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4'-Modified Nucleoside Analogs: Potent Inhibitors Active against Entecavir-resistant Hepatitis B Virus

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Abstract

Certain nucleoside/nucleotide reverse transcriptase (RT) inhibitors (NRTIs) are effective against HIV-1 and HBV. However, both viruses often acquire NRTI resistance, making it crucial to develop more potent agents that offer profound viral suppression. We report here that 4'-C-cyano-2-amino-2'-deoxyadenosine (CA_dA) is a novel highly potent inhibitor of both HBV

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Potential conflict of interest; S.K. and H.M. are among co-inventors on a patent for 4'-ethynyl-2-fluoro-2'-deoxyadenosine; all the rights, title, and interest to the patent have been assigned to Yamasa Corporation, Chiba, Japan. The other authors declare no competing financial interests.

Additional Supporting Information may be found in the online version of this article.

(IC₅₀=0.4 nM) and HIV-1 (IC₅₀=0.4 nM). In contrast, the approved anti-HBV NRTI entecavir (ETV) potently inhibits HBV (IC₅₀=0.7 nM) but is much less active against HIV-1 (IC₅₀=1,000 nM). Similarly, the highly potent HIV-1 inhibitor 4'-ethynyl-2-fluoro-2'-deoxyadenosine (EFdA) (IC₅₀=0.3 nM) is less active against HBV (IC₅₀=160 nM). Southern analysis using Huh-7 cells transfected with HBV-containing plasmids demonstrated that CAAdA was potent against both wild-type (IC₅₀=7.2 nM) and ETV-resistant HBV (IC₅₀=69.6 nM for HBV_{ETV-R}^{L180M/S202G/M204V}), whereas ETV failed to reduce HBV_{ETV-R}^{L180M/S202G/M204V} DNA even at 1 μM. Once daily peroral administration of CAAdA reduced HBV_{ETV-R}^{L180M/S202G/M204V} viremia (p=0.0005) in human-liver-chimeric/HBV_{ETV-R}^{L180M/S202G/M204V}-infected mice, while ETV completely failed to reduce HBV_{ETV-R}^{L180M/S202G/M204V} viremia. None of the mice had significant drug-related body-weight or serum human-albumin concentration changes. Molecular modeling suggests that a shallower HBV-RT hydrophobic pocket at the polymerase active site can better accommodate the slightly shorter 4'-cyano of CAAdA-triphosphate (TP), but not the longer 4'-ethynyl of EFdA-TP. In contrast, the deeper HIV-1-RT pocket can efficiently accommodate the 4'-substitutions of both NRTIs. The ETV-TP's cyclopentyl ring can bind more efficiently at the shallow HBV-RT binding pocket. *Conclusion:* These data provide insights on the structural and functional associations of HBV- and HIV-1-RTs and show that CAAdA may offer new therapeutic options for HBV patients.

The hepatitis B virus (HBV) is a double-stranded DNA virus, which belongs to the hepadnaviridae family, causes in humans acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma¹. In the United States, acute hepatitis B incidence has decreased by >80% from 1990 to 2007, a notable decline among children and adolescents predominantly due to the universal screening of pregnant women to prevent perinatal hepatitis B virus transmission with HBV vaccine^{2,3}. However, the prevalence of HBV infection still remains essentially unchanged among persons over 50 years old³. It has especially been a serious threat among people who have multiple sexual exposures, men who have sex with men, and intravenous drug users². Worse, such populations have a high risk for co-infection with human immunodeficiency virus-type 1 (HIV-1). Individuals co-infected with HIV-1 and HBV often have faster progression of liver fibrosis and an increased risk of end-stage liver diseases than patients with HBV infection alone^{4,5}. To date, various strategies have been used to treat HBV infection. Interferon has some advantages, such as its limited treatment duration, the absence of the occurrence of resistant mutants, and durable responses, although inherent side effects of interferon are vexing in a number of patients^{6,7}. In this regard, nucleoside/nucleotide reverse transcriptase (RT) inhibitors (NRTIs) have been used as a component of combined antiretroviral therapies (cART) for patients infected with HIV-1 and a few of such NRTIs are known to be also active against HBV. Those NRTIs that are currently being used to treat chronic HBV infections⁵ include lamivudine (3TC or LVD), adefovir (ADV), entecavir (ETV), telbivudine (LdT), and tenofovir (TDF). Such NRTIs act as HBV DNA polymerase [reverse transcriptase (RT)] inhibitors, and they have proven to be highly effective in reducing HBV viremia.

Currently, ETV is one of the most commonly used drugs against HBV because of its potent activity and low rate of HBV acquisition of drug resistance. However, certain HBV variants that acquired 3TC-associated amino acid substitutions such as M204V and L180M (HBV^{M204V} and HBV^{L180M/M204V}) are less susceptible to ETV by factors of 20 to 30 and

tend to develop high levels of ETV resistance⁸. Those harboring HBV^{M204V}, and/or HBV^{L180M/M204V} are, therefore, recommended to receive TDF or ADV; however, a long-term treatment with TDF or ADV has been associated with certain adverse effects such as renal toxicity^{9,10}. Since the cure of HBV infection or elimination of HBV in once HBV-infected individuals is as yet elusive, novel anti-HBV therapeutics that are potent against wild-type HBV (HBV^{WT}) and have no or less long-term adverse effects are urgently needed.

We previously reported 4'-ethynyl-2-fluoro-2'-deoxyadenosine (EFdA), which has a unique 4'-ethynyl moiety and retains the 3'-hydroxyl moiety in the ribose (Fig. 1), exerts highly potent anti-HIV-1 activity *in vitro* and *in vivo*¹¹⁻¹⁶ and is currently undergoing clinical trials¹⁷. In the present study, we examined ~150 nucleoside analogs including EFdA and its congeners, 4'-C-cyano-2-amino-2'-deoxyadenosine (CAAdA) and 4'-C-cyano-2'-deoxyguanosine (CdG) (Fig. 1)¹⁸ for their anti-HIV-1/HBV activity *in vitro* and *in vivo*.

Materials and Methods

Cells, viruses, and antiviral agents

Various cell lines, HIV and HBV isolates, and nucleoside analogs employed in the present study are described in detail in the Supporting Information.

Anti-HIV and -HBV assays, cytotoxicity and mitochondrial DNA assays, and quantification of HBV and mitochondrial DNA

In vitro anti-HIV and -HBV assays, real-time HBV-PCR and Southern blot assays, methods for determining drug-associated cytotoxicity and mitochondrial toxicity and methods for quantification of HBV and mitochondrial DNA (mtDNA) are described in detail in the Supporting Information.

HBV-DNA transfection and detection of core-associated HBV DNA from transfected cells using southern blot hybridization

Huh-7 cells were transfected with various HBV-containing plasmids and cultured in the presence of each drug and core-associated HBV DNA was extracted and subjected to the Southern blot assay. More detailed procedures are given in the Supporting Information.

HBV infection of human-liver-chimeric mice

Severe combined immunodeficiency mice transgenic for the urokinase-type plasminogen activator gene (uPA^{+/+}/SCID^{+/+} mice) with their liver partly replaced with human hepatocytes (hu-liver-chimeric-uPA^{+/+}/SCID^{+/+} mice) were infected with HBV and were treated with a test compound once a day over 14 days. More detailed procedures are given in the Supporting Information.

Molecular modeling

Structural models of ternary complexes of HBV- and HIV-1-RTs in complex with primer-templates (pt) and NRTI-triphosphates (TP) were built. Structural analysis, visualization, and figures were generated using SYBYL-X (Tripos Associates, St. Louis, MO) and Maestro

version 9.3 (Schrödinger, LLC, New York). More detailed experimental procedures are given in the Supporting Information.

Results

Determination of the activity of nucleoside analogs against HIV-1 using MT4 cells

We tested approximately 150 mostly newly designed and synthesized nucleoside analogs for antiviral activity against HIV-1. We also examined 13 NRTIs, which are or were in clinical use or under clinical trials as anti-HIV-1 and/or anti-HBV NRTIs for anti-HIV-1 activity as reference drugs. When examined with the MTT assay using MT-4 cells, 12 of the 13 reference drugs exerted moderate to highly potent activity against a wild-type strain HIV-1_{NL4-3}. As shown in Table 1, while ETV and ddI were moderately potent against HIV-1_{NL4-3} (IC₅₀ values of ~1 μM), 3TC, FTC, TDF, ADV, ABC, d4T, Ed4T, and ddC were substantially active with an IC₅₀ values ranging from 0.23 to 0.65 μM. AZT proved to be more active with an IC₅₀ value of 0.03 μM. EFdA showed the most potent activity with an IC₅₀ value of 0.0003 μM, as previously reported¹⁶. LdT, an FDA-approved anti-HBV therapeutic, showed no detectable anti-HIV-1 activity when examined at concentrations of up to 100 μM. Of note, two novel compounds, CAdA and CdG (Fig. 1), were identified among the ~150 nucleoside analogs to be very active against HIV-1_{NL4-3} (Tables 1 and S1). It is of note that CAdA and CdG exerted potent anti-HIV-1 activity against the wild-type HIV-1 isolate (HIV-1_{NL4-3}) with IC₅₀ values of 0.00016 and 0.000042 μM, respectively (Table S1). Both analogs had reduced activity by 8–97 fold against 3 multi-drug-resistant HIV-1 clinical isolates examined. Yet, if the absolute concentrations are considered, the activity of both CAdA and CdG was significantly potent against the 3 variants, and they also showed potent activity against HIV-2 strains (Table S1). In contrast, the activity of all the four FDA-approved anti-HIV-1 drugs examined was significantly decreased against the variants with the IC₅₀ values of 0.22 to >10 μM compared to that of CAdA and CdG (Table S1).

Determination of the activity of nucleoside analogs against HBV using HepG2 2.2.15 cells

We then attempted to evaluate the anti-HBV activity of all the compounds described above. In general, no *in vitro* inhibition assay of *de novo* HBV infection using hepatocyte-derived cells and infectious HBV particles has been established. In the present study, in order to evaluate whether a given test compound reduced the amount of intracellular HBV DNA in HepG2 2.2.15 cells, DNA isolated from HepG2 2.2.15 cells, which were cultured in the presence of each test compound over 12 days, was subjected to real-time HBV-PCR as described in the Materials & Methods section. A standard curve for HBV quantification, in which the C_T (cycle threshold) values for each reaction were obtained, was generated with serial dilutions of a plasmid (pHBV^{WT}) containing 1.24-fold HBV genome of a wild-type HBV (genotype C2/Ce:AB246345, HBV^{WT})¹⁹, and the C_T value of each test sample was converted to an HBV copy number. A representative data set of the HBV DNA decrease caused by ETV is illustrated in Supplementary Fig. 1. The HepG2 2.2.15 cells intracellularly contain HBV DNA in the forms of both integrated HBV DNA and episomal DNA. The amount of integrated HBV DNA does not change with the addition of anti-HBV agents (such as ETV) but episomal DNA amounts decrease if reverse transcription of HBV RNA is

blocked as seen in Fig. S1. When cultured alone, HepG2 2.2.15 cells proved to contain 9.0×10^5 copies/well (each well contains approximately 6.0×10^5 cells at the conclusion of the 12-day period of culture); however, in the presence of ETV, the number of HBV copies continued to decrease down to $\sim 0.9 \times 10^5$ copies/well in a dose-response fashion at up to 100 nM and no further decrease in the copy number was seen at and beyond 100 nM of ETV. The S.D. values were mostly within 5% of the mean values as determined in triplicate (Fig. S1). These data indicate that the assay system used in the present study is appropriately reproducible and quantitative and that each cell contains ~ 2 copies of HBV, in line with the initial report that a HepG2 2.2.15 cell has two copies integrated in cellular DNA in a head-to-head tandem fashion²⁰.

We consequently examined the ~ 150 nucleoside analogs plus the 13 reference NRTIs for activity against HBV in the real-time HBV-PCR assay described above. Anti-HBV activity of 5 selected reference nucleoside/nucleotide analogs is shown in Fig. 2A. All these 5 analogs significantly reduced the number of HBV copies in similar dose-response fashions. The order of the potency of the five analogs was: ETV > LdT > 3TC > TDF > ddC and their IC_{50} values were 0.0007, 0.007, 0.03, 0.04, and 0.75 μ M, respectively (Fig. 2A & Table 1). CAdA and CdG were found to be highly active against HBV both with an IC_{50} value of 0.0004 μ M.

In order to corroborate the anti-HBV activity observed using the real-time HBV-PCR assay, we also conducted a Southern blot assay and determined whether ETV, CAdA, and CdG would block HBV replication (Fig. S2A). Huh-7 cells were first transfected with pHBV^{WT}. The results revealed the presence of a single-stranded (SS) DNA signal as cultured in the absence of agents (Fig. S2A). The signal was clearly seen in the cells treated with a low concentration of ETV (1 nM; Fig. S2A). However, the signal weakened in a dose-dependent manner and was virtually lost in the cells exposed to 100 nM ETV. The IC_{50} value calculated by measuring the changes in the signal density was 14.1 nM. As examined under the same conditions, the IC_{50} values of CAdA and CdG against HBV^{WT} were 7.2 nM and 9.8 nM, respectively. Thus, the potency determined with the Southern blot assay was overall comparable among the three compounds, consistent with the data obtained with the real-time HBV-PCR assay, while the absolute IC_{50} values in the Southern blot assay were greater by 16 to 24-fold than in the real-time HBV-PCR assay.

Relationship of anti-HIV-1 & anti-HBV activity of nucleoside analogs examined

Approximately 65% of the ~ 150 nucleoside analogs examined in the present study showed no activity against HBV or HIV-1; $\sim 20\%$ were active against both HBV and HIV-1; $\sim 10\%$ active against HBV only; while $\sim 5\%$ active against HIV-1 only. Among the $\sim 20\%$ nucleoside analogs active against both viruses, CAdA and CdG were most potent against both viruses and were further examined in the present study. Fig. 2B illustrates the activity of selected NRTIs against HBV and HIV-1. The X-axis shows the potency of anti-HBV activity, while the Y-axis the potency of anti-HIV-1 activity. AZT, Ed4T, and d4T were all substantially potent against HIV-1, while these three drugs show virtually no activity against HBV. ABC, ddI, and ddC were substantially potent against HIV-1 and HBV, while ADV, 3TC, TDF, and FTC were substantially potent against HIV-1 but more potent against HBV.

LdT was substantially potent against HBV but was virtually inert against HIV-1. EFdA was extremely potent against HIV-1; however, the compound was not that potent against HBV. ETV proved to be highly potent against HBV but it was not that potent against HIV-1. Of note, both CAaA and CdG were highly potent against both HIV-1 and HBV (Fig. 2B).

CAaA and CdG block HBV_{ETV-R}^{L180M/S202G/M204V} and HBV_{ADV-R}^{A181T/N236T} replication

We subsequently asked whether CAaA and CdG blocked the replication of ETV-resistant HBV (HBV_{ETV-R}^{L180M/S202G/M204V}) and ADV-resistant HBV (HBV_{ADV-R}^{A181T/N236T}). As expected, ETV at 1 to 1,000 nM concentrations failed to block HBV_{ETV-R}^{L180M/S202G/M204V} replication; even the highest concentration (1,000 nM) of ETV failed to achieve 50% signal reduction as shown in Fig. S2B & Table 2. By contrast, both CAaA and CdG significantly blocked HBV_{ETV-R}^{L180M/S202G/M204V} replication in a dose-response fashion (Fig. S2B); the IC₅₀ values of CAaA and CdG were 70 ± 14 and 42 ± 34 nM, respectively (Table 2). The average fold-reductions of the potency of CAaA and CdG against HBV_{ETV-R}^{L180M/S202G/M204V} were only 9.7 and 4.3, respectively, compared with their IC₅₀ values against HBV^{WT} (Table 2). The data clearly indicate that HBV_{ETV-R}^{L180M/S202G/M204V} is highly susceptible to both CAaA and CdG. We also determined the activity of CAaA and CdG against HBV_{ADV-R}^{A181T/N236T}. As shown in Table 2 and Fig. S2, both CAaA and CdG were active against HBV_{ADV-R}^{A181T/N236T} with IC₅₀s of 0.09 and 0.04 μM, respectively, although ADV had a significantly greater IC₅₀ value of 39.5 μM.

CAaA and CdG Block HBV Replication in Hu-liver-chimeric SCID Mice

We then asked whether CAaA and CdG blocked the replication of HBV in hu-liver-chimeric-uPA^{+/+}/SCID^{+/+} mice. In 8 weeks following inoculation of these mice with HBV^{WT}, they were orally gavaged with ETV, CAaA, or CdG (prepared at a concentration of 0.002 mg/mL in saline) using oral sondes so that the dose administered resulted in 0.02 mg/kg/day. Just before the administration of ETV, the HBV copy numbers in their plasma were as high as ~5 × 10⁹; however, the viremia levels significantly went down on day 7 of ETV administration and the viremia reduction further went down even after the termination of ETV administration to the lowest (day 21) (Fig. 3A, upper-left panel). Around day 21 and beyond, the viremia went up slowly, but the viremia level on day 42 was still lower than the day 1 viremia level. Both CAaA and CdG also significantly blocked the replication of HBV under the same conditions (upper panel in Fig. 3A). The greatest magnitudes of serum HBV-DNA level reduction with CAaA and CdG were by -2.9 or -2.7 log₁₀ copies/ml, respectively. Over days 3–14, the decrease of HBV DNA copy numbers was significantly greater in CAaA-treated mice than in ETV-treated mice (p=0.013: repeated measures analysis of variance with two-tailed p values corrected for multiple comparisons). Over days 17–41, the decrease of HBV DNA copy numbers in CAaA-treated mice and CdG-treated mice were significantly greater than in ETV-treated mice (p=0.0026 and p=0.0015, respectively). This was thought to be partly due to CAaA- and CdG-treated mice starting from lower levels due to the effects during days 3–14.

In 8 weeks following inoculation of the hu-liver-chimeric-uPA^{+/+}/SCID^{+/+} mice with an HBV_{ETV-R}^{L180M/S202G/M204V}, the viremia levels had reached 8.6 × 10⁵ – 7.7 × 10⁶ (lower

panels in Fig. 3A). ETV, at a dose of 0.02 mg/kg/day, showed essentially no reduction in HBV_{ETV-R}^{L180M/S202G/M204V} viremia levels (lower panel, Fig. 3A). By contrast, CAdA and CdG at the same dose of 0.02 mg/kg/day, brought about a significant level of viremia reduction by day 7 of administration. The greatest magnitudes of viremia reduction with CAdA and CdG were $-1.4 \log_{10}$ copies/ml (average of -1.6 and -1.1) and $-1.2 \log_{10}$ copies/ml (average of -0.9 and -1.5), respectively (Fig. 3A). Over the 14-day period of administration, the viremia reduction in both CAdA- and CdG-receiving mice was significantly lower compared to that in ETV-receiving mice ($p=0.0005$).

ETV, CAdA, and CdG caused no significant changes in body-weights and serum human albumin levels in mice

Human albumin (h-albumin) levels in serum and body weights of the treated mice were also monitored during the treatment (Fig. 3B). HBV^{WT}-infected mice treated with CAdA or CdG had moderately decreased h-albumin levels; but mice treated with ETV also had a comparable decrease of h-albumin levels over the period of observation ($p=0.4-0.5$). HBV_{ETV-R}^{L180M/S202G/M204V}-infected mice treated with ETV, CAdA, or CdG also had a gradual decrease in their h-albumin levels; however, there was no significant difference observed between the three groups. There was no significant difference in body weights among ETV-treated, CAdA-treated, and CdG-treated groups (Fig. 3B).

CAdA and CdG appear not to have significant hepatotoxicity

Certain NRTIs are known to inhibit the activity of human DNA polymerase γ , an enzyme essential for mitochondrial DNA (mtDNA) replication and repair, and to cause a variety of adverse effects including peripheral neuropathy, myopathy, pancreatitis, and lactic acidosis with hepatic steatosis. Thus, we examined whether CAdA had caused any hepatotoxicity in the CAdA-receiving hu-liver-chimeric-uPA^{+/+}/SCID^{+/+} mice described above. The mice were sacrificed upon the conclusion of CAdA administration over 14 days and their liver tissues were histologically examined. Liver chimerism harboring human hepatocytes (pink area) and mouse hepatocytes (red area) was evident (Fig. S3A). No necrotic or steatotic changes were identified in the CAdA-receiving mice. In the portal region, bile ductule cell hyperplasia (open arrowheads) with a few mononuclear cell infiltration was noted, and single cell necrosis (closed arrowheads) and ceroid-laden macrophages (arrows) were sporadically observed in parenchyma (Fig. S3B); however, these minor changes were also seen in the mice with HBV infection but receiving no agents (vehicle only; Fig. S3C & S3D). Thus, it was concluded that CAdA did not cause any identifiable liver toxicities over the 14-day period of its administration.

We further examined the effects of CAdA and CdG on cellular mtDNA, L-lactate synthesis, and viable cell numbers using HepG2 cells at concentrations much higher than the circulation of the hu-liver-chimeric-uPA^{+/+}/SCID^{+/+} mice receiving CAdA or CdG. After 12 days of culture, cells and supernatants were harvested and subjected to DNA extraction (for real-time mtDNA-PCR) and determination of L-lactate levels in the culture medium. As shown in Fig. 4A, no reduction of mtDNA synthesis was observed and no L-lactate level changes were identified in the cells exposed to ETV (up to 100 μ M), although slight and moderate decreases in the cell viability were seen as determined in MTT assay at 10 and 100

μM , respectively. Another NRTI, ddC, which is known to inhibit the activity of DNA polymerase γ in humans²¹, significantly decreased mtDNA synthesis at 1 μM and beyond without causing any significant decrease in L-lactate amounts or viable cell numbers (Fig. 4B). CAdA and CdG at concentrations of 1 μM and beyond significantly lowered the levels of mtDNA (Fig. 4C & 4D). However, CAdA and CdG severely lowered the levels of L-lactate and viable cell numbers, in particular, at 10 and 100 μM , which was thought to be responsible for the lowered mtDNA levels.

Molecular Modeling of HIV-1-RT and HBV- RT Complexed with ETV, EFdA, and CAdA

We finally conducted molecular modeling of HIV-1-RT and HBV-RT complexed with ETV-TP, EFdA-TP, or CAdA-TP and primer/template (p/t). The sugar moiety of all three agents in the triphosphate form was found close to a hydrophobic pocket in both HIV-1-RT and HBV-RT. The pocket in HIV-1-RT is constructed by residues A114, Y115, P157, F160, M184, and D185 (Fig. 5A, C, E, 6A, C & E) and that in HBV-RT by residues A87, F88, P177, L180, M204, and D205 (Fig. 5B, D, F, 6B, D & F).

In the ternary complex of HIV-1-RT and ETV-TP, the cyclopentyl ring (pseudo-sugar) and exocyclic alkene ($=\text{CH}_2$) moieties of ETV-TP have some hydrophobic interactions with A114, Y115, and the aliphatic chain of D185 of HIV-1-RT (Fig. 6A). By contrast, in HBV-RT complexed with ETV-TP, the equivalent residues (A87, F88, and D205)(Fig. 6B) engage in tight hydrophobic interactions with ETV-TP. There are additional hydrophobic contacts involving L180 of HBV-RT and the ETV-TP's cyclopentyl ring, which are not present in HIV-1-RT since the amino acid residue equivalent to L180 of HBV-RT is F160, which does not appear to interact with ETV-TP in the HIV-1-RT/ETV-TP complex, explaining why ETV is highly potent against HBV but not against HIV-1. While EFdA-TP's 4'-ethynyl interacts very well with the hydrophobic pocket of HIV-1-RT as previously described¹⁵, we found that the 4'-ethynyl also interacts with the pocket of HBV-RT and that CAdA-TP's 4'-cyano interacts with the hydrophobic pockets of both HIV-1-RT and HBV-RT (Fig. 5C–F, shown as pink surface representation)(Fig. 6C–F). However, there is a clear and noticeable difference between the architectures of the hydrophobic pocket of the two enzymes. The HIV-1-RT's pocket is deep (Fig. 5C & Fig. 6C), in contrast to HBV-RT's pocket, which is considerably shallower, thus potentially limiting the position of certain NRTI-TPs (Fig. 5D & Fig. 6D). Indeed, the slightly longer 4'-ethynyl of EFdA-TP has reduced binding or misalignment of EFdA-TP in the HBV-RT pocket, although the same 4'-ethynyl attains strong binding to the HIV-1-RT pocket (Fig. 5C, 5D, 6C & 6D). On the other hand, the 4'-cyano of CAdA-TP is accommodated very well in HIV-1-RT as well as HBV-RT's shallower hydrophobic pocket (Fig. 5E, 5F, 6E & 6F). These findings should at least in part explain why EFdA is highly potent against HIV-1 but not against HBV, while CAdA is highly potent against both HIV-1 and HBV. Since 2,6-diaminopurine nucleoside analogs are prodrugs to 2'-deoxyguanosine nucleoside analogs²², we hypothesize that CAdA may get converted to CdG. Thus, the modeling insights gained from the complexes of HIV-1-RT and HBV-1-RT with CAdA-TP would apply to CdG as well.

Discussion

At the beginning of the present study, we found that EFdA, a highly potent anti-HIV-1 agent, was much less potent against HBV^{WT} in cell-based assays. On the other hand, ETV proved to be highly active against HBV^{WT}, while it was much less active against HIV-1. Furthermore, AZT, the first known nucleoside analog active against HIV-1^{23,24} was virtually inert against HBV^{WT}, while LdT that has been used for the treatment of HBV infection was essentially inert against HIV-1 (Fig. 2B). In this regard, HBV-RT, in terms of amino acid sequence homology, is close to HIV-1-RT and murine leukemia virus (MuLV)-RT, but with less than 25% amino acid sequence identity. Nevertheless, the study of sequence alignment performed by Das *et al.*²⁵ has demonstrated that the functionally important amino acid residues are highly conserved among the RTs of HBV, HIV-1, and MuLV, especially in the catalytic region of those RTs that include the conserved domains A-G^{26,27}. It was thought from the observed differential activity of EFdA, ETV, AZT, and LdT against HIV-1 and HBV that the catalytic regions of HIV-1 and HBV should sufficiently differ so that HIV-1-RT and HBV-RT differentially recognize those nucleosides as good or poor substrates. Thus, we hypothesized that we could successfully identify lead compounds active against HBV among modified nucleosides that we examined and found essentially inert against HIV-1 or inappropriate as anti-HIV-1 therapeutics in our initial *in vitro* screening assays conducted in the 1980s and 1990s. Indeed, both CA₄A and CdG were among the modified nucleoside analogs we previously reported to be active against HIV-1¹⁸.

Various 4'-modified nucleoside analogs were reported to be active against HIV-1 in the 1990s²⁸⁻³⁰, although most of those analogs were overly cytotoxic and none of them entered clinical trials. However, the addition of a fluorine atom at the 2-position in the purine moiety of potent but cytotoxic 4'-ethynyl-2'-deoxyadenosine³¹, generating EFdA, successfully conferred highly potent anti-HIV-1 activity on EFdA and reduced the potential toxicity, especially the inhibition of human DNA polymerase γ activity¹². Moreover, the half-life of EFdA-TP, the active form of EFdA, proved to be ~17 hours or longer¹². EFdA is currently under clinical trials as a potential therapeutic for treating HIV-1 infection. The mechanism, by which EFdA exerts potent activity against a wide spectrum of multi-NRTI-resistant HIV-1 variants and the emergence of HIV-1 variants resistant to EFdA is significantly delayed and the multitude of EFdA resistance is highly limited³², is thought to be derived from multiple mechanisms involved in its activity against HIV-1. EFdA-TP has been shown to block HIV-1-RT as a translocation defective NRTI, which significantly slows proviral DNA synthesis and acts as a *de facto* immediate chain terminator. Although non-translocated EFdA-monophosphate (MP)-terminated primers are unblocked, they are efficiently converted back to the EFdA-MP-terminated form¹⁵. Moreover, EFdA-TP functions as a delayed chain terminator, allowing incorporation of an additional physiological dNTP before blocking DNA extension, thereby probably enabling EFdA-MP-terminated to be free from excision. Moreover, EFdA-MP is highly efficiently misincorporated by HIV-1-RT, making the mismatched primers extremely difficult to be extended and efficiently disallowing the excision of EFdA-TP from the mismatched primers³³.

Considering that the multiple antiviral mechanisms involved represent a unique feature of the 4'-modified NRTIs, we hypothesized that CAAdA and CdG would also act as efficient HBV DNA chain terminators through the mechanism(s) mentioned above, which should differ from the mechanism(s) through which ETV exerts its activity against HBV and that CAAdA and CdG would also be active against HBV_{ETV-R}^{L180M/S202G/M204V}. As expected, both CAAdA and CdG proved to significantly reduce the production of both HBV_{WT} and HBV_{ETV-R}^{L180M/S202G/M204V} in cell-based assays and productively HBV-infected hu-liver-chimeric-uPA^{+/+}/SCID^{+/+} mice (Fig. 2 & Fig. 3). The IC₅₀ value difference in the HBV signal reduction in the Southern blot between HBV^{WT} and HBV_{ETV-R}^{L180M/S202G/M204V} was 9.7-fold for CAAdA treatment and 4.3-fold for CdG treatment (Table 2). In the mouse model, the difference in the viremia reduction magnitude between HBV^{WT} and HBV_{ETV-R}^{L180M/S202G/M204V} was 30-fold (−2.9 vs −1.4 log₁₀ copies/ml) for CAAdA treatment and 30-fold (−2.7 vs −1.2 log₁₀ copies/ml) for CdG treatment. However, it should be noted that the potential efficacy against HBV_{ETV-R}^{L180M/S202G/M204V} of CAAdA and CdG in the clinical settings is to be determined only in carefully managed clinical trials.

Certain NRTIs such as stavudine (d4T) and zalcitabine (ddC) serve as substrates of mitochondrial polymerase γ , resulting in mtDNA depletion, which leads to decreased synthesis of mitochondrial proteins that maintain oxidative phosphorylation pathways. In the early stages of mitochondrial toxicity, glycolysis is inhibited and the metabolism of pyruvate is shifted to L-lactate, causing a decrease of energy production and bringing about myopathy, peripheral neuropathy, and life-threatening hepatic steatosis with lactic acidosis. In the present study, the short-term administration of either of CAAdA or CdG showed no significant liver toxicity or general toxicity as examined with body-weight and serum human albumin level changes as indicators in the productively HBV-infected hu-liver-chimeric-uPA^{+/+}/SCID^{+/+} mice. In terms of the potential mitochondrial toxicity and hepatotoxicity of nucleoside analogs including CAAdA and CdG, it is noteworthy that clevudine, one of the recently developed anti-HBV NRTIs, reportedly attained sustained suppression of HBV-DNA for several months after cessation of therapy³⁴, was associated with myopathy characterized by the depletion of mtDNA³⁵. Potential toxicities such as mitochondrial toxicity of CAAdA or CdG have to be carefully examined in controlled clinical trials in humans.

It is noteworthy that we have previously determined the intracellular metabolism of EFdA, which is a nucleoside analog, is structurally related to CAAdA and CdG, and is highly potent against HIV-1^{12,14}. In the case of EFdA, the intraperitoneal administration (20 mg/kg) resulted in a maximal concentration of 4 μ g/ml in 30 min, more than 1,000-fold higher than its *in vitro* anti-HIV-1 IC₉₀ concentration (~5 nM)¹⁴. Since both CAAdA and CdG exerted highly potent activity *in vitro* and *in vivo* as shown in the present study, it is strongly suggested that both analogs well penetrate their target cells and are well triphosphorylated intracellularly, although detailed pharmacokinetics and pharmacodynamics of CAAdA and CdG remain to be determined.

In our structural modeling of HIV-1-RT and HBV-RT in complex with ETV-TP, EFdA-TP, or CAAdA-TP, there appear to be three major factors contributing to greater potency of ETV against HBV compared to HIV-1: (i) the shallower pocket in HBV-RT compared to HIV-1-

RT, (ii) the presence of a phenylalanine (F88) in HBV-RT in place of the slightly polar tyrosine (Y115) in HIV-1-RT (Fig. 6A & 6B) that appears to contribute to a stronger hydrophobic interaction with the exocyclic alkene-containing hydrophobic ring of ETV-TP and (iii) favorable interactions of L180 in HBV-RT with ethene-cyclopentyl ring in ETV-TP, which is absent in HIV-1-RT due to the slightly distal location of F160 at equivalent position. Therefore, the stronger hydrophobic interactions of the pseudo-sugar (exocyclic alkene containing cyclopentyl ring) of ETV-TP in HBV-RT compared to those in HIV-1-RT may contribute to the enhanced binding of ETV-TP leading to a greater inhibition of HBV compared to HIV-1 (Table 1)³⁶, although it is possible that other factors also contribute to the observed potency differences. In the models of EFdA-TP, the 4'-ethynyl binding pocket in HIV-1-RT (Fig. 5C & 6C) is deeper compared to that in HBV-RT (Fig. 5D & 6D), which may increase the interactions and affinity of EFdA-TP with HIV-1-RT compared to that in HBV-RT. The sugar ring of CAdA-TP has a 4'-cyano instead of the 4'-ethynyl in EFdA. The 4'-cyano group is slightly shorter than the 4'-ethynyl (by ~0.1 Å), and both moieties can be accommodated in the deeper hydrophobic pocket of HIV-1-RT (Fig. 6C, E & G), accounting for the similar antiviral potencies of EFdA and CAdA against HIV-1 (IC₅₀ values: 0.4 vs. 0.3 nM, Table 1). Moreover, the 4'-cyano-containing NRTI-TPs can be accommodated in the shallow pocket of HBV-RT, but the slightly longer 4'-ethynyl may lead to reduced binding or misalignment of EFdA in the same pocket. These subtle differences between lengths of 4'-cyano and 4'-ethynyl may explain to a certain extent the difference in the potency of EFdA-TP compared to that of CAdA in HBV-RT (Fig. 5D, F, 6D & F). However, differences in the catabolism of CAdA and EFdA to respective triphosphates in different cell types may also affect the potency of these NRTIs.

In conclusion, the data presented in this study suggest that CAdA and CdG represent promising candidates as novel therapeutics for HBV_{ETV-R^{L180M/S202G/M204V}} infection and that further structural optimization of 4'-modified nucleosides should help develop more novel NRTIs that have unique and better antiviral profiles against HBV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HBV	hepatitis B virus
HIV-1	human immunodeficiency virus-type 1
RT	reverse transcriptase

NRTI	nucleoside/nucleotide reverse transcriptase inhibitor
cART	combined antiretroviral therapies
mtDNA	mitochondrial DNA
SCID	severe combined immunodeficiency
uPA	urokinase-type plasminogen activator
TP	triphosphates
3TC	lamivudine
ADV	adefovir dipivoxil
ETV	entecavir
LdT	telbivudine
TDF	tenofovir disoproxil fumarate
HBV^{WT}	wild-type HBV
EFdA	4'-ethynyl-2-fluoro-2'-deoxyadenosine
CAdA	4'-C-cyano-2-amino-2'-deoxyadenosine
CdG	4'-C-cyano-2'-deoxyguanosine
Ed4T	2',3'-didehydro-3'-deoxy-4'-ethynylthymidine
FTC	emtricitabine
AZT	3'-azido-3'-deoxythymidine
ABC	abacavir
d4T	2',3'-didehydro-3'-deoxythymidine
ddI	2',3'-dideoxyinosine
ddC	2',3'-dideoxycytidine
C_T	cycle threshold
SS	single stranded
ETV-R	entecavir resistance
ADV-R	adefovir resistance

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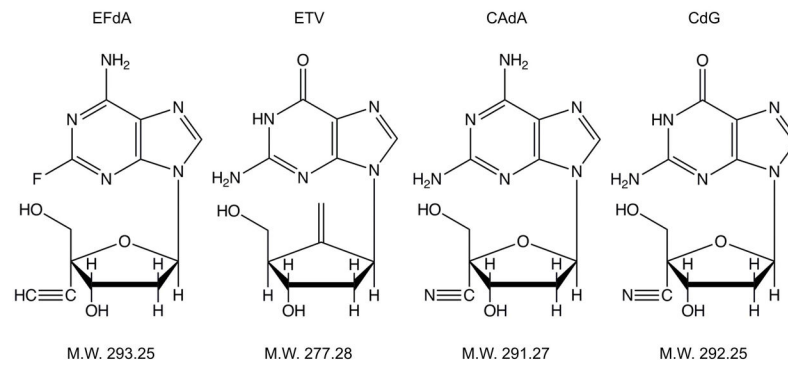
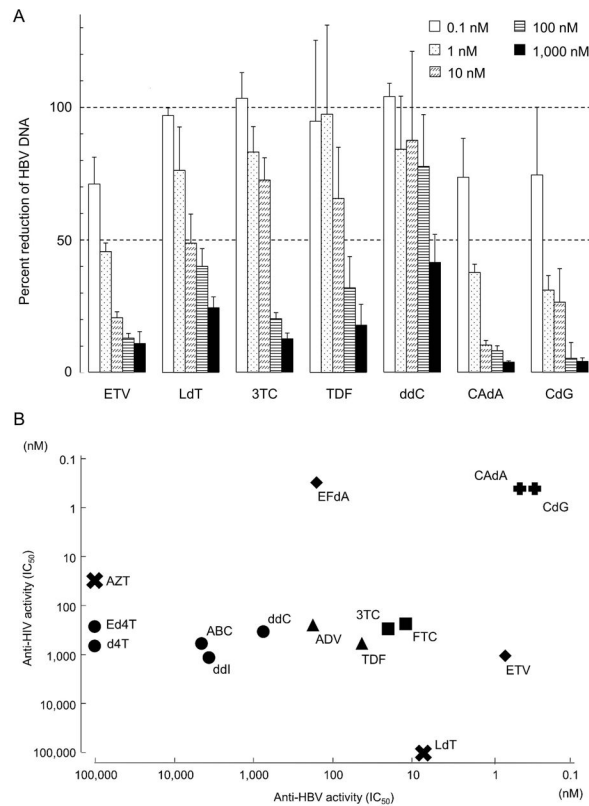


Fig. 1. Structures and molecular weights of 4'-ethynyl-2-fluoro-2'-deoxyadenosine (EFdA), entecavir (ETV), 4'-C-cyano-2-amino-2'-deoxyadenosine (CAAdA), and 4'-C-cyano-2'-deoxyguanosine (CdG).

**Fig. 2.**

(A) Reduction of HBV DNA levels in HepG2 2.2.15 cells with ETV, LdT, 3TC, TDF, ddC, CAdA, and CdG. Intracellular HBV-DNA levels in each well were quantified using real-time HBV-PCR and shown as percent controls compared to that without drug (control=100%). All data are shown as the average values obtained from at least 3 independent experiments. (B) Comparative analysis of anti-HBV and anti-HIV-1 activity of various NRTIs. The X-axis shows the potency (IC₅₀ values) of anti-HBV activity, while the Y-axis shows the potency of anti-HIV-1 activity. Of note, *bis*-pivaloyloxymethyl derivatives of acyclic nucleosides have 9–23 fold greater antiviral potency than their corresponding unmodified compounds such as PMPA. *Bis*-pivaloyloxymethyl-PMEA, known as adefovir dipivoxil, is also more potent than its corresponding unmodified compound (PMEA). This is due to their greater cellular uptake and intracellular formation of the active diphosphorylated metabolites³⁸. ADV and TDF have now been commercially used, and we thus used these prodrugs instead of their active forms.

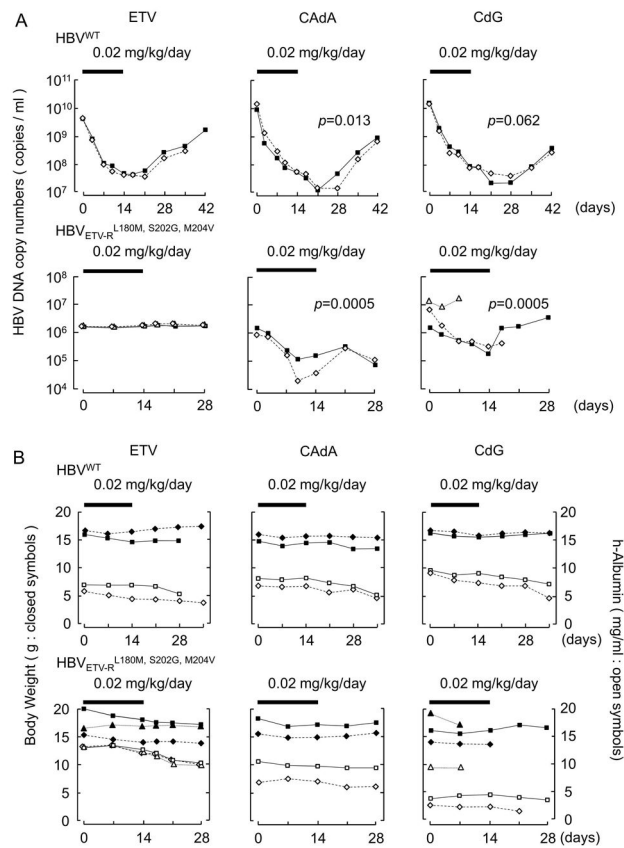


Fig. 3. Antiviral activity of NRTIs against HBV in hu-liver-chimeric-uPA^{+/+}/SCID^{+/+} mice. (A) In 8 weeks following inoculation of the mice with HBV^{WT} (upper panels) HBV_{ETV-R}^{L180M/S202G/M204V} (lower panels), ETV, CAaA, or CdG was administered (0.02 mg/kg/day). Serum HBV-DNA levels following indicated treatments were determined by real-time PCR (n=2–3 per group). Each line illustrates the changes in DNA copy numbers in an individual mouse. CAaA brought about significantly greater levels of HBV^{WT} viremia reduction over days 3–14 days (p=0.013) compared to ETV. CdG also brought about HBV^{WT} viremia reduction, which however was not significant compared to ETV (p=0.062). CAaA and CdG also elicited significantly greater levels of HBV_{ETV-R}^{L180M/S202G/M204V} viremia reduction by day 7 (p=0.0005 in both groups) and the reduction was seen even after the conclusion of the administration. Thick bars denote the administration period (14 days) with a dose of 0.02 mg/kg/day. Comparison was made between ETV and CAaA or CdG. (B) Human albumin levels in serum and body weights of the treated mice were monitored. Each line illustrates the changes in albumin levels and body weights in an individual mouse. There are no significant differences among ETV, CAaA, and CdG. Thick bars denote the administration period (14 days) with a dose of 0.02 mg/kg/day.

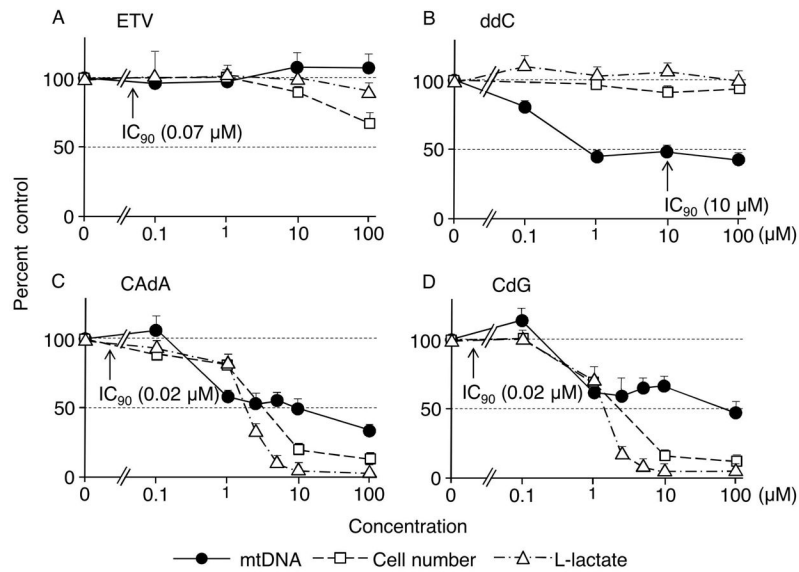


Fig. 4. Effects of ETV, ddC, CAdA, and CdG on mitochondrial DNA (mtDNA) synthesis, L-lactate levels, and viable cell numbers using HepG2 cells. HepG2 cells were cultured in the presence of various concentrations of a test agent. After 12 days of culture, cells were harvested and subjected to DNA extraction for mtDNA determination (real-time mtDNA-PCR) and supernatants harvested at the same time were subjected to the determination of L-lactate levels. The number of viable cells was determined using the MTT assay on day 5 of culture under the same conditions described above and the average numbers are shown as reference. Note that no reduction of mtDNA synthesis was observed with ETV (up to 100 μM), while ddC significantly decreased mtDNA synthesis at 1 μM and beyond. Arrows indicate the IC_{90} concentration for each agent against HBV^{WT} determined from the assays conducted for and illustrated in Table 1. No significant changes in L-lactate levels in the medium were seen with ETV or ddC (panels A and B). CAdA and CdG showed significant reduction in the amounts of mtDNA and L-lactate at 1 μM and beyond as assessed on day 12; however, a significant reduction of viable cell numbers had been noted as assessed on day 5 of culture (panels C and D).

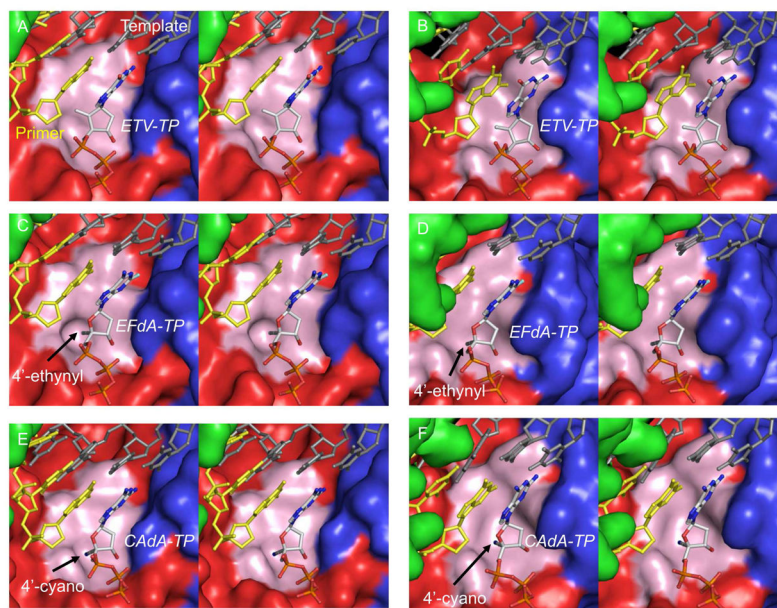


Fig. 5. Molecular models of HIV-1-reverse transcriptase (RT)/template-primer/NRTI- triphosphates (TP)s and HBV-polymerase (RT)/template-primer/NRTI-TPs. Stereoviews of the ternary complexes of HIV-1-RT/template-primer with ETV-TP (panel A), EFdA-TP (panel C), and CAdA-TP (panel E), are shown in surface representations: fingers, palm, and thumb of RT are colored in blue, red, and green, respectively. Stereoviews of HBV-RT/template-primer with ETV-TP (panel B), EFdA-TP (panel D), and CAdA-TP (panel F) are also shown. The template (dark gray), primer (yellow), and NRTI-TP are rendered as balls-and-sticks. Individual atoms in NRTI-TP are colored as carbon in white, nitrogen blue, oxygen red, phosphorus orange, and fluorine aquamarine. The arrows denote the positions of 4'-ethynyl (panels C and D) and 4'-cyano (panels E and F) moieties. The hydrophobic pocket comprises residues A114, Y115, P157, F160, M184, and D185 in HIV-1-RT and is shown in light pink. In HBV-RT, the pocket is formed by residues A87, F88, P177, L180, M204, and D205 and is shown in light pink. The hydrophobic pocket that accommodates the 4'-substituent is deeper in HIV-1-RT than in HBV-RT.

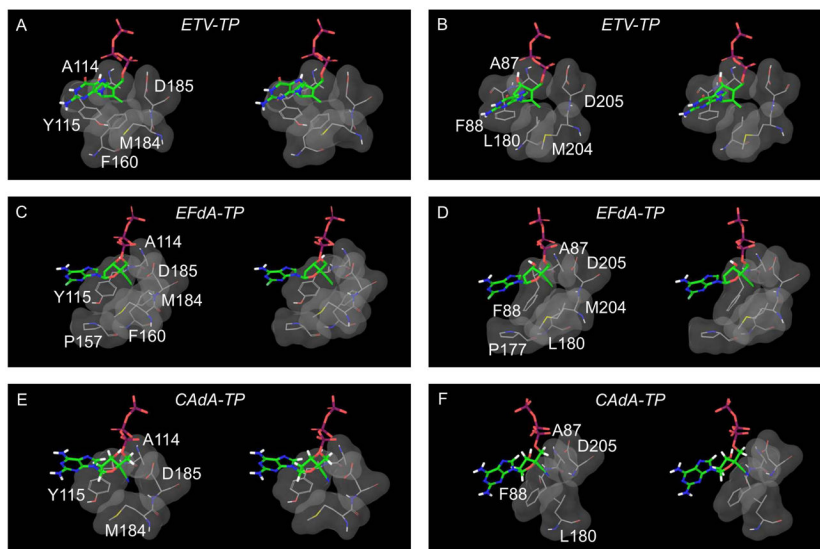


Fig. 6. Interactions of ETV-TP, EFdA-TP, and CAdA-TP with the hydrophobic pocket of HIV-1-RT and HBV-RT in stereoview. The cyclopentyl ring of ETV-TP does not have favorable interactions with the slightly polar Y115 of HIV-1-RT (panel A) but has strong hydrophobic interactions with the corresponding residue F88 of HBV-RT (panel B). The sugar ring of EFdA-TP has strong hydrophobic interactions with HIV-1-RT (panel C). The sugar ring of EFdA-TP seems to have favorable interactions with HBV-RT (panel D) but to a lesser extent compared to EFdA-TP with HIV-1-RT. The ethynyl group of EFdA-TP has good hydrophobic contact with F160 of HIV-1-RT (panel C); however, it is not accommodated well in the shallow cavity of HBV-RT (panel D) and is probably responsible for EFdA's lesser activity against HBV. The shorter 4'-cyano of CAdA-TP is accommodated equally well in the cavities of HIV-1-RT (panel E) and HBV-RT (panel F).

Table 1

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

Drug	IC ₅₀ (μM) against		CC ₅₀ (μM) in			S.I. with	
	Anti-HIV-1	Anti-HBV	MT-4	HepG2 2.2.15	Anti-HIV-1	Anti-HBV	
ETV	1.0 ± 0.4	0.0007 ± 0.0002	39.1 ± 8.7	> 100	38	> 140,000	
ddI	1.1 ± 0.04	3.7 ± 0.7	> 100	> 100	> 89	> 27	
3TC	0.29 ± 0.08	0.03 ± 0.01	> 100	> 100	> 340	> 3,300	
FTC	0.23 ± 0.10	0.02 ± 0.01	> 100	> 100	> 430	> 5,000	
TDF	0.58 ± 0.39	0.04 ± 0.01	40.4 ± 11.3	> 100	70	> 2,500	
ADV	0.24 ± 0.12	0.20 ± 0.08	5.1 ± 1.0	> 100	21	> 500	
ABC	0.58 ± 0.03	3.9 ± 1.0	> 100	> 100	> 170	> 26	
d4T	0.65 ± 0.03	> 100	> 100	> 100	> 150	N.D.	
Eg4T	0.26 ± 0.01	> 100	> 100	> 100	> 380	N.D.	
ddC	0.33 ± 0.13	0.75 ± 0.12	> 100	> 100	> 300	> 130	
AZT	0.03 ± 0.01	> 100	30.5 ± 3.1	> 100	1,020	N.D.	
EFdA	0.0003 ± 0.0001	0.16 ± 0.03	19.2 ± 5.9	> 100	64,000	> 630	
LdT	> 100	0.007 ± 0.001	> 100	> 100	N.D.	> 14,000	
CAdA	0.0004 ± 0.0001	0.0004 ± 0.0002	3.5 ± 1.2	3.8 ± 1.0	8,750	9,500	
CdG	0.0004 ± 0.0001	0.0004 ± 0.0001	2.8 ± 0.9	3.2 ± 0.7	7,000	8,000	

Abbreviations: IC₅₀: 50% inhibitory concentration, CC₅₀: 50% cytotoxic concentration, S.I.: selectivity index. (CC₅₀/IC₅₀), N.D.: not determined.

Anti-HIV-1 activity was determined using MT-4 cells, while anti-HBV activity was determined using HepG2 2.2.15 cells. Each assay was conducted in duplicate or triplicate. The values represent mean values (± 1 S.D.) out of two or three independent experiments.

In vitro susceptibility of the HBV^{WT}, HBV^{ETV-R^{L180M/S202G/M204V}}, and HBV^{ADV-R^{A181T/N236T}} to ETV, ADV, CAAdA, and CdG.

Table 2

Drug	IC ₅₀ (μM) against		
	HBV ^{WT}	HBV ^{ETV-R^{L180M/S202G/M204V}}	HBV ^{ADV-R^{A181T/N236T}}
ETV	0.014 ± 0.008 [n=2]	>1 (>71.4) [n=2]	0.18 (13.2) [n=1]
ADV	7.7 [n=1]*	36.4 ± 23.5 (4.7) [n=2]	39.5 ± 13.3 (5.1) [n=2]
CAAdA	0.007 ± 0.01 [n=2]	0.07 ± 0.01 (9.7) [n=2]	0.09 ± 0.07 (12.9) [n=2]
CdG	0.01 ± 0.002 [n=2]	0.04 ± 0.03 (4.3) [n=2]	0.04 ± 0.02 (4.0) [n=2]

* Referenced from Ref 37

The IC₅₀ numbers were obtained using Southern blotting assay. The IC₅₀ values represent average values (±1 S.D.) out of two experiments except for the IC₅₀ value of ETV against HBV^{ADV-R^{A181T/N236T}} and that of ADV against HBV^{WT}. The numbers in parentheses represent the fold changes of IC₅₀ values compared to those against HBV^{WT}. The numbers in brackets represent the replicate numbers.