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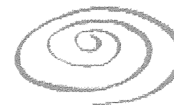

EFFECT OF TRANSLOCATION DEFECTIVE REVERSE TRANSCRIPTASE INHIBITORS ON THE ACTIVITY OF N348I, A CONNECTION SUBDOMAIN DRUG RESISTANT HIV-1 REVERSE TRANSCRIPTASE MUTANT

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Abstract

4'-Ethynyl-2-fluoro-2'-deoxyadenosine (EFdA) is a highly potent inhibitor of HIV-1 reverse transcriptase (RT). We have previously shown that its exceptional antiviral activity stems from a unique mechanism of action that is based primarily on blocking translocation of RT; therefore we named EFdA a Translocation Defective RT Inhibitor (TDRTI). The N348I mutation at the connection subdomain (CS) of HIV-1 RT confers clinically significant resistance to both nucleoside (NRTIs) and non-nucleoside RT inhibitors (NNRTIs). In this study we tested EFdA-triphosphate (TP) together with a related compound, ENdA-TP (4'-ethynyl-2-amino-2'-deoxyadenosine triphosphate) against HIV-1 RTs that carry clinically relevant drug resistance mutations: N348I,D67N/K70R/L210Q/T215F, D67N/K70R/L210Q/T215F/N348I, and A62V/V75I/F77L/F116Y/Q151M. We demonstrate that these enzymes remain susceptible to TDRTIs. Similar to WT RT, the N348I RT is inhibited by EFdA mainly at the point of incorporation through decreased translocation. In addition, the N348I substitution decreases the RNase H cleavage of DNA terminated with EFdA-MP ($T/P_{EFdA-MP}$). Moreover, N348I RT unblocks EFdA-terminated primers with similar efficiency as the WT enzyme, and further enhances EFdA unblocking in the background of AZT-resistance mutations. This study provides biochemical insights into the mechanism of inhibition of N348I RT by TDRTIs and highlights the excellent efficacy of this class of inhibitors against WT and drug-resistant HIV-1 RTs.

Key words: EFdA, ENdA, N348I, Translocation Defective Reverse Transcriptase Inhibitors, Reverse Transcriptase, HIV-1, Antivirals.

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INTRODUCTION

Human immunodeficiency type 1 reverse transcriptase (HIV-1 RT) is a key enzyme that converts single-stranded genomic RNA into double-stranded DNA, which in turn is transported to the nucleus and integrated into the host cellgenome. The HIV-1 RT catalyzes both the RNA-and DNA-dependent DNA polymerase, and RNase H activities (11).The functional form of HIV-1 RT is a heterodimer consisting of p66 and p51 polypeptides (16). The p66 subunit has both enzymatic activities and includes the polymerase and the RNase H domains. The polymerase domain consists of the fingers, palm and thumb subdomains which are analogous to a right hand connected to the RNase H domain through the connection subdomain (42) (Fig. 1).

The p51 subunit lacks the RNase H domain and has been proposed to play a structural role (42), although recent work from our laboratory has also shown that mutations in the p51 subunit affect the polymerase and RNase H enzymatic functions of RT (38).

Highly active antiretroviral therapies (HAART) have been very effective in suppressing viral loads and having a significant impact on the life expectancy of HIV patients. Key components of HAART are drugs that target HIV RT. The two classes of RT inhibitors currently used in the clinic are nucleoside-nucleotide RT inhibitors (NRTI) and non-nucleoside RT inhibitors (NNRTI). NRTIs inhibit RT by acting as chain-terminators after they are incorporated into the nascent DNA chain. NNRTIs act noncompetitively by binding to a hydrophobic pocket adjacent to, but distinct from the polymerase active site of RT and by imposing rigidity to the movements of thumb subdomain required for efficient polymerase function (20, 22, 35, 37, 39, 41).

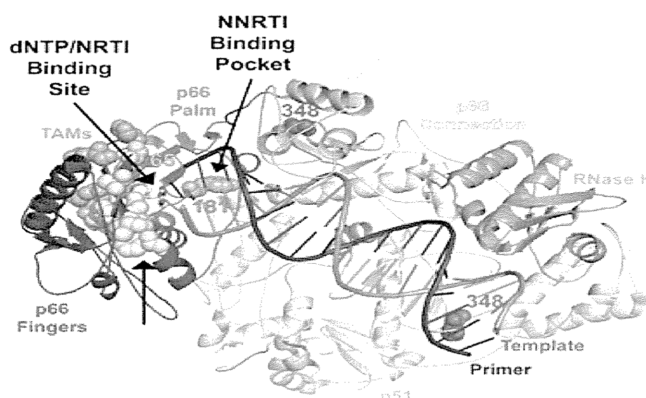


Figure 1. HIV-1 RT structure with highlighted residues of drug-resistance. The RT color scheme is as follows: fingers in blue, palm in red, thumb in green, connection in yellow, RNase H in orange, and p51 in gray. The Q151M complex is shown in cyan, TAMs in magenta, and 348 residue in purple. The RT coordinates are from PDB ID 1T05. The figure was made using PyMOL.

Despite the phenomenal success of HAART regimens, continuous use of antivirals leads to the emergence of viruses that are resistant to all known anti-AIDS drugs. The mutations associated with NNRTI resistanceare generally located at the NNRTI binding pocket (NNIBP). However, the mutations that cause resistance to NRTIs have been noted to be scattered in the polymerase domain (22, 39). While most NNRTI and NRTI resistance mutations are at the palm and fingers subdomains of HIV-1 RT, it has recently been shown that some mutations associated with NNRTI and NRTI resistance are at the connection and RNase H regions of RT (6, 8, 13, 15, 17, 29, 43). The most significant of these mutations is N348I, which confers moderate resistance to both NRTIs and NNRTIs, and is present in a significant number of clinical isolates, especially in the presence of other NRTI mutations.

In light of the new emerging drug resistance mutations, it is essential to identify inhibitors that are very potent and effective against viral strains that are resistant to all approved therapeutics. One such inhibitor is 4'-ethynyl-2-fluoro-2'-deoxyadenosine triphosphate (EFdA-TP) (18, 19). We have recently reported the mechanism of HIV inhibition by EFdA (26).In contrast to other approved NRTIs, which have a modification at 3'OH, EFdA contains a 3'OH moiety and blocks DNA synthesis by locking the primer terminus at the pre-translocation site of HIV-1 RT. In addition to EFdA, we have recently shown that ENdA also inhibits HIV RT potently acting as a TDRTI (data not shown).

Recently, using transient-state kinetic experiments we established the mechanism of NNRTI resistance of HIV-1 RT containing the N348I mutation at the connection subdomain of the enzyme (38).We showed that the resistance to the NNRTI nevirapine (NEV) is primarily the result of changes distant from the NNRTI binding pocket, which decrease inhibitor binding (increase $K_{d\text{-}NVP}$) by primarily decreasing the association rate of the inhibitor ($k_{on\text{-}NVP}$). Moreover, the N348I mutation increased nucleic acid binding affinity, enhanced processivity and lowered the catalytic turnover rate of the natural substrate. In this study we determine the ability of TDRTIs to block reverse transcription by the multi-drug resistant N348I HIV-1 RT as well as other NRTI resistant RTs, D67N/K70R/L210Q/T215F (resistant to AZT by the excision mechanism) D67N/K70R/L210Q/T215F/N348I, and A62V/V75I/F77L/F116Y/Q151M (multidrug resistant to AZT and dideoxynucleotide RT inhibitors).

MATERIALS AND METHODS

Enzymes and Nucleic acids

The RT genes coding for p66 and p51 subunits of BH10 HIV-1 were cloned in the pETDuet-1 vector (Novagen) using restriction sites *NcoI* and *SacI* for the p51 subunit, and *SacII* and *AvrII* for the p66 subunit (2, 38). The sequences coding for a hexa-histidine tag and the 3C protease recognition sequence were added at the N terminus of the p51 subunit. RT was expressed in BL21 (Invitrogen) and purified by nickel affinity chromatography and monoQ anion exchange chromatography (33). Oligonucleotides used in this study were chemically synthesized and purchased from Integrated DNA Technologies (Coralville, IA). Sequences of the DNA substrates are shown in Table 1. Deoxynucleotide triphosphates and dideoxynucleotide triphosphates were purchased from Fermentas (Glen Burnie, MD). EFdA and ENdA were synthesized by Yamasa Corporation (Chiba, Japan) as described before (30). Using EFdA and ENdA as starting material the triphosphate forms EFdA-TP and ENdA-TP were synthesized by TriLink BioTechnologies (San Diego, CA). Concentrations of nucleotides, EFdA-TP and ENdA-TP were calculated spectrophotometrically on the basis of absorption at 260 nm and their extinction coefficients. All nucleotides were treated with inorganic pyrophosphatase (Roche Diagnostics) as described previously (24) to remove traces of PPi contamination that might interfere with the rescue assay.

Primer extension assays

Inhibition of HIV-1 RT by TDRTIs

DNA template (T_{d31}) was annealed to 5'-Cy3 labeled DNA primer (P_{d18}). To monitor primer extension, the T_{d31}/5'Cy3-P_{d18} hybrid (20 nM) was incubated at 37°C with WT or drug-resistant HIV-1 RTs (20 nM) in a buffer containing 50 mM Tris (pH 7.8) and 50 mM NaCl (RT buffer). Varying amounts of EFdA-TP or ENdA-TP were added and the reactions were initiated by the addition of 6 mM $MgCl_2$to a final volume of 20 µl. All dNTPs were present at a final concentration of 1 µM. The reactions were terminated after 15 minutes by adding equal volume of 100% formamide containing traces of bromophenol blue. The products were resolved on a 15% polyacrylamide 7 M urea gel. In this and in subsequent assays, the gels were scanned with a PhosphorImager (FujiFilm FLA 5000), the bands for fully extended product were quantified using Multi Gauge (FujiFilm) and results were plotted using one

Table 1.DNA and RNA sequences used in this study.

Polymerization experiments	
T_{d31}	5'-CCA TAG ATA GCA TTG GTG CTC GAA CAG TGA C
T_{r31}	5'-CCA UAG AUA GCA UUG GUG CUC GAA CAG UGA C
P_{d18}	5'-Cy3-GTC ACT GTT CGA GCA CCA
Footprinting experiments	
T_{d43}	5'-Cy3-CCA TAG ATA GCA TTG GTG CTC GAA CAG TGA CAA TCA GTG TAGA
P_{d30}	5'-TCT ACA CTG ATT GTC ACT GTT CGA GCA CCA
RNase H experiments	
T_{r35}	5'-Cy3-GGA AAU CUC UAG CAG UGG CGC CCG AAC AGG GAC CU
P_{d25}	5'-AGG TCC CTG TTC GGG CGC CAC TGC T

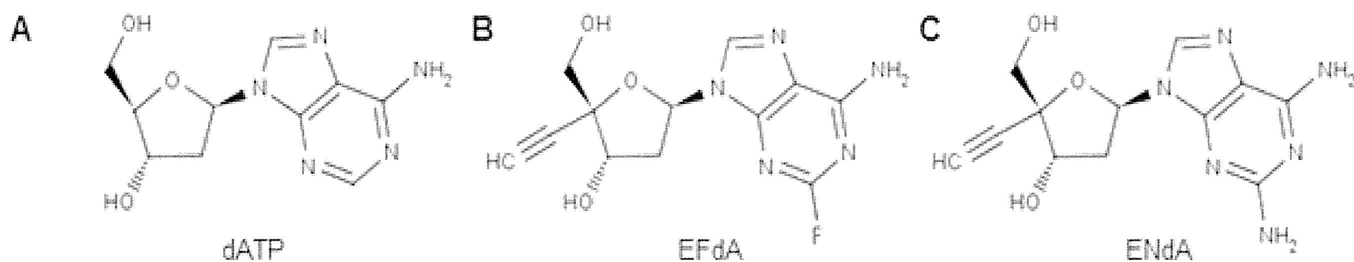


Figure 2. Chemical structures of dATP, EFdA and ENdA.

site competition equation on GraphPad Prism 4 to determine the IC_{50} for EFdA-TP and ENdA-TP.

Site-specific Fe^{2+} Footprinting Assay

Site-specific Fe^{2+} footprints were monitored on 5'-Cy3-labeled DNA templates. 100 nM of 5'Cy3-T_{d43}/P_{d20} was incubated with 600 nMWT or N348I RT in a buffer containing 120 mM sodium cacodylate (pH 7), 20 mM NaCl, 6 mM $MgCl_2$, and either of 5 μM ddATP or 1 μM EFdA-TP, to allow quantitative chain-termination. Prior to the treatment with Fe^{2+}, complexes were pre-incubated for 7 min with increasing concentrations of the next incoming nucleotide (dTTP). The complexes were treated with ammonium iron sulfate (1 mM) as previously described (21). This reaction relies on autoxidation of Fe^{2+}(3) to create a local concentration of hydroxyl radical which cleaves the DNA at the nucleotide closest to the Fe^{2+} specifically bound to the RNase H active site.

ATP-dependent Excision and Rescue assay

20 nM of purified T_{d31}/$P_{d18\text{-}EFdA\text{-}MP}$ or T_{r31}/$P_{d18\text{-}EFdA\text{-}MP}$ were incubated with 60 nM WT, N348I, D67N/K70R/L210Q/T215F or D67N/K70R/L210Q/T215F/N348I RT in the presence of 3.5 mM ATP, 100 M dATP, 0.5 μM dTTP, and 10 μM ddGTP in RT buffer and 10 mM $MgCl_2$. Aliquots of the reaction were stopped at different time points (0-90 min) and analyzed as described above.

RNase H Assays

RNase H assays were performed by incubating the RNA/DNA duplex 5'Cy3-T_{r35}/P_{d25} or 5'Cy3-T_{r35}/$P_{d25\text{-}ddAMP}$ or 5'Cy3-T_{r35}/$P_{d25\text{-}EFdA\text{-}MP}$ (50 nM) with WT or N348I RT (50 nM) in RT buffer at 37 °C with $MgCl_2$ (6 mM). Reactions were quenched after incubation (1–5 min) with equal volumes of formamide containing trace amounts of bromophenol blue. Reaction products were analyzed as before. The primary RNase H cleavage product is mainly 18 nucleotides from the 3'-end of the DNA primer (18 nucleotides), and the secondary cleavage product is mainly 12 nucleotides from the 3'-end of the primer (12 nucleotides) as reported previously (10, 12, 38).

RESULTS

The inhibitors used here to characterize the susceptibility of N348I to various drugs are adenosine analogs. The structures of these analogs are shown in Fig. 2. The normal deoxynucleotide dATP is shown in Fig. 2A. EFdA and ENdA are shown in Figs. 2B and 2C, respectively.It can be seen in these figures that unlike other anti-HIV NRTIs both EFdA and ENdA have a 3'OH. These compounds also contain an ethynyl group at the 4' position. EFdA and ENdA differ in their substitutions at the 2 position of the purine ring. EFdA at this position has fluorine whereas ENdA has an amino group.

Inhibition of WT and N348I mutant of HIV-1 RT

The inhibition of WT, N348I, D67N/K70R/L210Q/T215F, D67N/K70R/L210Q/T215F/N348I mutants of HIV-1 RT by EFdA-TP and ENdA-TP was assessed by a primer extension assay. As shown in Fig. 3A-3D, EFdA-TP and ENdA-TP suppressed RT-catalyzed DNA synthesis in a dose-dependent manner. The IC_{50}values for both analogs are shown in Table 2. N348I, D67N/K70R/L210Q/T215F and D67N/K70R/L210Q/T215F/N348I RTs were inhibited by EFdA-TP and ENdA-TP with similar efficiency compared to the WT enzyme. In addition, another mutant HIV-1 RT (A62V/V75I/F77L/F116Y/Q151M) was included in drug susceptibility assays (Fig. 3E-3G).

We have previously shown that EFdA inhibits DNA synthesis at the point of incorporation. Thus, we examined here the stopping patterns after incorporation products of the primer extension assay for the stopping patterns (Fig. 3). The primer synthesis shown in Fig. 3 clearly demonstrates that the stopping pattern follows the incorporation of adenosine analogs. Three distinct bands at positions 1, 6 and 10 indicate that both analogs inhibit RT mainly at the point of incorporation. Therefore, these compounds act primarily as obligate chain terminators. There is also an additional band at position 7, suggesting that in some

Table 2. IC_{50} values of EFdA-TP and ENdA-TP against WT and drug-resistant HIV-1 RTs.

Inhibitor/Enzyme	WT	N348I	D67N/K70R/ L210Q/T215F	D67N/K70R/L210Q/ T215F/N348I	A62V/V75I/F77L/ F116Y/Q151M
EFdA-TP (nM)	130	122	157	217	121
ENdA-TP (nM)	71	54	98	110	85

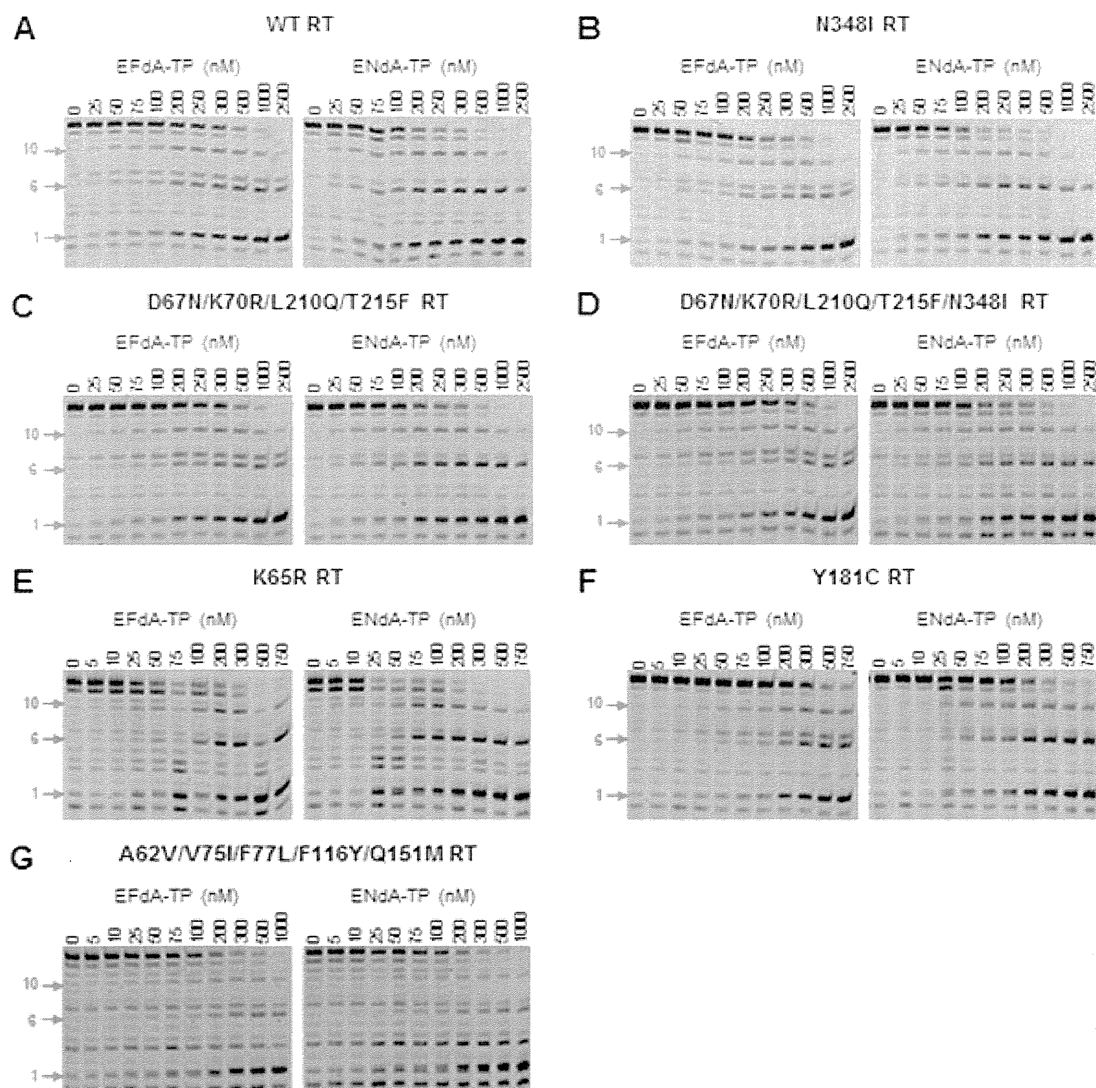


Figure 3. EFdA-TP and ENdA-TP inhibit WT and drug-resistant HIV-1 RTs. (A) T_{d31}/P_{d18} was incubated with various HIV-1 RTs for 15 minutes in the presence of 1μM dNTPs, $MgCl_2$ and increasing concentrations of EFdA-TP or ENdA-TP. The products synthesized by HIV-1 RT were quantified and plotted against increasing concentrations of the inhibitors. The IC_{50}values of the nucleotide analogs were determined by quantifying the percent of full extension and fitting the data points to GraphPad Prism 4 using one-site competition nonlinear regression (shown in Table 2). Arrows indicate the positions where dATP or dATP analogs are expected to be incorporated.

instances EFdA may allow addition of one nucleotide after its incorporation, thus acting as a delayed chain terminator (Fig. 3). This type of inhibition is far less common and is sequence-dependent. These finding agrees with our previous studies on WT RT (26).

Effect of EFdA-MP on Translocation of WT and N348I mutant of HIV-1 RT

The connection subdomain mutation N348I has been related to the altered DNA binding affinity and processivity of the mutant enzyme compared to the WT RT (4, 38). Since EFdA is a TDRTI and its incorporation is assumed to affect the translocation and thereby DNA binding and processivity, we investigated the translocation of EFdA-containing template-primers using the hydroxyl radical site-specific footprinting assay (21). The results of the footprinting assay shown in Fig. 4 demonstrate that the presence of EFdA-MP at the 3'end of the DNA primer blocks translocation and prevents incorporation of the next incoming dNTP. Therefore, similar to WT RT, the mutant N348I RT is also inhibited by EFdA-TP *via* the same mechanism.

Effect of EFdA-MP on RNase H activity of WT and N348I RTs

The template/primers containing EFdA-MP, ddAMP, or without inhibitor incorporated at the 3'end of the primer were used in RNase H assays with WT and N348I RTs in a time dependent manner. As previously noted, Fig. 5 shows that N348I mutant RT has decreased RNase H activity for all substrates used in this assay. The RNase H assays carried out in presence of T/P trap showed the disappearance of the secondary cuts for both enzymes used here. This is likely due to a defect in translocation that EFdA imposes on the enzyme. Interestingly, the primary cut of EFdA-teminated primers is a single band when the T/P has EFdA, but not ddA at the 3' primer terminus. Moreover, the RNA cleavage of $T_{r35}/P_{d25\text{-EFdA-MP}}$ was less than that of $T_{r35}/P_{d25\text{-ddAMP}}$ or T_{r35}/P_{d25} possibly because of less favorable positioning at the RNase H of T/P with EFdA at the 3' terminus.

ATP-dependent unblocking of EFdA-MP terminated primers by WT and N348I RTs

Since EFdA-MP-terminated primers bind predominantly in a pre-translocation mode we expected that EFdA-MP will be efficiently unblocked by both WT and N348I RTs.

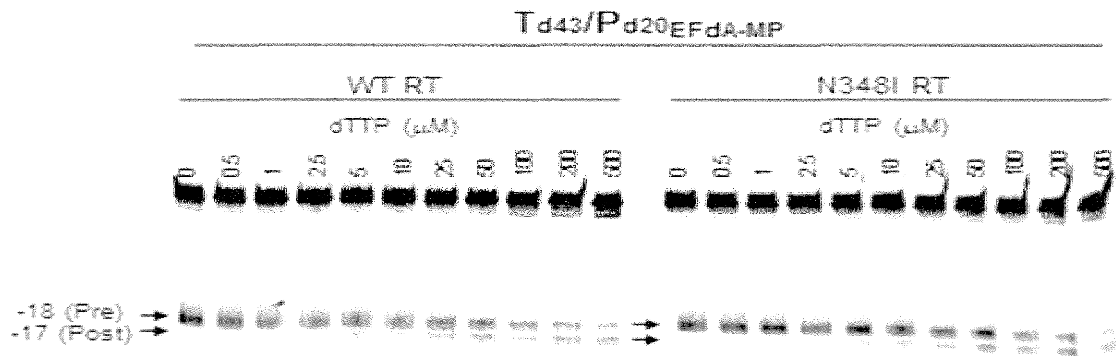


Figure 4. Determination of the translocation state of WT or N348I HIV-1 RT bound to $T_{d43}/P_{20\text{-EFdA-MP}}$. The translocation state of RT after EFdA-MP incorporation was determined using site-specific Fe^{2+}footprinting. $T_{d43}/P_{d30\text{-EFdA-MP}}$ (100 nM) with 5'-Cy3 label on the DNA template was incubated with HIV-1 RT (600 nM) and various concentrations of the next incoming nucleotide (dTTP). The complexes were treated for 5 min with ammonium iron sulfate (1 mM) and resolved on a polyacrylamide 7 M urea gel. An excision at position 18 indicates a pre-translocation complex, whereas the excision at position 17 represents a post-translocation complex. In both WT and N348I RT EFdA-MP prevents translocation with similar efficiency.

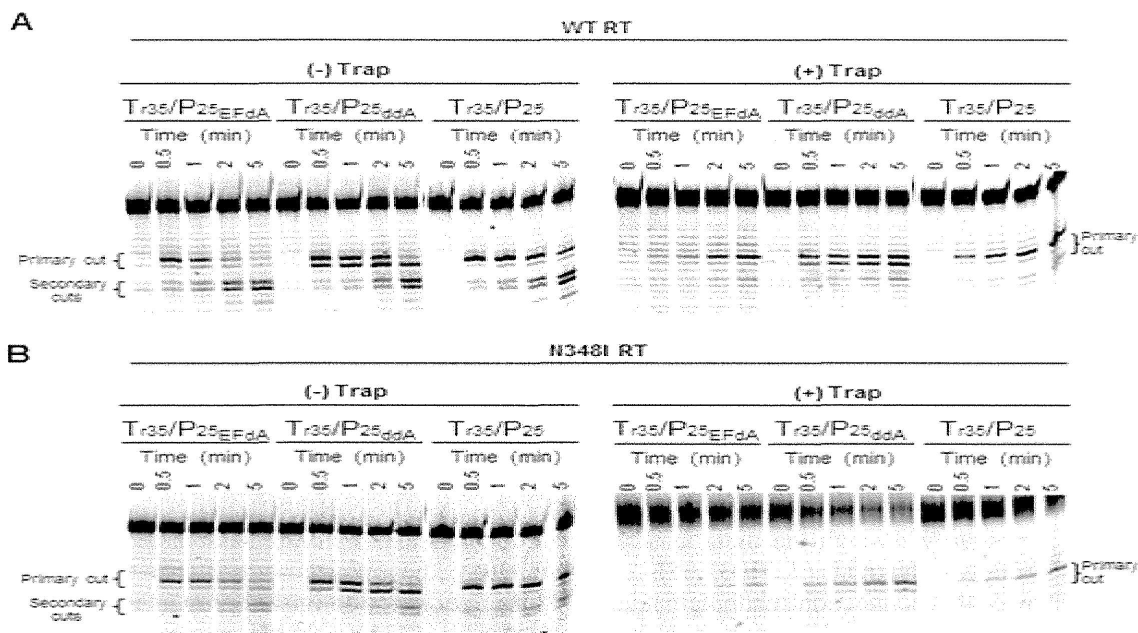


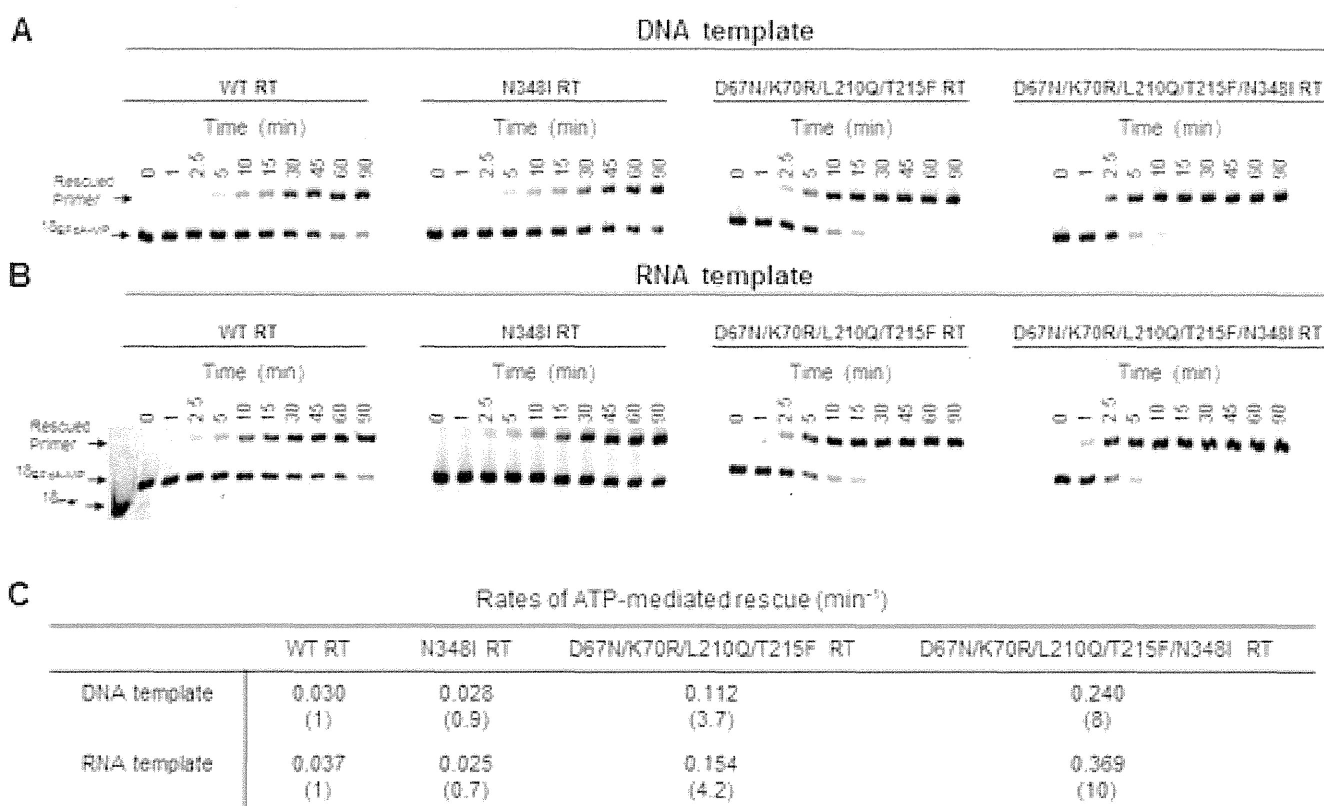
Figure 5. Effect of EFdA on RNase H activity of WT and N348I HIV-1 RTs. 50 nM Cy3-$T_{r35}/P_{d25\text{-EFdA-MP}}$ or Cy3-$T_{r35}/P_{d25\text{-ddAMP}}$ or Cy3-T_{r35}/P_{d25} was incubated with 50 nMWT (A) or N348I (B) HIV-1 RT for varying times (0-5 minutes) at 37°C in RT buffer. The experiment was carried out in the presence or absence of non-labeled T_{d35}/P_{d25} trap (25 μM). Reactions were initiated with the addition of $MgCl_2$ and stopped with formamide. The primary and secondary cuts are indicated in the gel images.

The ATP-dependent excision and subsequent rescue of EFdA-MP primers is shown in Fig. 6. The bands marked as 'Rescued Primer" have comparable product for the WT and N348I mutant enzyme for both DNA (Fig. 6A) and RNA (Fig. 6B) templates suggesting that resistance mutant N348I does not have any significant effect on the unblocking of EFdA-MP containing primers (RNA *vs.* DNA) (Fig. 6). However, the N348I mutation in the background of AZT resistance mutations D67N, K70R, L210Q and T215F showed a 2-fold increase in unblocking EFdA-MP containing primers both with DNA and RNA templates (Fig. 6).

DISCUSSION

There are currently more than 20 antiretrovirals that have been approved by the US Food and Drug Administration for the treatment of HIV infection. They fall into four categories, targeting HIV RT, protease, integrase, the entry step, and the fusion of the viral and cell membranes. RT inhibitors are either NRTIs or NNRTIs.The NRTIs-such zidovudine (AZT) and lamivudine compete with the natural substrates and get incorporated into the nascent DNA chain, blocking further polymerization because they lack a 3'OH group required for DNA synthesis. NNRTIs such as nevirapine and efavirenz inhibit the polymerase activity of RT by binding at a hydrophobic pocket nearly 10 Å away from the polymerase active site (Fig. 1). This pocket is created after the binding of NNRTIs. The highly active antiretroviral therapy (HAART) introduced in the mid-90s contains the combination of antivirals (generally a protease inhibitor and two NRTIs or an NNRTI and two NRTIs) targets the replication of the resistant virus.

Extended or incomplete treatments with antiretrovirals result in the emergence of drug resistance mutations. In the case of drugs that target RT, most of the resistance mutations were found to be present in the polymerase domain of RT. These resistance mutations against NRTIs function primarily with two mechanisms: (i) they reduce the binding affinity/incorporation of NRTI (34, 40) or (ii) enhance



	WT RT	N348I RT	D67N/K70R/L210Q/T215F RT	D67N/K70R/L210Q/T215F/N348I RT
DNA template	0.030 (1)	0.028 (0.9)	0.112 (3.7)	0.240 (8)
RNA template	0.037 (1)	0.025 (0.7)	0.154 (4.2)	0.369 (10)

Figure 6. ATP-dependent unblocking of EFdA-MP terminated primers. ATP-dependent rescue of $T_{d31}/P_{d18\text{-}EFdA\text{-}MP}$ (A) and $T_{r31}/P_{d18\text{-}EFdA\text{-}MP}$ (B). Purified $T/P_{EFdA\text{-}MP}$ was incubated with WT, N348I, D67N/K70R/L210Q/T215F or D67N/K70R/L210Q/T215F/N348I HIV-1 RT in the presence of ATP (3.5 mM), dATP (100 μM), dTTP (0.5 μM), ddGTP (10 μM) and 10 mM $MgCl_2$ at 37 °C. Aliquots of the reaction were stopped at the indicated time points (0-90 min). (C) The rates of the ATP-dependent rescue of EFdA-MP terminated primers were calculated after quantifying the rescued products and plotting to the burst equation in GraphPad Prism 4.

the selective excision of incorporated NRTI from a chain-terminated primer terminus (9, 23-25, 36). The resistance against NNRTIs is primarily through the mutations that reduce the binding affinity of NNRTIs (7, 31, 32,35).

Recent studies showed that connection subdomain mutations can confer resistance to NRTIs. Nikolenko et al. suggested that some of these mutations increase AZT resistance by reducing template RNA degradation, thereby preserving the RNA template and providing additional time for RT to excise AZT monophosphate (27, 28). Hachiya et al., (13) as well as another research group (43) identified a clinical isolatewith phenotypic resistance to nevirapine (NVP) in the absence of known NNRTI mutations. This resistance was shown to be caused by N348I, a mutation at the connection subdomain of HIV-1RT. This mutation is not a polymorphism, as it exists in more than 10% of drug-treated, but not drug-naïve HIV patients. The connection subdomain mutation N348I has been related to the altered DNA binding affinity and processivity of the mutant enzyme compared to the WT RT (4, 38). Ehteshami et al. showed that N348I enhances resistance to AZT through both RNase H-dependent and -independent mechanisms (10). Since EFdA is a TDRTI and its incorporation is assumed to affect the translocation and thereby DNA binding and processivity, we investigated the susceptibility of two highly potent antiretrovirals EFdA and ENdA.

We report that both EFdA-TP and ENdA-TP are very potent inhibitors of N348I, D67N/K70R/L210Q/T215F, D67N/K70R/L210Q/T215F/N348I, and A62V/V75I/F77L/F116Y/Q151M RTs. They inhibit RT primarily at the point of incorporation and since they prevent enzyme translocation they both belong to the TDRTI class of NRTIs. The D67N/K70R/L210Q/T215F set of mutations are the classical thymidine-associated mutations (TAMs), which are known to cause resistance to AZT by enhancing excision of AZT-terminated primers (1, 5, 23). The A62V/V75I/F77L/F116Y/Q151M set of mutations is known as the "Q151M" complex RT, and has been known as a multidrug-resistance mutation, since the latter mutations are known to be involved in resistant variants with reduced susceptibility to dideoxynucleotides and to AZT. Unlike D67N/K70R/L210Q/T215F RT, the Q151M complex decreases susceptibility to NRTIs by decreasing incorporation efficiency of the inhibitors rather than increasing excision and unblocking of chain-terminated primers (14). Finally, N348I is known to cause resistance to both NRTIs and NNRTIs. Hence, collectively, these mutants represent all mechanisms by which RT becomes resistant to available antivirals. Importantly, we find that they are all susceptible to the EFdA and ENdA TDRTIs.

Hence, this new class of RT inhibitors should be able to efficiently block viruses that carry clinically relevant mutations, including the new connection domain mutation N348I.

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Other articles in this theme issue include references (44-71).

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総　論

新しい抗ウイルス薬開発の考え方

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要　旨　宿主代謝経路依存度の高いウイルスに対する治療薬開発は困難であったが，特異的抗ヘルペス薬 acyclovir の同定後，抗ウイルス薬の概念は一変する．効率的な開発のためのスクリーニング法，unmet medical needs，特許戦略について述べる．

はじめに

インターフェロンなどに抗ウイルス効果があることは知られていたが，狭義の意味では 1977 年に同定された抗ヘルペス薬である acyclovir[1]が初めての抗ウイルス薬であろう．その後，HIV に対する dideoxynucleoside 誘導体[2]，インフルエンザに対するノイラミニダーゼ阻害剤[3]が開発され，ウイルス感染症に対する有効な治療薬として広く認められるようになった．しかし，上記のウイルス疾患でも安全に，そして完全に治療できる状況には至っていない．その理由の 1 つとして薬剤耐性の獲得があげられる．ウイルスは複製時に正確性に乏しい核酸合成酵素を用いることから変異しやすく，結果として耐性を獲得してしまう．例えば，HIV 感染症では 20 以上の強力な阻害剤が開発されても耐性が克服できないだけでなく，耐性 HIV の伝播まで起こっている[4]．インフルエンザにおいても耐性株が未治療患者からも分離されている[5]．これまでの抗ウイルス薬開発から取り残されたウイルスへの対応も迫られている一方で，新たなウイルス（SARS コロナウイルスなど）も同定されている．本項では次世代型抗ウイルス薬開発の方向について，筆者らの意見を述べてみたい．

■薬剤標的分子は決めるべきか

世界的にみても 10 万を超える化合物を有するアカデミアは少ないが，企業では通常多くの化合物を有している．これらのライブラリーから目的の化合物を見出す方法として，あえて標的を決めずにすべて検討するランダムスクリーニングがまずあげられる．化合物のウイルス複製阻止効果を判定することで，あらゆる複製ステップに対する阻害剤を見出せる．しかし，細胞を用いたアッセイは数日単位の時間を要する medium through put レベルに留まりやすく，莫大な労力や費用がかかってしまう．

一方，標的以外の分子に効果を示す薬剤は見出すことができなくなるが，ウイルス由来酵素など標的分子を絞ればランダムスクリーニングであっても効率をあげることができる．つまり，ウイルス由来酵素活性を指標にすることで，感染性ウイルスを使わず，短時間に，かつ細胞毒性があってもヒット化合物を同定できる．また，386 プレー

トでの自動化も比較的容易である．標的分子構造が明らかにされていれば，さらに効率化が図れる．具体的には標的分子に結合してその機能を阻害する化合物を *in silico* シミュレーションから選別し，ウイルス蛋白との結合アッセイやウイルス感染系等の *in vitro* 効果判定を行う方法である．例えば，抗インフルエンザ薬のノイラミニダーゼ阻害剤[6]やHIV-1プロテアーゼ阻害剤[7]などに応用されている．この方法の問題点として，揺らぎや動きをもって機能している分子も多く，結晶解析から得られた構造が生理的な蛋白構造を代表するとは限らない．最近では共結晶化による既存の薬剤との結合様式の解明によって克服されつつある．さらなる効率化を目指し，化合物合成前にコンピュータ上で標的分子結合に最適化された化合物を創生するバーチャルケミストリーも応用されている．そのために「京」に代表されるスーパーコンピュータが活用されている．いうまでもないが，これらの方法では安定性や細胞内への取り込み効率は反映されないため，適宜合成展開と *in vitro* または *in vivo* アッセイでの確認が必要である．

■スクリーニング法の選択

対象化合物が比較的少なければ効率にこだわらずに，その研究室が現有するアッセイでスクリーニングを開始した方がよいと思われる．我々は細胞を用いたMTT比色法による生存細胞数から検討しており，薬剤濃度を段階的に数点検討しても，他の研究の合間に100化合物/weekのスクリーニングが可能である[8]．ELISAを用いた分子間結合阻害スクリーニングであれば，反応時間を入れても最短2〜3時間で検討でき[9]，大量のスクリーニングが可能となる．分子合成研究者の立場からすれば，せっかく創製したことから余力があればいくつかのウイルスに対する効果を検討したいところである[10]．一方，検体が多い場合，スクリーニングの選択は熟考が必須である．使用するスクリーニング法には一長一短があるため（表1），どのような薬剤を同定したいのか，コンセプトが大切である．また現実的な労力，時間，技術，資金から方法を選択せざるを得ない場合もある．

■新しい特徴のあるアッセイ系を使う

これまでHIVやヘルペス等に関しては膨大な数の化合物がランダムスクリーニングされている．そのため，組合せ論的に構成されるコンビナトリアルケミストリーでカバーできなかった独自の化合物以外はすでに試されたと考えるべきである．つまり，これまでと同じスクリーニングや合成で新たな化合物を見出す可能性は少ない．それでは，どのようなスクリーニングや化合物がいいのであろうか．例えば，既存のアッセイ系では見逃される化合物を検出できるスクリーニングである．生体内で重要なHIVレセプターの1つであるCCR5は株化細胞ではほとんど発現しておらず，主なHIV実験室株はCCR5非依存的に感染できる．そのためCCR5発現細胞とCCR5依存性HIVを用いることでCCR5阻害剤は見出されている[11]．他にも臨床抗ヘルペス薬のadelavin（araA）はその効果を検出できない細胞株があることから[12]，細胞の選択も重要である．

治療が確立されたウイルス，例えばヘルペスではTK欠損株，HIVでは薬剤耐性株に効果を示す新薬が必要とされている．今後，耐性ウイルスは増加するため，初めから耐性ウイルスを使用したスクリーニングも考えられる．多少効果が劣っていても耐性ウイルスに効果を示すことは意義がある．事実，我々が開発したEFdA[13]は，耐性ウイルスを用いたスクリーニングで同定している．

■アイディアは異分野融合から生まれる

新しいアッセイや化学合成法などを確立させるためには異分野研究者の協力が必要である．標的蛋白の立体構造の解析，生物学的活性ドメインや基質結合部位の同定からの最適化を図り，臨床薬が開発されているが[6]，これらは生物学者と化学合成者に構造学，そしてシミュレーションといった異分野研究者の協力で成し遂げられている．他

表1　スクリーニング法の比較

	標的分子を限定しない (cell-based)	標的分子を限定 (protein-based)
長所	見落としが少ない. 毒性の検討が可能.	迅速・簡便. ウイルス取扱い不要. 毒性にかかわらず検出可能.
短所	長期間・労力を要する. 毒性のあるものや細胞透過性のないものを見落とす. 作用機序があいまい.	標的分子以外は検討できない*1. 毒性・細胞透過性*2を評価できない. ↓ 化合物の合成展開も必要.
スクリーニング例	プラーク法 MTT 法 レポーターアッセイ（MAGI 法など）	ELISA *in vitro* 酵素アッセイ α-スクリーン

*1：標的分子特異的スクリーニングであっても，異なるアッセイを用いて確認する必要がある．HIV インテグラーゼ阻害剤と考えられていたものが吸着阻害剤であった例もある[15]．
*2：侵入阻害剤には必ずしも要求されない．また，透過性がなくとも活性を検出できるため利点でもある．

にもペプチドは経口吸収がなく薬剤になりにくいと考えられてきたが，ペプチド研究者の参入によって HIV 融合阻害剤は臨床応用に至った[14]．最も臨床薬剤が開発された HIV でも，複製に必須な蛋白の半分も標的分子として利用されておらず，まだ開発余地が残されている．研究費の有効活用のためにも，異分野融合から既存の方法を打破すべきである．

■抗ウイルス薬の unmet medical needs とは

長期間の服用が必要な慢性感染症では1日1回の薬剤で，かつ副作用のないものが望まれるが，開発の初期段階から考慮することは難しい．また，耐性化への対応も迫られる．治癒が見込まれないウイルス性疾患では，新規感染が起こらない状態を維持する，もしくは感染細胞を排除する，という2つの方針がある．特に癌ウイルスに対する方針としては後者が優れていると思われる．例えば，HBV は通常の逆転写酵素阻害剤だけでは潜伏 HBV ゲノムを取り除くことができない．また，日本に多い HTLV-1 に関しても，これまでほとんど開発されていない．他に，熱帯出血性ウイルスなどに代表される neglected infectious diseases も標的となろう（**表2**）．個々のウイルスについてこれから何が求められていくかは，本特集の各論を参照いただきたいが，上記したように研究費をお互いにつぎ込みながら競争する領域は避け，新たなスクリーニング系をもつ，もしくは新たな標的分子に対する薬剤を開発するべきである．

■抗ウイルス薬におけるコンパニオン診断法の確立

Ribavirin のような非特異的に幅広いウイルスに対して効果を示す薬剤もあるが，基本的に個々のウイルスに特異的な薬剤開発が必要となることから診断法の確立も同時に進める必要がある．HIV や肝炎ウイルス等は慢性感染症に分類されるため診断に数日を要する抗体，もしくは遺伝子検査でも対応できるが，急性ウイルス感染症では簡便な診断キットがなければ適切な治療時期を逃すことになり，せっかくの薬効を発揮させることができない．インフルエンザ薬は迅速診断キット（コンパニオン診断薬）があることも広く普及した理由の1つになっている．併せて耐性検査の迅速化や簡便化も必要となっていくであろう．

表2　Unmet medical needsとしてのウイルス疾患

	関連ウイルス	疾患	現状
急性感染症[*1]	粘膜感染系ウイルス	風邪・胃腸炎など	対応なし
	熱帯ウイルス[*2]	出血熱など	対応なし
	多岐にわたるウイルス	脳炎・髄膜炎	ヘルペス以外対応なし
慢性感染症	HIV	HIV感染症	予後が改善中
	麻疹ウイルス	SSPE[*3]	予後不良
	HBV HCV HTLV-1 EBV HPV	ウイルス発癌	HBVに逆転写酵素阻害剤が有効．HCVが併用療法で排除可能となる．HPVにcidofovir[*4]が有効．他は対応なし

＊1：迅速診断法（コンパニオン試薬）の開発が必須．
＊2：海外渡航の増加とともに危険性が増加しており，感染制御の概念との併用も考慮すべきである．
＊3：subacute sclerosing panencephalitis（亜急性硬化性全脳炎；麻疹ウイルス変異株で引き起こされる）
＊4：acyclovir耐性HSVやCMVに効果を示す．アデノウイルスにも効果があると報告されているが，本邦では認可されていない．

■論文発表と創薬特許

創薬にとって特許は絶対に不可欠であるが，アカデミアにおいては学会・論文発表と相反する難しい問題である．日本や米国などでは発表後であっても一定期間以内に特許出願を行えば新規性喪失の例外規定が適用され，発明の新規性が認められるが，一部の国ではそのような特例は認められず，すでに発明が公知になったものとみなされてしまう可能性がある．また，大学内での学位発表が公知とみなされる場合も多いことから，大学院生の研究テーマとしては不適切である．さらに最終化合物において，「動物レベルで効果が出ない」「分解が早い」「毒性が出る」などの不測の事態に備え，バックアップ化合物，周辺化合物を含めた十分な特許戦略が必要である．なぜならば，特許出願を行うと自らの情報を開示することになり，不十分な特許の隙をついて，特許権を行使する際に障害となるような特許を他者にとられることもあり，有用な薬剤シーズが葬られる可能性すらある．Chemical biologyのためのツールとしての化合物開発を出口に設定している場合であっても，本当にツールにしか使えないか，検討すべきである．

■臨床応用という出口を見据えているか

短期間投与になるインフルエンザやヘルペスのような毎年一定数の患者の発生が見込まれる，もしくはHIVのような症例数が少なくとも投与が長期にわたる感染症に対する薬剤は利益を上げやすい．そのため，早い時点で製薬企業と共同研究を開始すれば，臨床応用への加速化が期待できる．一方でオーファンに代表されるような患者数が少ない疾患の場合，アカデミアでの開発を強いられることが多いが，非営利団体であるアカデミアは，これらの疾患に対する開発を積極的にすべきという考えもある．米国NIHで確立されているGMPレベルでの合成，精製ができるような環境を日本でも整備すべきである．そのため共同開発先のマッチングを含めて，これらのシーズを開拓できる法整備や補助が必要である．どちらにせよ，迷走を招かないために出口を研究開始時期に見据えるべきであろう．

おわりに

スクリーニング法には一長一短があり，どれが正解ということはない．それ以上に独自の方法を編み出すことが大事である．一部ウイルス性疾患に対しての治療法が確立しつつあるが，medical

needsを満たすべきウイルス疾患は依然残されたままである．これまで日本発の抗ウイルス薬は非常に少なく，米国に大きな後れを取っている．論文発表を出口とした研究ではなく，社会的意義を意識した創薬につなげたいと願う研究者の一助となれば幸いである．

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Mechanism of the dependence of hepatitis B virus genotype G on co-infection with other genotypes for viral replication

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SUMMARY. Hepatitis B virus (HBV) is classified into several genotypes. Genotype G (HBV/G) is characterised by worldwide dispersion, low intragenotypic diversity and a peculiar sequence of the precore and core region (stop codon and 36-nucleotide insertion). As a rule, HBV/G is detected in co-infection with another genotype, most frequently HBV/A2. In a previous *in vivo* study, viral replication of HBV/G was significantly enhanced by co-infection with HBV/A2. However, the mechanism by which co-infection with HBV/A2 enhances HBV/G replication is not fully understood. In this study, we employed 1.24-fold HBV/A2 clones that selectively expressed each viral protein and revealed that the core protein expressing construct significantly enhanced the replication of HBV/G in Huh7 cells. The introduction of the HBV/A2 core promoter or core protein or both genomic regions into the HBV/G genome showed that both the core promoter and core protein are required for efficient HBV/G replication. The effect of genotype on the interaction between foreign core protein and HBV/G showed that HBV/A2 was the strongest enhancer of HBV/G replication. Furthermore, Western blot analysis of Dane particles isolated from cultures of Huh7 cells co-transfected by HBV/G and a cytomegalovirus (CMV) promoter–driven HBV/A2 core protein expression construct indicated that HBV/G employed HBV/A2 core protein during particle assembly. In conclusion, HBV/G could take advantage of core proteins from other genotypes during co-infection to replicate efficiently and to effectively package HBV DNA into virions.

Keywords: co-transfection, core protein, genotype A, genotype G, hepatitis B virus, replication.

INTRODUCTION

Hepatitis B virus (HBV) infection affects more than 350 million people and is one of the major causes of acute and chronic liver disease. Acute HBV infection in adults is usually self-limiting, while chronic HBV infection can cause chronic hepatitis, liver cirrhosis or hepatocellular carcinoma [1]. As the clinical course in infected individuals depends on a complex interplay among various factors including viral, host and environmental factors, molecular characteristics of HBV including the genotype could become increasingly important in our understanding of HBV clinical implications [2].

Abbreviations: CMV, cytomegalovirus; CP, core promoter; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; SEAP, secreted alkaline phosphatase.

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Eight major HBV genotypes (A–H) have been identified by a sequence divergence >8% in the entire HBV genome [3,4] and have a relatively distinct geographical distribution, which may be associated with anthropological history [5]. Hepatitis B virus genotype G (HBV/G) was first described in 2000 by studies carried out in France [6]. It is usually detected during co-infection with other genotypes, most frequently with HBV/A2 [7,8]. Co-infection with HBV/C and H has also been reported [9–11]. One of the features distinguishing HBV/G from other genotypes is the 36-nucleotide (nt) insertion in its core gene [6,12]. Recent studies indicated that the 36-nt insertion increased core protein translation without enhancing mRNA abundance [13], and insertion of the 36-nt in the core region of genotypes A and D impaired genome replication, despite upregulation of core protein expression, indicating that the 36-nt insertion could alter core protein expression without altering the mRNA expression [14]. The other feature of the HBV/G genome that is unique is the possession of two stop codons in the precore region that prevents the expression of hepatitis B e antigen (HBeAg) [6,12]. Nevertheless, some HBV/G carriers are

HBeAg positive, which is explained by co-infection with an HBeAg-expressing HBV/A strain [7].

As previously reported, HBV/G monoinfection in uPA/SCID mice that had been transplanted with human hepatocytes (hereafter referred to as chimeric mice) resulted in very low level viral replication, but HBV replication increased markedly when the animals were co-infected with HBV/A2, C or H [11,15]. Furthermore, the co-infection induced more pronounced fibrosis, which concurs with findings from studies of immunosuppressed patients [16]. However, as it is still unclear how the interaction between HBV/G and other genotypes enhances the replication of HBV/G and affects the virological and clinical manifestation within an individual, we conducted *in vitro* studies using 1.24-fold HBV clones to elucidate the mechanism of HBV/G replication during co-infection.

MATERIALS AND METHODS

Plasmid constructs of HBV DNA and sequencing

Hepatitis B virus DNA was extracted from 100 μL of serum using the QIAamp DNA blood kit (Qiagen GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments (A and B) covering the entire HBV/G genome. PCR with nested primers was performed using TaKaRa LA Taq polymerase (Takara Biochemicals, Kyoto, Japan) for 35 cycles (30 s at 95° C, 30 s at 60° C and 2 min at 72° C). The primer pairs and protocols for plasmid construction are outlined in the Supporting Information. As reported previously [17], these fragments were added to the pUC19 vector, which had been deprived of promoters (Invitrogen Corp., Carlsbad, CA, USA), by digestion with *Hind*III and *Eco*RI, resulting in the 1.24-fold HBV genome – required to transcribe the oversized pregenome and precore messenger RNA. Cloned HBV DNA sequences were confirmed with Prism BigDye (Applied Biosystems, Foster City, CA, USA) using the ABI 3100 automated sequencer.

HBV DNA mutagenesis and construct design

HBV/A2 and HBV/G clones containing the 1.24-fold HBV genome were constructed using isolates obtained from a co-infected Caucasian patient from the San Francisco cohort described in our previous study (patient #1) [7]. The study design conformed to the 1975 Declaration of Helsinki and was approved by our institutional ethics committee. Written informed consent was obtained from the patient. The HBV/A2 clones isolated from the patient's blood specimen did not possess any precore or core promoter mutations that are known to affect HBeAg expression. To study the interaction between the different genotype isolates, the following viral protein expression constructs were prepared (outlined in Fig. 1) in HBV/A2 recombinant plasmids: HBV/A2-S, HBV/A2-core, HBV/A2-pol and HBV/A2-X and were each able to selectively translate one of the four viral proteins (the large surface, precore/core, polymerase and X proteins, respectively), whereas translation of the other three was prevented by the introduction of point mutations that produced corresponding stop codons (Fig. 1a). The following stop codons were used: (i) for surface protein: change from TTA to TAG in the 15th codon of the S gene (T198A) [18], (ii) for core protein: change from AAG to TAG in the 96th codon of the core gene (A2186T), (iii) for polymerase: change from CAACAA to TAATAA in the 283rd and 284th codons of the pol gene (C2558T/C2592T) and (iv) for X protein: change from CAA to TAA in the 7th codon of the HBx gene (C1395T) [19]. All of the above HBV/A2 recombinant plasmids possessed a TCTG motif after nucleotide position 1876, which abolished genome replication by altering the ε loop (CTGT to TCTG, nt 1877–1880) [20]. The 'HBV/A2-N' clone contained all six mutations and was used as an experimental negative control. All of the mutations in this study (substitutions, insertions and deletions) were created by overlapping PCR extension followed by the exchange of endonuclease enzyme-restricted fragments, as described previously [13,21].

Three cytomegalovirus (CMV) promoter-driven expression clones were constructed containing the whole core genes (not including the precore section) of HBV/G (nt 1901–2488), HBV/A2 (nt 1901–2458) and HBV/C (nt 1901–2452): CMV-HBV/G/core, CMV-HBV/A2/core and CMV-HBV/C/core, respectively (Fig. 1b).

Three replicating recombinant constructs were created by recombination of different genomic sections of HBV/G and HBV/A2 (Fig. 1c). The 'HBV-G/A2-CP' clone was a HBV/G-based construct in which the leading fragment containing the core promoter (CP) region (nt 1413–1806) was replaced with that of HBV/A2. The 'HBV-G/A2-CP +core' clone was also an HBV/G-based construct, in which the leading fragment containing the core promoter (CP), precore and core region (nt 1413–2821) of HBV/G was replaced with those of HBV/A2. The 'HBV-G/A2-core' clone was an HBV/G-based construct in which the fragment of the precore and core region (nt 1806–2821) was replaced with those of HBV/A2.

Cell culture and transfection

After 16 h of culture, Huh7 cells were transfected with 5 μg of DNA construct per 10-cm diameter dish using the Fugene 6 transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol and harvested 3 days later. Transfection efficiency was measured by co-transfection with 0.5 μg of a reporter plasmid expressing secreted alkaline phosphatase (SEAP) and normalised with subsequent SEAP measurement from culture supernatant using a SEAP reporter assay kit (Toyobo, Osaka, Japan) [17]. Three experiments were conducted for each clone.

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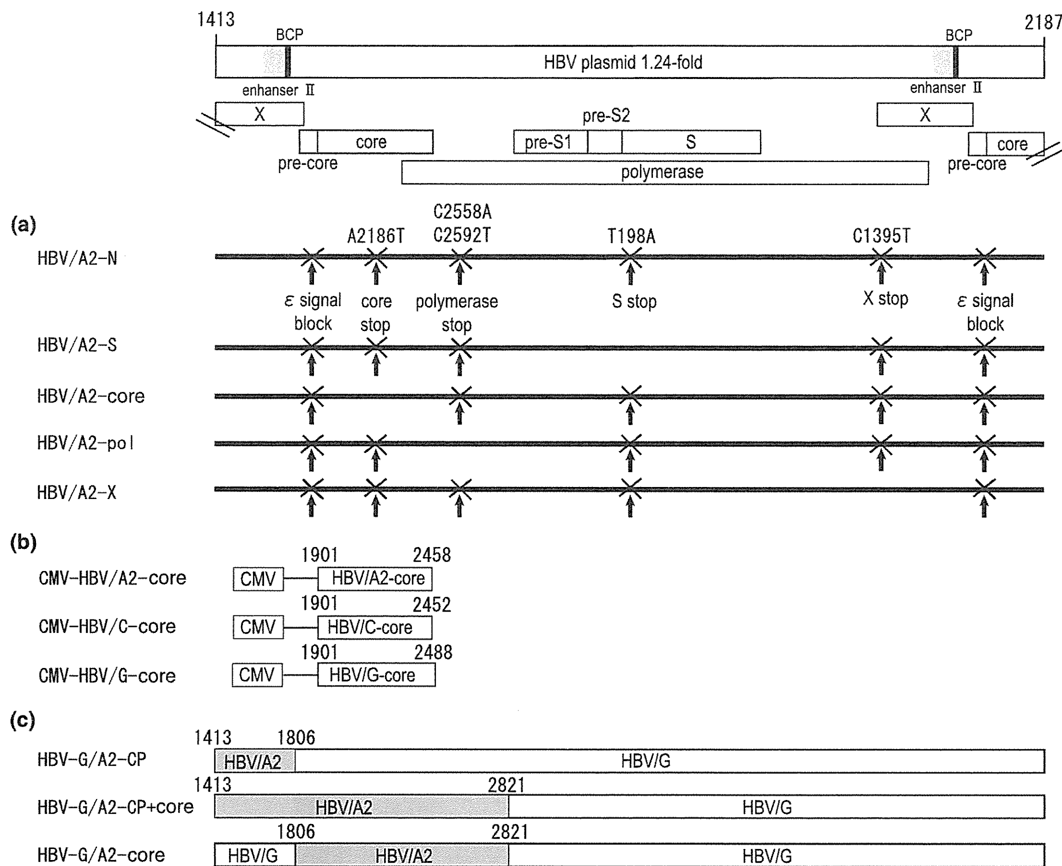

Fig. 1 HBV constructs (1.24-fold) and CMV-driven HBV core protein expression constructs used for the present study. CP, core promoter; BCP, basal core promoter; CMV, cytomegalovirus promoter. Stop codons for the corresponding HBV protein are indicated by crosses and arrows. All HBV/A2 recombinant plasmids consisted of the packaging-negative mutation (ε signal block). In three recombinant constructs between HBV/A2 and HBV/G, the corresponding recombinant genomic parts are shown by the grey bar. CMV-core constructs produce core protein without generating HBeAg in the absence of the preceding ε signal.

Determination of HBV markers

The expression levels of hepatitis B surface antigen (HBsAg) and HBeAg were determined by chemiluminescent enzyme immunoassay using commercial assay kits (Fujirebio Inc., Tokyo, Japan). The detection limit of the HBsAg assay is 0.05 IU/mL. HBV core-related antigen (HBcrAg) was measured in serum using a previously described chemiluminescent enzyme immunoassay [22]. The detection limit of the HBcrAg assay is 1.0 kU/mL.

Southern blot hybridisation

Southern blot hybridisation was performed with full-length probes for each genotype/subgenotype according to previously described methods [23]. In brief, cells were harvested and lysed in 1.5 mL of lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA and 1% NP-40. Half of the cell lysate was treated with 100 μg/mL of RNase A and 200 μg/mL of DNase I for 2 h at 37° C, in the presence of 6 mM Mg acetate. Then, HBV DNA was released by proteinase K digestion, extracted with phenol and precipitated with ethanol after the addition of 20 μg of glycogen. DNA was separated on a 1.2% agarose gel, transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) and hybridised with an alkaline phosphatase-labelled full-length HBV/G or HBV/A2 fragment generated with a Gene Images AlkPhos direct labelling module (GE Healthcare, Hertfordshire, UK). The detection was performed with CDP-Star, ready-to use (Roche Diagnostics GmbH). The signals were analysed by using a LAS-3000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Western blot analysis

Serum or culture medium samples were subjected to SDS-PAGE under 15–25% polyacrylamide gel electrophoresis conditions. The proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA) at 15 V for 45 min. The

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membrane was then blocked and probed using alkaline phosphatase-conjugated HB50 (for HBcAg) or HB91 (for HBcrAg) monoclonal antibody [22] at room temperature for 1 h, before being washed and incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution (KPL, Gaithersburg, MD, USA) for 15 min (for HBcrAg) or 90 min (for HBcAg).

Sucrose density gradient ultracentrifugation

Aliquots (1.7 mL) of 10%, 20%, 30%, 40%, 50% or 60% (w/w) sucrose in 10 mM Tris–HCl, 150 mM NaCl and 1 mM EDTA (pH 7.5) were carefully layered in a 12–mL ultracentrifuge tube and left at room temperature for 6 h. The culture supernatant of Huh7 cells that had been co-transfected with the 1.24-fold HBV genome construct (HBV/G or HBV/A) and/or the CMV-HBV/A2-core plasmid was layered onto this sucrose gradient, and ultracentrifugation was performed at 200 000 × g for 15 h at 4° C in a Beckman Sw40Ti rotor (Beckman Coulter, Chaska, MN, USA). Fractions were collected from the top to the bottom of the gradient. The density of each fraction was calculated from its weight and volume. Each fraction was diluted 10-fold and tested for HBcrAg, HBsAg, HBeAg and HBV DNA.

Immunoprecipitation

Immunoprecipitation was carried out using magnetic beads coated with monoclonal anti-HBs from the 'Magnosphere™ MS300/Caboxyl' kit (JSR Corp., Tokyo, Japan) [24]. A 100-μL aliquot of sample was mixed with 100 μL of a magnetic bead suspension. The mixture was then incubated for 1 h at room temperature under gentle agitation and then magnetically separated. The core protein in the precipitate was analysed by Western blotting.

RESULTS

The replication of HBV/G is enhanced by HBV/A2 in co-transfection experiments

In this study, HBV/G and HBV/A2 genome clones (1.24-fold) were constructed from the serum of a HBV carrier that had been co-infected with HBV/G and HBV/A2. The HBV/G-d36 clone is a HBV/G genome-based construct in which the genotype-specific 36-nt insertion was deleted. We performed co-transfection with HBV/A2 and HBV/G clones and assessed virological features. Because of an over 12% sequence divergence between genotype A and G at the nucleotide level [12], the blot was hybridised successively with genotype-specific probes to DNA of each genotype. However, due to the unbiased binding of each probe at lower efficiency in Southern blot analysis [although the replication of HBV/A2 was higher than that of HBV/G, relative value of HBV/A2 with probe G became lower (0.63), as well as the detection of HBV/G with probe A was very weak (0.24)], each probe of genotype G or A was used for hybridisation with the HBV/A2 and HBV/G clones (Fig. 2a). The density of single-strand HBV DNA detected by the genotype-specific probes in Southern blot analysis revealed that co-transfection with HBV/A2 resulted in increased replication of the wild-type HBV/G

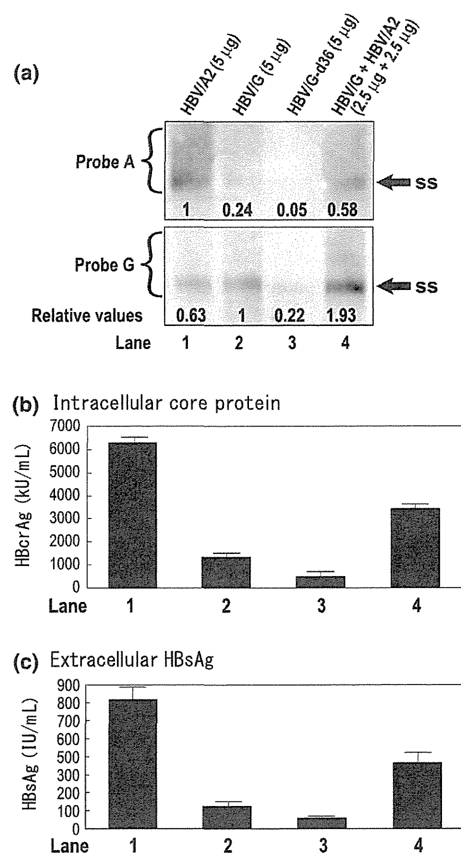

Fig. 2 (a) Southern blot analysis for replicative activity among HBV/G monotransfection, HBV/G-d36 monotransfection, HBV/A2 monotransfection and co-transfection with HBV/A2 and HBV/G (3 days after transfection). HBV/G-d36 clone was a deletion mutant lacking the 36-nt unique insertion in the core gene of the wild-type HBV/G clone. Hybridisation of the blot with genotype-specific probes of genotype A2 (upper) and G (lower). The density values shown at the bottom were measured to the probe-specific DNA sample. Single-stranded (SS) DNA is indicated by arrows. (b) Intracellular expression of core protein was estimated by detecting HBV-core-related antigen (HBcrAg) [22] as measured by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, n = 3). (c) HBsAg levels in the supernatant as detected by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, n = 3). All experiments were tested at least three times.

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