Fig. 5C). HBV_{WT}^{Ce}-infected mice treated with CMCdG had slightly decreased h-albumin levels, but mice treated with ETV also had a slight decrease in h-albumin levels over the period of observation. HBV_{ETV-R}^{L180M/S202G/M204V}-infected mice treated with ETV and CMCdG also had a slight

TABLE 2 *In vitro* susceptibility of HBV_{WT}^{Ce}, HBV_{ETV-R}^{L180M/S202G/M204V}, and HBV_{ADV-R}^{A181T/N236T} to ETV, ADV, TDF, and CMCdG in HBV plasmid-transfected Huh7 cells

Drug	IC_{50} ^a (nM) against:		
	HBV _{WT} ^{Ce}	HBV _{ETV-R} ^{L180M/S202G/M204V}	HBV _{ADV-R} ^{A181T/N236T}
ETV	16 ± 12 ($n = 2$)	$64,037 \pm 15,482$ (4,002.3, $n = 2$)	105 ± 113 (6.6, $n = 2$)
ADV	$1,163 \pm 469.6$ ($n = 2$)	$30,774 \pm 16,119$ (26.5, $n = 4$)	$120,870 \pm 125,904$ (103.9, $n = 4$)
TDF	177 ± 69.8 ($n = 3$)	406 ± 103 (2.3, $n = 3$)	218 ± 14.0 (1.2, $n = 2$)
CMCdG	206 ± 62.6 ($n = 3$)	$2,657 \pm 1,273$ (12.9, $n = 2$)	96 ± 77 (0.5, $n = 2$)

^aThe IC_{50} values were obtained using Southern blotting assay. The IC_{50} values represent average numbers (± 1 SD) from two to four independent experiments. The numbers in parentheses represent the number of replicated assays (n) for HBV_{WT}^{Ce} and the fold change of the IC_{50} value compared to that against HBV_{WT}^{Ce}, number of replicated assays (n), for HBV_{ETV-R}^{L180M/S202G/M204V} and HBV_{ADV-R}^{A181T/N236T}.

FIG 5 Reduction of serum HBV_{WT}^{Ce} and HBV_{ETV-R}^{L180M/S202G/M204V} DNA amounts by CMCdG in human liver-chimeric mice. (A) At 8 weeks following the inoculation of human liver-chimeric mice with HBV_{WT}^{Ce} (top) or HBV_{ETV-R}^{L180M/S202G/M204V} (bottom), ETV or CMCdG was intragastrically administered (1 mg/kg/day). After the indicated treatments, serum HBV DNA levels were determined using real-time PCR ($n = 2$ to 3 mice per group). Each line illustrates the changes in DNA copy numbers in each mouse. Note that CMCdG reduced the level of HBV_{WT}^{Ce} viremia comparably to ETV ($P = 0.10$).

(Continued on next page)

decrease in their h-albumin levels; however, there was no significant difference observed between the two groups. There was no significant difference in body weights between the ETV-treated and CMCdG-treated groups (Fig. 5C). The mice were sacrificed upon the conclusion of CMCdG administration, and their liver tissues were histologically examined. Of note, liver chimerism harboring human hepatocytes (light pink area) and murine hepatocytes (magenta area) was evident (see Fig. S1A and B in the supplemental material). No necrotic or fibrotic changes were identified in the mice receiving CMCdG. Vacuolar degeneration of the cytoplasm was clearly observed at a high magnification; however, these changes were also seen in the similarly HBV-infected mice receiving no agents (vehicle only; Fig. S1C and D). Thus, it was concluded that CMCdG did not cause any identifiable liver toxicities over the 14-day period of its administration.

Structural analyses of CMCdG-TP interactions with HBV_{WT} RT and HBV_{ETV-R}^{L180M/S202G/M204V} RT. Finally, we analyzed the structural interactions of ETV triphosphate (TP), CdG-TP, and CMCdG-TP using the homology model of HBV_{WT} RT described in Materials and Methods (Fig. 6a to d). There were multiple strong polar interactions between ETV-TP and HBV_{WT} RT (Fig. 6a). The amino substituent of the purine of ETV-TP formed a hydrogen bond interaction with the backbone carbonyl of M171. The 3'-hydroxy substituent of the cyclopentyl group formed a hydrogen bond with the backbone nitrogen of F88. The triphosphate group of ETV-TP formed polar interactions with multiple amino acid residues of the RT of HBV_{WT}, S85, A86, and A87. The triphosphate group also interacted with a magnesium ion, which also interacted with D83 and V84. These networks of strong polar interactions should be responsible for stabilizing the binding of ETV-TP in the active site of RT of HBV_{WT}. Similar polar interactions were seen for CMCdG-TP with HBV_{WT} RT (Fig. 6b). Besides these polar interactions, both ETV-TP and CMCdG-TP formed good van der Waals interactions with M204 and D205 in the active site of HBV_{WT} RT, as seen from the analysis of the Connolly surface interactions (Fig. 6c and d, Fig. 7a and c, and Fig. S2a, c, i, and k). CdG does not have a moiety capable of interacting with Met204 (Fig. 7b and S2e to h), while the 4'-cyano group of CMCdG and CdG formed good van der Waals interactions with L180 of the RT of HBV_{WT} (Fig. 6h, Fig. 7b and c, and Fig. S2-e, -g, -i, and -k). The L180 interaction was also present in the complex of CMCdG with the RT of HBV_{ETV-R}^{L180M/S202G/M204V} (Fig. 6h and S2j and l). However, there was a distinct difference in the nonpolar interactions of ETV-TP with the RT of HBV_{ETV-R}^{L180M/S202G/M204V} from those of CMCdG with the RT of HBV_{ETV-R}^{L180M/S202G/M204V}. As shown in Fig. 6g and S2b and d, the ethylene group (methylene plus a carbon of the cyclopentyl) maintains some nonpolar interactions with the substituted V204; however, the nonpolar interactions with the substituted M180 were completely lost for ETV-TP complexed with the RT of HBV_{ETV-R}^{L180M/S202G/M204V} (Fig. 6g and S2b and d). This was apparently because of the lack of the 4'-cyano group in ETV compared to CMCdG (Fig. 6h and S2j and l). Thus, from the observations that the interactions with V204 and D205 persist in both complexes of CMCdG-TP and ETV-TP with the RT of HBV_{ETV-R}^{L180M/S202G/M204V}, it is most likely that the difference in the interac-

FIG 5 Legend (Continued)

However, the reduction of the level of HBV_{ETV-R}^{L180M/S202G/M204V} viremia by CMCdG was significantly greater than that by ETV over 14 days ($P < 0.0001$). Of note, the initial HBV_{ETV-R}^{L180M/S202G/M204V} viremia levels in HBV_{ETV-R}^{L180M/S202G/M204V}-infected mice ($n = 2$) were relatively greater than the initial HBV_{WT}^{Ce} viremia levels in HBV_{WT}^{Ce}-infected mice ($n = 2$) since these two experiments were conducted on two different occasions, although the conditions used were the same. The reduction seen with CMCdG persisted over a week following the conclusion of administration. (B) Serum hepatitis B surface antigen (HBsAg) levels were monitored. Each line illustrates the changes in HBsAg levels in an individual mouse. There were no significant changes in HBsAg levels during the period of CMCdG or ETV administration in either HBV_{WT}^{Ce}- or HBV_{ETV-R}^{L180M/S202G/M204V}-exposed human liver-chimeric mice. (C) The serum human albumin level and body weight of each mouse were monitored. Each line denotes an individual mouse. There were no significant differences between the groups receiving ETV and CMCdG. The thick bar in each panel denotes the period of administration (14 days) of a dose of 1 mg/kg/day.

FIG 6 Polar and nonpolar interactions between HBV_{WT} reverse transcriptase (RT) and HBV_{ETV-R}^{L180M/S202G/M204V} RT complexed with ETV triphosphate (TP) or CMCdG-TP. (a and e) Polar interactions of ETV-TP with HBV_{WT} RT (a) and HBV_{ETV-R}^{L180M/S202G/M204V} RT (e); (b and f) polar interactions of CMCdG-TP with HBV_{WT} RT (b) and HBV_{ETV-R}^{L180M/S202G/M204V} RT (f); (c and g) Connolly surface interactions of ETV-TP with HBV_{WT} RT (c) and HBV_{ETV-R}^{L180M/S202G/M204V} RT (g); (d and h) Connolly surface interactions of CMCdG-TP with HBV_{WT} RT (d) and HBV_{ETV-R}^{L180M/S202G/M204V} RT (h). ETV-TP and CMCdG-TP are shown as thick sticks and green carbons. Selected HBV_{WT} RT amino acids are shown as thin sticks and gray carbons. Hydrogen bonds are shown as yellow dotted lines. The magnesium ion is shown as a magenta sphere. Surfaces for ETV-TP and CMCdG-TP are shown in gray, while those for residues 180, 204, and 205 are shown in magenta, orange, and yellow, respectively.

tions of CMCdG-TP and ETV-TP with the RT of HBV_{ETV-R}^{L180M/S202G/M204V} arises because of the sustained interactions of the 4'-cyano group of CMCdG-TP with L180 in the RT of HBV_{WT} as well as with the substituted amino acid M180 in the RT of HBV_{ETV-R}^{L180M/S202G/M204V}.

FIG 7 Interactions of ETV-TP, CdG-TP, and CMCdG-TP with the hydrophobic pockets of HBV_{WT} RT. The Connolly surface interactions of important substituents of the inhibitors with L180 and M204 of HBV_{WT} RT are shown. Transparent surfaces of the selected inhibitor regions are in gray; L180 and M204 surfaces are shown in maroon and orange, respectively. (a) The ethylene group of ETV-TP has van der Waals interactions with M204, whereas the hydrogen occupying the 4'-substituent position does not have much interaction with L180. (b) The oxygen (at a position corresponding to the ethylene of ETV) in CdG-TP has no interaction with M204, whereas the 4'-cyano substituent has a good interaction with L180. (c) Only CMCdG-TP has substituents with good van der Waals surface interactions with both L180 (through its 4'-cyano moiety) and M204 (through its ethylene moiety).

DISCUSSION

In the present study, we demonstrated that a novel 4'-modified nucleoside HBV reverse transcriptase inhibitor (NRTI), CMCdG, blocks the activity of RT of both HBV and HIV-1, thereby suppressing the infectivity and replication of HBV and HIV-1 (Tables 1 and 2). Unlike the approved conventional anti-HIV-1 NRTIs bearing the 2'-3'-dideoxy configuration, CMCdG retains a 3'-hydroxyl and is assumed to act as a chain-

terminating agent by diminishing translocation from the pretranslocation nucleotide-binding site to the posttranslocation primer-binding site due to the presence of the 4' substitute, the 4'-cyano moiety, as we have shown in the case of EFdA (22). We have previously reported that two 4'-modified NRTIs, CAdA and CdG, both of which bear the 4'-cyano moiety, highly potently block HBV DNA production in HepG2.2.15 cells with IC_{50} values of ~ 0.4 nM, and ETV showed a similar potency with an IC_{50} value of ~ 0.7 nM (19). Southern blot analysis employing HBV-containing plasmid-transfected Huh7 cells showed that CAdA exerted potent activity against wild-type HBV (IC_{50} , ~ 7 nM) as well as ETV-resistant HBV (IC_{50} , ~ 70 nM for HBV_{ETV-R}^{L180M/S202G/M204V}), while ETV did not significantly reduce the synthesis of HBV_{ETV-R}^{L180M/S202G/M204V} DNA even at 1 μ M. In this regard, it is noteworthy that once-daily peroral CAdA administration decreased the level of viremia of HBV_{ETV-R}^{L180M/S202G/M204V} in human liver-chimeric, HBV_{ETV-R}^{L180M/S202G/M204V}-infected mice; however, ETV showed no reduction of the level of HBV_{ETV-R}^{L180M/S202G/M204V} viremia in those mice (19). As the viremia levels were statistically compared using the two-tailed *P* values corrected for multiple comparisons by the Hochberg method, there was a significant difference with a *P* value of 0.0005 (19). Nevertheless, both CAdA and CdG proved to be significantly more toxic in test tubes (CC_{50} values, 3.2 to 3.8 μ M) than ETV (CC_{50} values, >100 μ M), as assessed using HepG2.2.15 cells (19); thus, CAdA and CdG were dropped from the preclinical development pipeline.

Thus, we redesigned and newly synthesized a series of analogs of CAdA and CdG. In the redesign, in an attempt to potentially reduce the cytotoxicity seen in CAdA and CdG, we changed the 2'-deoxy-ribose moiety of CAdA and CdG into the 5-hydroxy-1-(hydroxymethyl)-2-methylenecyclopentane (HHMMC) moiety, since it has been shown that entecavir containing the HHMMC moiety has very favorable safety features in treating patients with HBV infection (23, 24). At the same time, in an attempt to preserve the potency of CAdA and CdG against HBV_{WT}^{Ce} and HBV_{ETV-R}^{L180M/S202G/M204V} as well as the potentially high genetic barrier against the emergence of drug-resistant HBV variants, as seen in the case of the potent activity of EFdA against various multi-NRTI-resistant HIV-1 variants (25), we retained the 4'-cyano moiety in the series of analogs that we newly synthesized. Indeed, the CMCdG that we newly developed potently blocked the production of HBV_{WT}^{Ce} DNA comparably to ETV in HepG2.2.15 cells and HBV_{WT}^{Ce} plasmid-transfected Huh7 cells. Importantly, CMCdG efficiently suppressed ETV-resistant HBV_{ETV-R}^{L180M/S202G/M204V} with an IC_{50} value of 2,657 nM, while under the same conditions, the IC_{50} values of ETV, ADV, and TDF were 64,037, 30,774, and 406 nM, respectively. Although TDF suppressed HBV_{ETV-R}^{L180M/S202G/M204V} at a lower dose (IC_{50} , 406 nM) than CMCdG (IC_{50} , 2,657 nM), TDF is known to cause problematic adverse effects, including nephropathy and bone abnormalities, in both HIV-infected patients (26) and HBV-infected patients (27, 28). In this regard, TDF's CC_{50} values spanned from 31 to 56 μ M (Table 1), and its SI turned out to be as low as 250; TAF's CC_{50} values ranged from 6.3 to 35 μ M, and its SI was 648. It was noted that the CC_{50} values of TDF and TAF in fresh human hepatocytes (PXB cells) were as low as 31 and 6.3 μ M, respectively (Table 1). However, CMCdG's CC_{50} values were >500 μ M in 4 of 5 cell types examined, including PXB cells, and its SI was $>21,739$ (Table 1). Considering that it is currently thought that therapy of HBV infection is life long, these data may suggest that CMCdG could have advantages in its safety features over TDF and TAF. Moreover, CMCdG was also potent against ADV-resistant HBV_{ADV-R}^{A181T/N236T} with an IC_{50} of 96 nM, whereas the IC_{50} values were 105, 120,870, and 218 nM for ETV, ADV, and TDF, respectively (Table 2). CMCdG comparably and potently blocked the production of HBV DNA in chronically HBV_{WT}^{Ce}-infected human liver-chimeric mice over 2 weeks (*P* = 0.10) and statistically significantly more potently blocked the production of HBV DNA in HBV_{ETV-R}^{L180M/S202G/M204V}-infected human liver-chimeric mice (*P* < 0.0001) than ETV (Fig. 5A). Interestingly, the toxicity of CMCdG was quite mitigated, as assessed using multiple human long-term-cultured hepatocytic cell lines (HepG2.2.15, HepG2, and Huh7), nonhepatocytic cell lines (MT-2, MOLT-4, and Daudi), as well as human primary hepatocytes (PXB cells) (Table 1 and Fig. 3). In fact, the CC_{50}

values of CMCdG were much greater than those of any of the currently available anti-HBV therapeutics examined (Table 1). Moreover, when CMCdG was intragastrically administered to HBV_{WT}^{Ce}- or HBV_{ETV-R}^{L180M/S202G/M204V}-infected human liver-chimeric mice, no significant changes in their body weights or serum human albumin levels were found (Fig. 5C). In the present study, we used human albumin levels for monitoring the potential toxicity of the test compounds since in the human hepatocyte-chimeric mice that we employed in the present work, the murine serum alanine transaminase (ALT) levels were constantly elevated (100 to 200 U/liter), because murine hepatocytes are designed to be continuously destroyed due to uPA accumulation in the endoplasmic reticulum of murine hepatocytes (29). In addition, the ALT assays that are routinely used cannot distinguish human ALT from murine ALT. Therefore, monitoring the possible toxicity of test compounds cannot be done with ALT levels. These data strongly suggest that the safety of CMCdG compared to that of the currently available anti-HBV therapeutics would be highly favorable, although the safety of agents such as CMCdG has to be carefully monitored in well-controlled preclinical and clinical trials.

We have previously reported that another 4'-cyano-bearing NRTI, EFdA, blocks HIV-1's RT as a translocation-defective inhibitor that highly effectively slows DNA synthesis, acting as a *de facto* immediate chain terminator (22, 30, 31). EFdA-TP has been shown to function as a delayed chain terminator, allowing incorporation of an additional deoxynucleoside triphosphate before blocking DNA synthesis (22). In such a case, EFdA monophosphate (MP)-terminated primers are protected from excision (31), a major NRTI resistance mechanism of HIV-1 RT (32). EFdA-MP can be efficiently misincorporated by HIV-1's RT, leading to mismatched primers that are extremely hard to extend and that are also protected from excision. Such multiple inhibitory features of EFdA should explain why HIV-1's resistance to EFdA hardly emerges (24). It should be noted, however, that CMCdG structurally bears both the 4'-cyano moiety that EFdA, CAdA, and CdG bear and the methylidenecyclopentyl ring with a hydroxyl group that ETV contains (Fig. 1). It has been shown that ETV's 3'-hydroxyl group allows the addition of several nucleotides following ETV-MP incorporation before chain termination. The phenomenon of RT inhibition by ETV occurs in both HIV-1 and HBV (33, 34) and is referred to as "delayed chain termination," which enables evasion of incorporated NRTI phosphorolytic excision. The presence of the 4' modification should be the key to the high genetic barrier seen in a nucleoside HIV-1 reverse transcriptase inhibitor, EFdA (25); CMCdG is also expected to have a high genetic barrier to the emergence of CMDdG-resistant HBV variants. However, currently, no robust system for evaluation of the emergence of drug-resistant HBV variants is available, and the issue of the emergence of CMCdG-resistant HBV has to be further examined as a critical future topic.

The structural analyses in the present study revealed that CMCdG-TP forms strong hydrogen bond interactions with a variety of amino acid residues upon and/or in the vicinity of the active site of the reverse transcriptase of HBV_{WT}, as ETV-TP similarly does (Fig. 6a and b). The ethylene (methylene plus a carbon of the cyclopentyl) of ETV-TP forms favorable van der Waals contacts with M204 of the HBV_{WT} RT, while ETV's 4'-hydrogen atom is relatively distant from L180 and apparently forms only moderately good contacts with L180 (Fig. 6a and 7a). The oxygen atom of CdG-TP is distant from M204, but its 4'-cyano moiety forms good contacts with L180 (Fig. 7b). In contrast, both the ethylene and 4'-cyano of CMCdG-TP form good van der Waals contacts with M204 and L180, respectively (Fig. 6b and 7c). It is also noteworthy that CMCdG-TP also forms highly favorable van der Waals interactions with three active-site amino acid residues, L180, M204, and M205, as ETV-TP does (Fig. 6c and d). Both of the favorable polar and nonpolar interactions seen in ETV and CMCdG should be, at least in part, the reason why both agents exert potent activity against HBV_{WT}^{Ce}, although it is not clear from these modeling analyses why ETV is comparably or more potent than CMCdG (IC₅₀ values, 16 ± 12 nM and 206 ± 62.6 nM for ETV and CMCdG, respectively; Table 2) and CdG exerts comparably or more potent activity against HBV than ETV (18). Importantly,

CMCdG-TP maintains very good van der Waals interactions with the mutated reverse transcriptase of ETV-resistant HBV containing L180M, S202G, and M204V, while ETV-TP virtually fully lost its contacts with L180M. This loss of ETV-TP's contact with L180M at least in part should reduce the activity of ETV-TP against HBV_{ETV-R}^{L180M/S202G/M204V} (Fig. 4a to c and Fig. 5A; Table 2). We previously reported that CAdA and CdG highly potently suppressed the replication of HBV_{WT}^D in HepG2.2.15 cells. These compounds also suppressed the production of the HBV_{WT}^{Ce} in the HBV_{WT}^{Ce} plasmid-transfected Huh7 cells and decreased the HBV_{WT}^{Ce} viremia level in HBV_{WT}^{Ce}-exposed human liver-chimeric mice.

We have also reported that a variety of 4'-modified nucleoside analogs, such as 4'-ethynyl-2'-deoxycytidine, 4'-ethynylarabinofuranosylcytosine, 4'-ethynyl-2'-deoxyadenosine, and 4'-ethynyl-2'-deoxyribofuranosyl-2,6-diaminopurine, were highly to moderately cytotoxic (11), although certain 4'-ethynyl-containing nucleosides, such as EFdA, were not toxic (12). Indeed, EFdA/MK8591 has been administered to individuals with HIV-1 infection and shown to be highly active against HIV-1 without bringing about significant toxicities and is presently in phase 2b clinical trials (17, 18). The reason why certain 4'-modified nucleosides are toxic while other 4'-modified compounds are not toxic is not known at this time.

Taken together, the present data on CMCdG warrant further studies toward its clinical development as a potential therapeutic for infection with wild-type and/or drug-resistant HBV and should shed light on the further optimization and development of more potent and less toxic anti-HBV therapeutics with a high genetic barrier to the emergence of drug-resistant HBV variants.

MATERIALS AND METHODS

Cells, viruses, and antiviral agents. The actively HBV-producing HepG2.2.15 human hepatoblastoma cell line (35) was a kind gift from B. Korba of Georgetown University. The HepG2.2.15 cell line carries four 5'-3' tandem copies of the HBV genotype D genome positioned such that two dimers of the genomic DNA are 3'-3' with respect to each other at chromosomally integrated sequences. The HepG2.2.15 cells were cultured with Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS; Gemini Bio-Products, West Sacramento, CA) and 1% G-418 solution (Roche Diagnostics GmbH, Mannheim, Germany) (36). The HepG2, Huh7, MT-2, MOLT-4, and Daudi cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), while PXB cells were purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). The HepG2 and Huh7 cells were grown in DMEM with 10% FCS. The MT-2, MOLT-4, and Daudi cells were grown in RPMI 1640-based culture medium supplemented with 10% FCS. HIV-1_{LAI} was also used for the anti-HIV drug susceptibility assay. CMCdG was newly designed and synthesized, and its purity was determined to be >99.8% by high-performance liquid chromatography. CdG was synthesized as previously described (37). ETV was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). ADV was purchased from Sigma-Aldrich (St. Louis, MO, USA). TDF was purchased from Toronto Research Chemicals (North York, ON, Canada), while TAF was purchased from MedChemExpress (Monmouth Junction, NJ, USA). All the compounds tested in the present study were initially dissolved in 100% dimethyl sulfoxide (DMSO), followed by further dilution to the indicated concentrations.

Anti-HBV and anti-HIV assays and cytotoxicity assay. Anti-HBV assays were conducted using the HepG2.2.15 cell line, which contains both integrated HBV_{WT}^D DNA and actively produced episomal HBV_{WT}^D DNA. The amount of integrated HBV_{WT}^D DNA does not change with the addition of an anti-HBV drug, while the amount of actively produced episomal DNA decreases with treatment. Thus, the quantity of episomal HBV_{WT}^D DNA is used as an indicator of active HBV production by cells and the inhibition of HBV_{WT}^D production by a test compound (38, 39). The cells were seeded in 96-well microtiter culture plates at a density of 4×10^3 cells in 200 μ l per well together with various concentrations of a compound. On days 3 and 7 after plating, the culture medium was removed and fresh medium and a drug were replenished. On day 14, the cells were harvested for DNA collection, and the DNA samples were extracted from the cells using a QIAamp DNA blood minikit (Qiagen, Valencia, CA), according to the manufacturer's instructions, and resuspended in 100 μ l Tris-EDTA buffer. PCR was conducted using a genesig standard kit for HBV core protein region (Primerdesign Ltd., Southampton, UK). The amount of HBV DNA in each assay sample with a test compound was compared to that in no-compound control samples.

Anti-HIV-1 assays were conducted as previously described (11). Briefly, MT-2 cells were exposed to a wild-type HIV-1_{LAI} strain at a 50% tissue culture infectious dose (HIV-1_{LAIV} 2 ng/ml p24). After viral exposure, the cell suspension (5×10^3 cells in 100 ml) was plated in each well of a 96-well flat microtiter culture plate containing various concentrations of a compound. After incubation for 7 days, the number of viable cells in each well was measured using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The potency of HIV-1 inhibition by a compound was determined based on its

inhibitory effect on virally induced cytopathicity in MT-2 cells, and the results are given as IC_{50} values. All assays were conducted in triplicate.

The cytotoxicity of a compound in HepG2.2.15 cells, Huh7 cells, HepG2 cells, PXB cells, and MT-2 cells was also determined. Cells were plated in a 96-well plate at a density of 2×10^4 (HepG2.2.15 cells, Huh7 cells, and HepG2 cells), 7×10^4 (PXB cells), or 2×10^4 (MT-2 cells) cells/ml and were continuously exposed to various concentrations of a compound throughout the period of the culture. The number of viable cells in each well was determined using a Cell Counting Kit-8, and the results are given as CC_{50} values.

Cell viability, cell growth, and mitochondrial toxicity assays. The MOLT-4 or Daudi cells were seeded in 96-well cell culture plates at a density of 4×10^3 cells in 200 μ l per well in the presence of various concentrations of ETV or CMCDg over a period of 7 days. Cell viability was determined with a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)-based assay, and cell growth was determined using 0.5% trypan blue stain solution and a TC20 automated cell counter (Bio-Rad Laboratories, Inc., Tokyo, Japan). The cells were subsequently subjected to a mitochondrial toxicity assay as previously described, with modifications (40–43). After DNA extraction from the cells using a QIAamp DNA blood minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions, real-time PCR was performed using Premix *Ex Taq* (TaKaRa Bio, Shiga, Japan) with the following primers and probe for detecting cytochrome *b* (cyt *b*) mitochondrial DNA (mtDNA): cyt-2s (5'-GCC TGC ATC CTC CAA AT-3'), cyt-2as (5'-AAG GTA GCG GAT GAT TCA GCC-3'), and cyt-2p (5'-FAM-CAC CAG ACG CCT CAA CCG CCT T-BHQ1-3', where FAM represents 6-carboxyfluorescein and BHQ1 represents black hole quencher 1) (44). The PCR preparations were incubated at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The data were analyzed using 7500 software (Applied Biosystems).

HBV DNA transfection and detection of core-associated HBV DNA from transfected cells using Southern blot hybridization. Plasmids carrying 1.24-fold the HBV genome of a wild-type strain (pHBV_{WT}^{Ce}), an entecavir-resistant strain (pHBV_{ETV-R}^{L180M/S202G/M204V}), and an adefovir-resistant strain (pHBV_{ADV-R}^{A181T/N236T}) were constructed for *in vitro* study as previously described (35). Huh7 cells (2×10^5) were plated onto a 6-cm-diameter dish, and 24 h later, the cells were transfected with 1 μ g of the plasmid using the FuGENE 6 transfection reagent (Promega, Madison, WI) according to the manufacturer's instructions. At 5 to 6 h after transfection, the transfected cells were further cultured in the presence of 0.1% DMSO irrespective of the different agents and their concentrations and were harvested at 72 h after transfection. Transfection efficiency was measured with cotransfection with 0.1 μ g of a reporter plasmid expressing secreted embryonic alkaline phosphatase (SEAP) and was normalized with subsequent SEAP measurements from culture supernatants using a SEAP reporter gene assay (Roche Diagnostics GmbH, Mannheim, Germany) (36). After DNA extraction, Southern blot hybridization was performed as previously described (35). Briefly, the harvested cells were lysed in 750 μ l of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1% IGEPAL CA-630 (Sigma-Aldrich, Japan). Total cell lysates were treated with 120 μ g/ml of RNase A and 30 μ g/ml of DNase I for 3 h at 37°C in the presence of 6 mM Mg^{2+} acetate. HBV DNA was then subjected to proteinase K digestion and extracted using phenol and ethanol. DNA was separated on a 1% agarose gel, transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany), hybridized with a digoxigenin (DIG)-dUTP-labeled full-length HBV genotype C fragment, and labeled using a DIG High Prime DNA labeling and detection starter kit II (Roche Diagnostics GmbH), and then the HBV DNA was detected by alkaline phosphatase-labeled anti-DIG antibody according to the manufacturer's instructions. The detection was performed with CDP-Star ready-to-use substrate (Roche Diagnostics GmbH). The signals were analyzed by using an ImageQuant LAS 4000 minisystem (GE Healthcare UK Ltd., Buckinghamshire, UK). For each compound, the relative amount of single-stranded replicative intermediate DNA (ssDNA) in treated cells was determined by Southern blot analysis using a chemiluminescence detection system (LAS 4000 mini-biomolecular imager; GE Healthcare). The IC_{50} values were determined as the drug concentration at which a 50% decrease in the amount of intracellular HBV ssDNA compared with that for untreated cells was achieved after treatment and were determined using the Forecast function of Microsoft Excel software.

HBV infection of human liver-chimeric mice. Human liver-chimeric mice were purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). Human serum albumin was measured by enzyme-linked immunosorbent assay using commercial kits (Eiken Chemical Co. Ltd., Tokyo, Japan). The serum levels of human albumins and body weight were required to be virtually identical among all of the mice to provide a reliable comparison. HBV DNA sequences spanning the S gene were amplified using real-time PCR employing the method of Abe et al. (45). Hepatitis B surface antigen (HBsAg) levels were measured using a commercial chemiluminescent enzyme immunoassay kit (Fujirebio Inc., Tokyo, Japan) as previously described (36). The mice were infected with HBV recovered from serum from preinfected mice as described in our previous report (36). Two recombinant infectious HBV clones, HBV_{WT}^{Ce} and HBV_{ETV-R}^{L180M/S202G/M204V}, were used in this study. At 8 weeks after inoculation, HBV-infected mice were treated with ETV or CMCDg at a dose of 1 mg/kg once a day over 14 days.

All animal protocols described in this study were performed in accord with the *Guide for the Care and Use of Laboratory Animals* (46) and approved by the Animal Welfare Committee of Phoenix Bio Co., Ltd. (approval no. 1540, 1592, 1883, and 2056).

Statistical analysis of changes in viral loads, body weights, and serum human albumin levels of treated mice. The changes in the viral loads, body weights, and serum human albumin levels from

the day 0 level of each mouse treated with ETV or CMCdG were compared using repeated-measures analysis of variance, with two-tailed *P* values corrected for multiple comparisons by the Hochberg method.

Molecular modeling. To gain insights into the atomic details of the inhibitory mechanism of ETV, CdG, and CMCdG, we built initial structural models of the ternary complexes of HBV reverse transcriptase (RT) with primer templates (pt) and in complex with ETV triphosphate (TP) and NRTI-TP using a homology model of the HBV_{WT} RT domain, semiempirical quantum chemical methods, and molecular dynamics, as previously described (19). Structural manipulations to generate CdG and CMCdG as well as to generate different mutated RT residues were performed using Maestro (version 10.7.015) software (Schrödinger, LLC, New York, NY). Correct bond orders of the residues, including zero-order bonds from the Mg²⁺ to the phosphate groups, were assigned, and the termini were capped. Restrained minimization using the OPLS3 force field was performed. A cutoff distance of 3.0 Å between a polar hydrogen and an oxygen or nitrogen atom and a minimum donor angle of 60° between D-H-A and a minimum acceptor angle of 90° between H-A-B (where H, D, A, and B are hydrogen, the donor, the acceptor, and the atom connected to the acceptor, respectively) were used to define the presence of hydrogen bonds. Connolly molecular surfaces for the inhibitors and selected RT residues from the active site were generated using a water sphere with a radius of 1.4 Å as a probe. Structural figures were generated using Maestro (version 10.7.015) software (Schrödinger, LLC, New York, NY).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02143-18>.

SUPPLEMENTAL FILE 1, PDF file, 7.3 MB.

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