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Effects of Nucleoside Analogs on Native and Site-Directed Mutants of HTLV Type 1 Reverse Transcriptase

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A bacterial assay was developed for testing HTLV-1 reverse transcriptase sensitivity to common nucleoside analog inhibitors in an *Escherichia coli* strain characterized by a temperature sensitive PolI/RecA deletion phenotype. This genetic complementation assay exploits the ability of HTLV-1 reverse transcriptase to functionally replace these missing activities at nonpermissive temperatures. The four inhibitors tested, dideoxyinosine, dideoxyadenosine, deoxythymidine, and didehydrodeoxythymidine are well-known inhibitors of HIV reverse transcriptase. All except dideoxyadenosine showed a strong activity against HTLV-1 reverse transcriptase with IC_{50} in the nanomolar range. Sequence alignments were used to identify amino acid residues in HTLV-1 reverse transcriptase, which correspond to those identified as important for drug-resistance in HIV reverse transcriptase. Mutations of some of these HTLV-1 residues altered the IC_{50} for the inhibitors as expected, which suggests that these amino acids have a function in HTLV-1 reverse transcriptase similar to that of their homologs in HIV reverse transcriptase.

INTRODUCTION

Human T-cell Leukemia Virus Type 1 (HTLV-1) is the first human retrovirus to be associated with a malignancy (*1–3*). A Type C retrovirus, it was initially isolated in 1980 by Poiesz *et al.* (*3*) from patients with adult T-cell leukemia (ATL), and the virus has since been shown to be clinically associated with ATL (*4–7*). HTLV-1 infection has also been linked to an increased susceptibility to opportunistic infections (*8*). Furthermore, it has been implicated in some degenerative neurological diseases including tropical spastic paraparesis (*9*), polymyositis, and multiple sclerosis (*8,10*).

The oncogenic potential of HTLV-1 is reflected in its capacity to transform T4-positive lymphocytes into immortal cell lines (*8*). Thus, most research efforts have focussed on elucidation of the ability of HTLV-1 to modulate the immune system (*11*). HTLV-1-infected individuals do not always develop disease, and disease progression itself varies among individuals. It is not known if a critical mass of infected lymphocytes is needed to develop disease, and it is not clear what overall role viral

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replication may play in disease progression. The involvement of replicating virus or viral replication enzymes in HTLV-1 pathogenesis might be addressed by selective inhibition of HTLV-1 RT, if possible.

Treatments for ATL have included chemotherapeutic regimens of etoposide, vincristine, methotrexate, and others (*12,13*). Limited success has been reported with treatment by either foscarnet (*14*) or a combination of AZT and interferon α (*15,16*). Most recently, it has been reported that the combination of AZT and interferon α does not act by a direct cytotoxic effect (*17*). Since both foscarnet and AZT are known RT inhibitors, these observations suggest that replicating virus may be involved in disease progression.

In Human Immunodeficiency Virus (HIV), the etiological agent of AIDS, the reverse transcriptase (HIV RT) has been the focus of efforts to develop therapeutic agents against AIDS (*18*). Although the role of actively replicating virus is not clear in all types of HTLV-1 disease, HTLV-1 RT may also represent a therapeutic target for some of these. More important, however, the degree of activity of known HIV RT inhibitors against HTLV-1 RT could provide useful information about structure-function relationships in HTLV-1 RT. Many HIV reverse transcriptase inhibitors have been studied extensively, and, in general, their mechanism of action is well understood (for reviews see *19–21*). However, such information is not yet available for HTLV-1 RT. Since the initial study by Rho *et al.* (*22*), which characterized the enzyme isolated from the virion, there have been two reports attempting to identify the location of the HTLV-1 RT gene (*23*) and to identify the quaternary structure of the mature RT (*24*). No specific HTLV-1 RT inhibitors have been identified, and no existing RT inhibitors have been evaluated for efficacy against HTLV-1 RT. The ability to evaluate potential HTLV-1 RT inhibitors could yield information as to the mechanism of action, particularly in the case of known HIV RT inhibitors; and this information, in turn, could be used to infer as yet unknown structural or functional properties of HTLV-1 RT. Ultimately, such inhibitors might be useful in adding to our knowledge about HTLV-1 pathogenesis.

At present, there is no established screening system to evaluate the effects of potential inhibitors on HTLV-1 RT. HIV RT has previously been demonstrated to facilitate the growth of Pol I-deficient *Escherichia coli* strains (*25*). Results reported here indicated that HTLV-1 RT can also compliment for missing Pol I, and this ability is the basis of a bacterial screening assay. This assay was used to assess the sensitivity of native HTLV-1 RT to four known RT inhibitors. The assay was also used to investigate the effects of mutations in HTLV-1 RT at positions which correspond to commonly reported drug resistant phenotypes of HIV-1 RT.

MATERIALS AND METHODS

Materials. *E. coli* strain KL414 (*26*) was obtained from the *E. coli* Genetic Stock Center (Yale University). Plasmid pHSG576 (*27*) was obtained from the National Institute of Genetics (Japan). Plasmid pRCH containing the cDNA from a replication competent clone of HTLV-1 CH (*28*) was generously provided by Dr. Lee Ratner (Washington University, St. Louis, MO). The HB101 cell line was used for cloning the HTLV-1 RT coding sequence into pHSG576. Dideoxyinosine, dideoxyadenosine,

didehydrodeoxythymidine, and deoxythymidine were obtained from Sigma. All other chemical reagents were purchased from Fisher Scientific.

Construction of RT clone for complementation assays. *E. coli* strain KL414 has the genotype F-, *ara-14, leuB6, lacZ36, proC32, LAM-, hisF860, recA56, thyA54, rpsE2115, rpsL109 (strR), xylA5, mtlA2, polA12 (ts), thi-1* (*26*). As described for a similar bacterial strain used by Kim and Loeb (*25*), the mutations *recA56 polA12 (ts)* confer KL414 with a RecA negative, temperature-sensitive DNA polymerase I phenotype (*26*). This strain is unable to grow at temperatures higher than 42°C at low density because the polymerase becomes totally defective in nick translation. Plasmid pHSG576 has a low copy number and a PolI-independent pSC101 origin (*27*). This plasmid also has a chloramphenicol resistance gene, a *lac* promoter, and the required restriction sites (BamHI and EcoRI) in the correct orientation.

The coding sequence for HTLV-1 RT was amplified from pRCH such that the frameshift was incorporated into the amplified DNA at the position identified by Nam *et al.* (*29*). This modified coding sequence was inserted into pHSG576 in three steps as shown in Fig. 1. A linker **$^{5'}$TCG ACA GGA GGT TAA TTA AAA ATG$^{3'}$** with a ribosome binding site (RBS) and a start codon in the same frame as the RT was inserted in front of the RT construct. Positive clones of pHSG576-RT were picked from LB plates with 30 μg/ml chloramphenicol and screened for incorporation of the RT construct. One of these clones, RT11, was used for the complementation assays.

Complementation assays. KL414 was plated on LB plates with 25 μg/ml streptomycin. KL414 was transformed with either pHSG576 or RT11 and was plated on LBSCI (LB with 25 μg/ml streptomycin, 30 μg/ml chloramphenicol, and 1 mM IPTG). The cultures were grown to log phase in LBSCI media at 30°C unless otherwise stated. Colony forming units (CFUs) were then plated in triplicate and incubated either at 30 or 45°C.

Inhibitor studies. KL414 transformed with either RT11 or a mutant RT was plated on LBSCI plates containing drugs, and incubated at either 30 or 45°C. KL414 transformed with pHSG576 was plated on drug-free LB plates and incubated at either 30 or 45°C as negative controls. For screening of mutant RTs, KL414 with native RT11 was used as a positive control.

Sequence alignment. Sequence alignment of HTLV-1 RT, p66 of HIV-1 RT, FIV RT, and reverse transcriptase of baboon endogenous virus were aligned using ClustalW (*30*), and the known conserved motifs of reverse transcriptase in the finger and palm regions were assigned (*31*).

Mutagenesis. Site-directed mutants I69L, H70V, and the double mutant were constructed using the QuikChange Mutagenesis kit from Stratagene. A 300-bp region of the HTLV-1 RT coding sequence, containing the target sequence for mutation, was digested out of RT11, ligated into pUC18, and used for site-directed mutagenesis. After site-directed mutagenesis, the 300-bp region was sequenced, using a Silver Sequence DNA sequencing kit (Promega), to verify the presence of the mutation (data not shown). This 300-bp region containing the mutation was ligated back into appropriately digested RT11 to incorporate the mutant sequence into the RT assay system for screening.

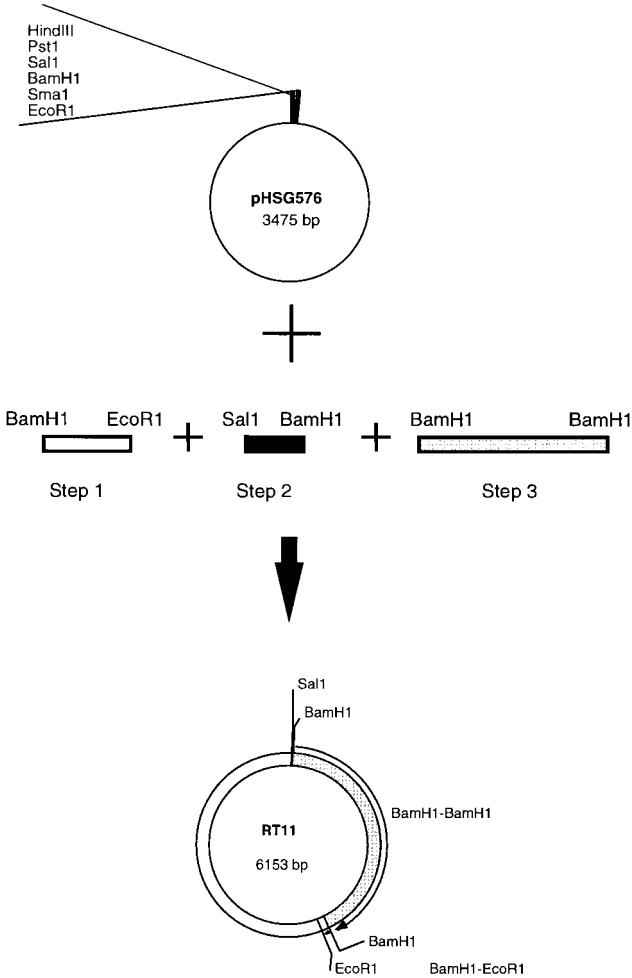

FIG. 1. Construction of RT clone in pHSG576. HTLV-1 RT was cloned into pHSG576 in three steps: First, the BamHI–EcoRI gene fragment was inserted; second the RBS linker SalI–BamHI was inserted 5′ of it; and third the BamHI–BamHI gene fragment was inserted.

RESULTS

Assay development. The ability of the HTLV-1 RT construct to complement the cell density dependent temperature sensitive phenotype of KL414 was qualitatively tested by streaking the cultures in spirals on LBSCI plates. As shown in Fig. 2A (left), controls of KL414 transformed with pHSG576 (no RT) grew normally at 30°C at all densities. However, as shown in Fig. 2A (right), at 45°C these same control plates grew only in the center where the colony density was high. They failed to grow on the outer arms of the spiral where the density was low. These results are similar to those seen for KL414 without plasmid or RT (data not shown). In contrast, Fig. 2B shows the results of the complementation with the RT, with the growth of

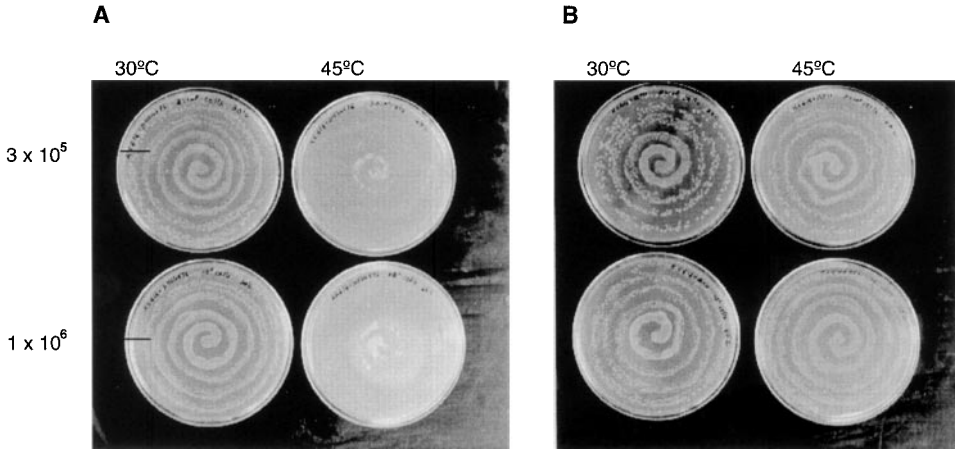

FIG. 2. Qualitative analysis. Two cultures of different concentrations were plated as indicated. (A) KL414 with pHSG576 (no RT gene) was streaked as spirals and incubated at 30°C (left) and 45°C (right). (B) KL414 with RT11 was streaked as spirals and incubated at 30°C (left) and 45°C (right).

KL414 transformed with RT11 at 30 and 45°C, respectively. Unlike the control plates in Fig. 2A, these plates are virtually identical at either temperature, indicating the ability of HTLV-1 RT to complement *E. coli* DNA polymerase I. The experiment was carried out at two different concentrations (top and bottom rows) and the results were same for both.

Quantitative analysis. 5000 and 1000 colony forming units (CFUs) were plated in triplicate on LBSCI plates and incubated at either 30 or 45°C. The plating efficiency for the control (KL414 with pHSG576) at 45°C was 2% ± 1 and 17% ± 4 for the 5000 and 1000 CFU plates, respectively. In contrast, the plating efficiency for KL414 carrying RT11 was found to be 100% with no detectable standard error and 94% ± 2 for 5000 and 1000 CFUs, respectively. In both cases, the plating efficiency of the control is consistently lower than that for KL414 with RT11 at both the dilutions plated, as one might expect from the density dependent aspect of the temperature sensitive phenotype.

Reproducibility. The plating efficiencies seemed more consistent at the 5000 CFUs per plate density. So, to test the reproducibility of the assay system, the 5000 CFUs per plate experiment was repeated two additional times in triplicate each time. As summarized in Table 1, the results for each experiment were found to be reproducible, although the percentage of survivors in the control plates varied slightly.

Factors affecting reproducibility. Early in the development of this assay system, our reproducibility was significantly poorer. We investigated the factors affecting reproducibility, as they are essential to the utility of this assay. We found that the major factor was the incubator settings at the nonpermissive temperature; the temperature on the digital read out was maintained between 45 and 45.5°C. Better reproducibility is obtained when assay plates are placed on top of two empty plates instead of being placed directly on the metal grill, and growing the cultures to log phase at 30°C was important as well.

TABLE 1

Reproducibility of the Bacterial Assay[a]

5000 Cells per plate	KL414+pHSG576	KL414+RT11
Expt. 1	2% (±1)	100% (±ND)
Expt. 2	1% (±1)	95% (±6)
Expt. 3	8% (±4)	100% (±ND)

[a] Bacteria were grown to log phase at 30°C and plated as described under Materials and Methods. Plating efficiency is given as percentage of the number of colony forming units (CFU) at 45°C compared to the number of CFU at 30°C. Errors are shown as Standard Errors (±SE). ND, Not Detectable.

Inhibitor studies on native RT. 2′,3′-dideoxyinosine (didanosine, ddI) is a known nucleoside inhibitor of HIV RT, which has proven useful in the treatment of AIDS. It is active in the triphosphate form, lacks a 3′-hydroxyl group and acts by chain termination of viral DNA synthesis (*32*). Other well-known nucleoside analogs, dideoxyadenosine (ddA), didehydrodeoxythymidine (D4T), and deoxythymidine (ddT), act by a similar mechanism (*33–35*).

KL414 transformed with RT11 was plated on LBSCI supplemented with the appropriate inhibitor and incubated at either 30 or 45°C as described under Materials and Methods. A negative control was also performed, where KL414 transformed with pHSG576 was incubated at either 30 or 45°C. As expected, the negative control grew normally at 30°C and failed to grow at 45°C. In all of the assays (Fig. 3), the numbers of bacterial colonies on the positive control plates, those incubated at 30°C and independent of the presence of the RT, were unaffected at any of the inhibitor concentrations. This result demonstrates that at the concentrations assayed, the inhibitors are not toxic to KL414, and they do not affect any endogenous bacterial enzymes. In contrast to these controls, the plating efficiency of the assay plates, those grown at 45°C and dependent upon the RT, began to decrease as the concentration of inhibitor increased. This decrease in plating efficiency would be expected if the enzymatic activity of HTLV-1 RT was inhibited by any of these compounds. Thus, each of these inhibitors disrupted the ability of the RT to complement for the lethal mutation at the nonpermissive temperature.

As shown in Fig. 3, the inhibition curves for D4T and ddT are similar and appear to be logarithmic. The curve for ddI was also logarithmic, but steeper than those for D4T and ddT, suggesting a more effective inhibition of HTLV-1 RT. In contrast to ddI, D4T, and ddT, the inhibition curve for ddA is linear and comparatively higher concentrations of ddA were needed for a response. The unique behavior of HTLV-1 RT with respect to ddA could be due to the lack of adenosine kinase in *E. coli* (*36*). In the absence of this enzyme, adenosine must be deaminated to inosine before it can be phosphorylated to the active form, and the apparent inhibition could be limited by the rate of deamination.

The 50% inhibitory concentration (IC_{50}) was calculated for each inhibitor to allow a more quantitative comparison with each other and with the reported values for HIV RT. As shown in Table 2, the values for ddT and D4T are very close, which suggests a similar mode of interaction of HTLV-1 RT. Notably, the IC_{50} of ddI was particularly