Research Report

Nonradioactive Detection of Retroviral-Associated RNase H Activity in a Microplate-Based, High-Throughput Format

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ABSTRACT

None of the available antiretroviral drugs that are currently used in the clinic to treat infection with HIV-1 is directed against the RNase H active site of the reverse transcriptase. Here we developed a nonradioactive, 96-well plate assay designed to be used for high-throughput screening of compounds capable of inhibiting the RNase H activity of HIV-1 reverse transcriptase. We employed a tRNA as substrate that was labeled with digoxygenin-modified reporter residues. The labeled tRNA was prehybridized with a DNA oligonucleotide that contained a single biotinylated residue at its 5′-terminus to ensure its attachment to streptavidin-coated microplates. The uncleaved, immobilized DNA/tRNA substrate was detected through the use of established ELISA protocols. Incubation with purified HIV-1 reverse transcriptase initiated RNase H degradation and caused a signal reduction to negligible background levels. In contrast, the signal intensity remained unaffected when using an RNase H deficient mutant enzyme. The assay was validated using the hydrazone derivative BBNH that was previously shown to inhibit RNase H degradation below concentrations of 10 μ*M.*

INTRODUCTION

The reverse transcriptase of HIV-1 is a prime target for the development of antiretroviral drugs that interfere with the retroviral life cycle. Retroviral reverse transcriptases are multifunctional enzymes possessing DNA polymerase activities on RNA and DNA templates, as well as RNase H activity that specifically cleaves the RNA moiety of RNA/DNA hybrids (24). Although both activities are essentially required to convert the ssRNA into proviral dsDNA, potent drugs that block RNase H degradation have as yet not been developed. All existing reverse transcriptase inhibitors, used in the clinic, are directed against the DNA polymerase activity (17).

Previous studies have shown that the metal chelators 1,10-phenanthroline (9), and N-(4-tert-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) (1) inhibit RNase H degradation in cell-free assays. The latter compound was shown to block both RNase H and the DNA polymerase activity of HIV-1 reverse transcriptase with similar IC_{50} values of approximately 3 μM. In contrast, 1,10-phenanthroline and other inhibitors, including 3′-azido-3′-deoxythymidine-monophosphate and derivatives of naphthylenesulfonic acid (11,13,22), affected RNase H degradation at much higher (millimolar) concentrations. More recently, it has been demonstrated that the 4-chlorophenylhydrazone of mesoxalic acid (CPHM) specifically antagonizes the RNase H activity of HIV-1 reverse transcriptase in the micromolar range (2,3). The inhibitory effect of CPHM was discovered through the use of a 96-well plate assay for the identification of DNA strand transfer inhibitors (3). In this assay, the RNase H activity is measured indirectly. A biotinylated DNA primer, bound to a model RNA template, was extended in the presence of HIV-1 reverse transcriptase and cold dNTPs. The reverse transcriptase-associated RNase H activity degrades the template as DNA synthesis proceeds towards the end of the RNA, which facilitates the ensuing transfer of newly synthesized DNA to an acceptor template. Since radiolabeled nucleotides are specifically incorporated into the DNA product that was successfully transferred, this assay enables the detection of compounds that inhibit either the DNA polymerase or the RNase H active site or both. Here we developed a microplate-based assay that detects RNase H degradation independent of DNA synthesis through the use of nonradioactively modified nucleotides and established ELISA protocols.

MATERIALS AND METHODS

Enzymes, Nucleic Acids, and Chemicals

Recombinant HIV-1 reverse transcriptase (p66/p51) and the RNase H-deficient mutant enzyme (p66E478Q/p51) were expressed in *E. coli* and purified essentially as previously described (10). All non-biotinylated oligodeoxynucleotides (ODNs) were synthesized by Invitrogen (Carlsbad, CA, USA). The biotinylated ODN, with the modified residue at its 5′-end,

was obtained from Sigma-Genosys (The Woodlands, TX, USA). Nonradioactively labeled $tRNA^{Lys3}$ was prepared as described below. BBNH (Calbiochem-Novabiochem, San Diego, CA, USA) was dissolved in DMSO and stored at 1 mM stock solutions at -20°C. Standard chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

In Vitro Transcription of Non-Radioactively Labeled RNA

Synthetic $tRNA^{Lys3}$ was synthesized in vitro using the plasmid pT7hLys3 as a template (26). A *Fok*I restriction site was introduced downstream of the $tRNA^{Lys3}$ gene to generate a DNA template that ensures run-off transcription of the tRNA with the correct 3′-CCA terminus. The *Fok*I-treated plasmid served as a template to synthesize nonradioactively labeled tRNA species. In vitro transcription of $tRNA^{Lys3}$ was conducted in the presence of mixtures of UTP-, digoxygenin (DIG)-, and/or biotin-modified UTP, as specified in Figures 1 and 2. The transcription mixture contained a final concentration of 10 mM for each of the nucleotides, including UTP (modified and unmodified), as well as a buffer containing 40 mM Tris-HCl (pH 7.8), 50 mM $MgCl_2$, 10 mM NaCl, 1 mM spermidine, and 5 mM DTT. The reaction was conducted with T7 RNA polymerase purchased from Ambion (Austin, TX, USA), and allowed to proceed overnight at 37°C, in a reaction volume of 40 µL. The tRNA was subsequently purified on an 8% polyacrylamide gel. RNA bands were visualized by UV shadowing. The fragments were excised from the gel and eluted with a buffer containing 0.5 M ammonium acetate/0.01% SDS. Following ethanol precipitation, the labeled tRNA was resuspended in water and stored at -20°C for up to 12 months.

96-Well Plate RNase H Assay

The labeled tRNA was hybridized to an excess of complementary ODNs to ensure its availability in the DNA/RNA duplex form. For this purpose, 10 ng labeled tRNA were incubated in a buffer containing 50 mM Tris-HCl (pH 7.8) and 50 mM NaCl, as well as each of the following ODNs: ODN76-59 (5′-TGGCGCCCGAACAGGGAC-3′), ODN58-41 (5′-TTGAACCCTGGACCCTCA-3′), ODN40-23 (5′-GATTAAAAGTCTGATGCT-3′), and ODN18-1 (5′-CGACTGAGCTATCCGGGC-3′). The four ODNs bind to adjacent regions of the tRNA; the numbering refers to the corresponding positions of $tRNA^{Lys3}$ (Figure 1A). Hybridization of ODNs and $tRNA^{Lys3}$ was conducted in a total reaction volume of 30 µL. The mixture was heated at 97°C for 3 min to denature the tRNA. Annealing was subsequently performed at 76°C for 15 min, followed by a 30-min incubation at room temperature. The primer/template complex was then transferred into the wells of a streptavidin pre-coated microplate to immobilize the DNA/tRNA substrate. A 24-µL solution containing 140 ng purified HIV-1 reverse transcriptase in 50 mM Tris-HCl (pH 7.8) and 50 mM NaCl was added after a pre-incubation period of 10 min with or without the RNase H inhibitor BBNH. RNase H cleavage was initiated by the addition of 6 µL 60 mM $MgCl_2$. The reaction was allowed to proceed for 1 h at 37°C. RNase H cleavage was detected by decreased signal intensities in a standard ELISA using anti-DIG antibodies coupled with peroxidase (Roche Diagnostics, Laval, PQ, Canada). Signal reduction to negligible background levels was observed after 40 min in the presence of a minimum of 15 ng HIV-1 reverse transcriptase. The plate was incubated with ABTS® substrate solution for 45 min following the manufacturer's recommendations (Roche Diagnostics). The color reaction was subsequently quantified using a photospectrometer (Titertek Multiscan® MCC/340). Absorbance was measured at 405 nm with a reference wavelength at 492 nm.

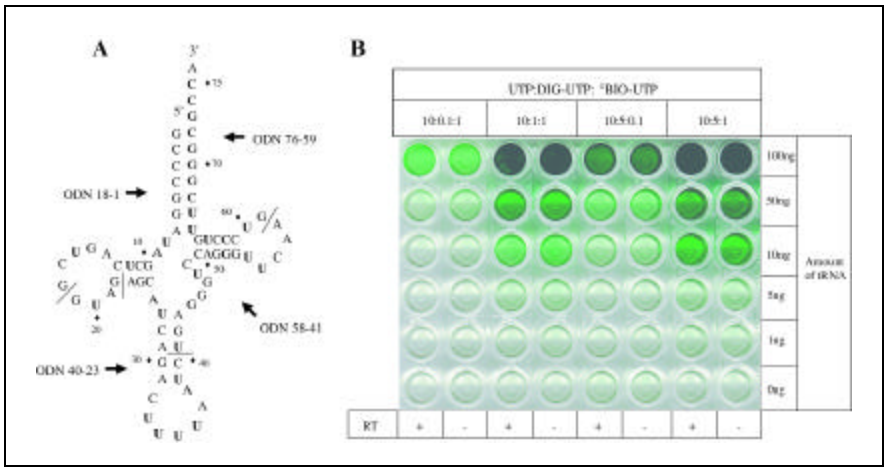

Figure 1. Detection of RNase H activity through use of $tRNA^{Lys3}$ labeled with biotin- and DIG-modified residues. (A) Sequence and secondary structure of $tRNA^{Lys3}$. Sequences that are complementary to the ODNs used in this study are delineated by slashes through the tRNA (positions 19–22 are not hybridized with a complementary ODN). The nomenclature of ODNs is based on the complementary tRNA sequence (26). Positions of $tRNA^{Lys3}$ that contain a uridine residue are shown in bold. These positions are possible sites for DIG-UTP and biotin-UTP (BIO-UTP) incorporation during transcription. (B) Detection of RNase H activity using $tRNA^{Lys3}$ molecules that have been transcribed with differing ratios of DIG-UTP, biotin-UTP, and UTP, as indicated. All reactions were performed in the presence and absence of reverse transcriptase, as described in the Materials and Methods section.

RESULTS AND DISCUSSION

Choice of Primer/Template Substrate

Many of the different stages during the complex process of reverse transcription that involve RNase H degradation were successfully reconstituted in cell-free, gel-based assays using either radioactively or nonradioactively labeled nucleic acid substrates (8,12, 23). These include minus-strand DNA synthesis (4,6), initiation of plus-strand DNA synthesis (5,18), first- and second-strand transfer events (14,16,21, 27), and reactions that involve the removal of RNA primers (5,18-20,22) or formerly uncleaved fragments (25). The RNase H domain is also implicated

in processive DNA synthesis (7). Each of these assays may be translated in a microplate format for the high-throughput screening of compounds that potentially block RNase H degradation. Here we employed a tRNA as substrate to detect RNase H degradation largely independent of sequence-specific effects and to reduce nonspecific background hydrolysis to a minimum. Because of their compact structures, tRNAs are relatively stable and may be handled without significant degradation over protracted periods of time.

We have recently demonstrated that tRNAs are capable of forming stable complexes with complementary ODNs, provided that binding of the ODNs interfered with the formation of intramolecular interactions of the tRNA (26). Complexes composed of $tRNA^{Lys3}$ and two ODNs that were complementary to the 3′-terminal residues in the acceptor stem (ODN76-59) and the TΨC-stem loop (ODN58-41) were efficiently recognized by HIV-1 reverse transcriptase. We had shown, through use of gel-based assays involving radiolabeled, in vitro synthesized $tRNA^{Lys3}$ that the tRNA served as a template for the reverse transcriptase and undergoes RNase H degradation. It was the aim of this study to translate this assay into a nonradioactive, 96-well plate format.

Nonradioactive ELISA-Based Detection of Reverse Transcriptase-Associated RNase H Activity

The DNA polymerase activity of HIV-1 reverse transcriptase can be detected in nonradioactive fashion

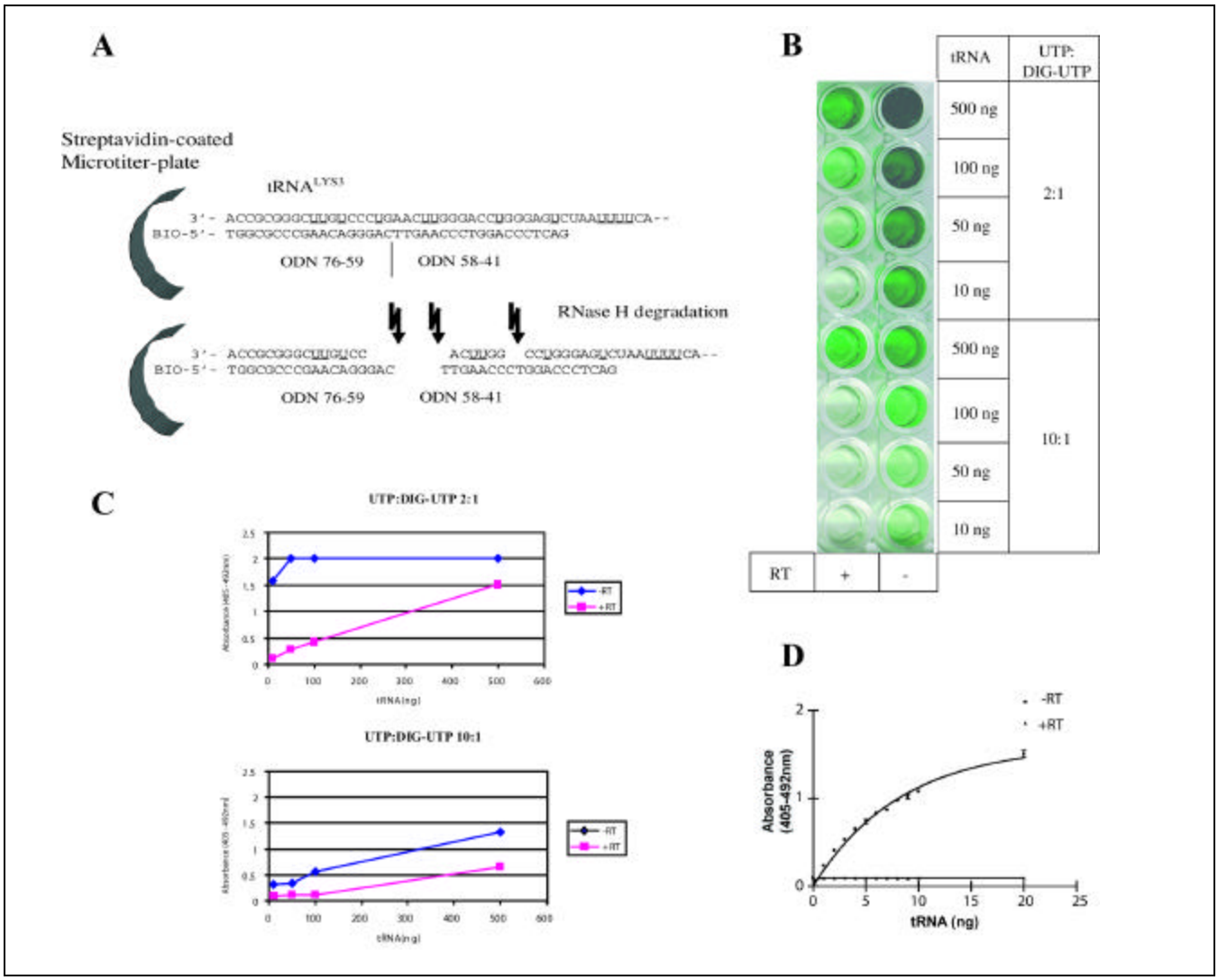

Figure 2. Detection of RNase H activity through the use of a biotinylated ODN and DIG-labeled $tRNA^{Lys3}$. (A) Immobilization of the tRNA/DNA complex that contains biotinylated ODN76-59. The figure shows the attachment of the nucleic acid substrate to the streptavidin-coated microplate. RNase H cleavage is indicated schematically. Possible sites for DIG-modified residues are underlined. (B) Detection of RNase H activity. The tRNA used in these reactions was transcribed with differing ratios of DIG-UTP and UTP, as indicated. Reactions were conducted with various concentrations of the labeled tRNA (10 ng to 1 µg). (C) Quantification of ELISA shown in panel B. Absorbance levels above 2 are above the limit, which can be read accurately. Thus, ELISA results above this limit are shown as 2, even though the actual number value may be higher. (D) Quantification of an ELISA based on reactions performed in the presence of lower concentrations of the labeled tRNA (0–20 ng). RT, reverse transcriptase.

through the incorporation of modified nucleotides that act as reporters for active DNA synthesis. DIG-modified nucleotides are effectively incorporated by HIV-1 reverse transcriptase and allow the nonradioactive detection of newly synthesized DNA using established ELISA techniques (21). In a typical assay, biotinylated nucleotides are incorporated concomitantly with reporter residues to immobilize the newly synthesized DNA to a streptavidin-coated microplate. To test whether a nonradioactive RNase H assay may function analogously, we labeled $tRNA^{Lys3}$ during in vitro transcription with T7 RNA polymerase by including DIG-UTP and biotin-UTP in the reaction mixture (see Materials and Methods). The labeled tRNA was pre-hybridized to four complementary ODNs and subsequently transferred to a 96-well plate (Figure 1A). In theory, incubation with HIV-1 reverse transcriptase initiates RNase H cleavage, which gives rise to small fragments that dissociate from the plate. The dissociation of labeled RNA fragments should result in signal reductions in the following ELISA. To analyze how the concentrations of modified nucleotides affect the sensitivity of the assay, we initially utilized different amounts of transcripts with different combinations of DIG and biotinylated residues (Figure 1B).

The data show that 10 ng purified the tRNA is the lower limit that yields a robust signal. The highest signal intensities were seen with tRNA transcripts, synthesized in the presence of UTP and the two modified nucleotides (DIG-UTP and biotin-UTP) at ratios between 10:5:1 and 10:1:1. However, despite the clear detection of the substrate, the difference among reactions conducted in the presence and absence of HIV-1 reverse transcriptase is negligible under each of the conditions tested. A reproducible decrease in signal intensity was not observed.

It is conceivable that the nonradioactively labeled tRNA may not be quantitatively cleaved into mono-, di-, or trinucleotides, as seen in the corresponding gel-based assay that involved radiolabeled $tRNA^{Lys3}$ (26). Larger fragments containing both biotin- and DIG-modified nucleotides that remain bound to the plate likely contribute to the strong signal. The chemical modifications and/or the immobilization of the tRNA via the biotin/streptavidin interaction may compromise both the binding and activity of the enzyme. Such problems associated with the nonradioactive detection of RNase H degradation have been described earlier (15). In a similar setup, HIV-1 reverse transcriptase utilized only 60% of the substrate, and the remaining immobilized nucleic acid limited the sensitivity of the assay (15).

To overcome this problem, we next attempted to facilitate RNase H degradation and the subsequent release of cleaved fragments by separating the two modified nucleotides. The tRNA was labeled during in vitro transcription with DIG-UTP, and the biotin label was covalently attached to the 5′-terminus of ODN76-59 that binds to the 3′-end of the acceptor stem. The principal of this assay is shown in Figure 2A. Only those RNA fragments that remain bound to the biotinylated ODN will contribute to the signal in the following ELISA. Any cleavage upstream from these 18 3′-terminal nucleotides of the tRNA will most likely cause the release of DIG-labeled fragments from the microplate. Fragments produced by cleavages close to and downstream from position 18 can also be released, if they do not form stable complexes with ODN76-59.

Figure 2B shows that this approach significantly improved the nonradioactive detection of RNase H activity. There is a clear difference in signal intensities among reactions performed in the presence of HIV-1 reverse transcriptase, as compared to reactions in the absence of the enzyme. The data show that the absence of HIV-1 reverse transcriptase generates a strong signal with similar intensity to that seen in the previous experiments using dually labeled $tRNA^{Lys3}$. Thus, a single biotin label is sufficient to immobilize the ODN/tRNA complex effectively. The presence of HIV-1 reverse transcriptase decreased the signal dramatically. The most pronounced difference between reactions performed in the presence and absence of enzyme were obtained with approximately 10 ng $tRNA^{Lys3}$ that was labeled using a 2:1 molar ratio UTP and DIG-UTP (Figure 2C).

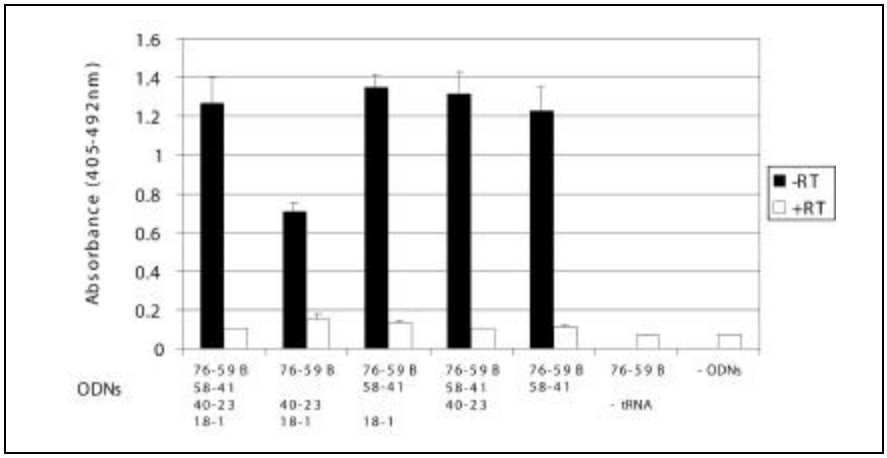

Figure 3. Quantification of an ELISA in which individual ODNs were omitted from the reaction mixture to determine if each ODN is required for efficient RNase H detection. Controls were conducted either in the absence of tRNA or in the absence the biotinylated ODN (76-59 B).

ODN Requirements

To validate the assay further, we next asked whether it is necessary to include each of the four ODNs in the reaction mixture to maintain a robust signal in the absence of reverse transcriptase and to detect a significant reduction in the presence of enzyme. Thus, we analyzed the efficiency of RNase H degradation in reactions from which ODN58-41, ODN40-23, or ODN18-1 was selectively excluded. The results show that ODN40-23 and ODN18-1 are dispensable (Figure 3). Incubation with HIV-1 reverse transcriptase, in the presence of all four ODNs, as well as in the absence of ODN40-23 and/or ODN18-1 caused strong signal reductions similar to the

values measured in the control reactions in the absence of the biotinylated ODN76-59 or in the absence of DIG-labeled $tRNA^{Lys3}$. In contrast, ODN58-41 is identified as an important component in the assay. The exclusion of ODN58-41 caused a slight decrease in the signal in the absence of reverse transcriptase. It is conceivable that ODN58-41 facilitates the binding of the biotinylated ODN76-59 to the 3′-end of the tRNA. This interpretation is consistent with our previous results showing that ODN58-41 forms a stable complex with $tRNA^{Lys3}$, most likely because it interferes effectively with the intramolecular interactions that stabilize the L-shaped structure of the tRNA (26). Thus, binding of ODN76-59 and ODN58-41 may significantly reduce refolding of $tRNA^{Lys3}$ to its native conformation. We also noticed that the signal reduction in the presence of reverse transcriptase is less pronounced when excluding ODN58-41. Thus, it appears that binding of ODN58-41 provides an important target for RNase H degradation to facilitate the release of the DIG-labeled tRNA fragments from the biotinylated ODN76-59. Together, these data show that the presence of ODN76-59 and ODN58-41 is necessary and sufficient to immobilize the labeled tRNA and to generate a substrate that is completely released through RNase H degradation.

Specificity

We next compared the efficiency of cleavage in a time course experiment using both wild-type HIV-1 reverse transcriptase and an RNase H-deficient mutant enzyme (i.e., HIV-1 reverse transciptase E478Q), in which one of the two metal binding sites required for catalysis is selectively altered (16). The time course shows that a significant signal reduction in the ELISA is exclusively seen when wild-type reverse transcriptase (Figure 4). Even a long reaction time of 60 min did not produce significant signal reductions with the RNase H-deficient E(478)→Q mutant. These data clearly demonstrate that the effects seen with the wild-type enzyme are specifically attributable to the reverse transcriptase-associated RNase H activity. This represents an important control to ensure that signal reductions are not caused by either bacterial nucleases that might have contaminated the enzyme preparations.

Inhibition of RNase H Degradation

We finally analyzed whether this assay allows the detection of inhibition of RNase H activity. To address this issue, we have studied the effects of different concentrations of BBNH in the 96-well plate-based assay. Diminished signal reductions are seen in the low micromolar range. The presence of BBNH caused a complete block of RNase H degradation at concentrations greater than or equal to 10 µM (Figure 5). These data are in good agreement with previous measurements using radiolabeled poly ([^{3}H]rG)-poly(dC) substrates in a classical assay based on the separation of cleaved and uncleaved RNA through precipitation with cold perchloric acid (1).

CONCLUSIONS

Here we developed and validated a 96-well plate assay for the nonradioactive detection of retroviral RNase H activity. The immobilized nucleic acid substrate, composed of a biotinylated ODN and a DIG-labeled tRNA, produced a robust signal using commercially available ELISA techniques. Incubation with purified HIV-1 reverse transcriptase initiated RNase H degradation, which resulted in signal reductions down to background levels seen with control reactions in the absence of nucleic acid substrates. The high ratio of uncleaved, immobilized substrate to the cleaved and released products renders this assay highly sensitive. The assay is flexible and DIG-modified nucleotides may also be replaced by other reporters and alternative detection methods. Taken together, the nonradioactive microplate assay described in this paper is a sensitive, safe, and convenient tool that can be utilized for high-throughput screening of compounds with the potential to antagonize RNase H degradation.

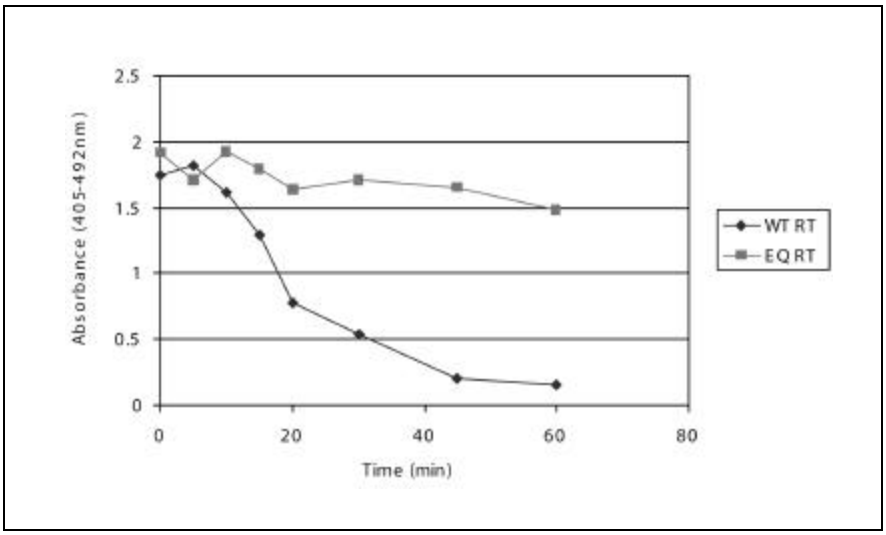

Figure 4. Time course of the RNase H activity quantified through an ELISA, using wild-type reverse transcriptase (RT) and the RNase H-deficient E(478)Q (EQ) mutant. The reaction was performed in the presence of biotinylated ODN76-59 and ODN58-41, as described in Figures 2 and 3.

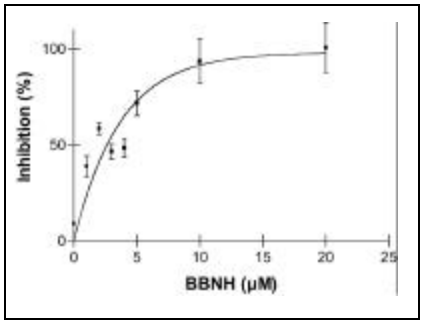

Figure 5. Inhibition of RNase H degradation in the presence of different concentrations of BBNH. Reactions were conducted as described in the Materials and Methods section. Each concentration point was analyzed in triplicate.

ACKNOWLEDGMENTS

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