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Non-nucleoside Reverse Transcriptase Inhibitors Inhibit Reverse Transcriptase through a Mutually Exclusive Interaction with Divalent Cation-dNTP Complexes

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Abstract

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are considered noncompetitive inhibitors that structurally alter reverse transcriptase (RT) and dramatically decrease catalysis. In this report, biochemical analysis with various divalent cations was used to demonstrate that NNRTIs and divalent cation–dNTP complexes are mutually exclusive, inhibiting each other's binding to RT/primer template (RT-P/T) complexes. The binding of catalytically competent divalent cation–dNTP complexes to RT-P/T was measured with Mg^{2+} , Mn^{2+} , Zn^{2+} , Co^{2+} , and Ni^{2+} using Ca^{2+} , a noncatalytic cation, for displacement. Binding strength order was $\text{Mn}^{2+} \approx \text{Zn}^{2+} \gg \text{Co}^{2+} > \text{Mg}^{2+} \approx \text{Ni}^{2+}$. Consistent with but not exclusive to mutually exclusive binding, primer extension assays showed that stronger divalent cation–dNTP complexes were more resistant to NNRTIs (efavirenz (EFV), rilpivirine (RPV), and nevirapine (NVP)). Filtration assays demonstrated that divalent cation–dNTP complexes inhibited the binding of ^{14}C -labeled EFV to RT-P/T with stronger binding complexes formed with Mn^{2+} inhibiting more potently than those with Mg^{2+} . Conversely, filter binding assays demonstrated that EFV inhibited ^3H -labeled dNTP binding to RT-P/T complexes with displacement of Mn^{2+} –dNTP complexes requiring much greater concentrations of EFV than the more weakly bound Mg^{2+} –dNTP complexes. EFV bound relatively weakly to the NNRTI resistant K103N RT; but, binding was modestly enhanced in the presence of P/T, and EFV was easily displaced by divalent cation–dNTP complexes. This suggests that K103N overcomes EFV inhibition mostly by binding more weakly to the drug and is in contrast to other reports that indicate K103N has little to no effect on drug or dNTP binding. Overall, this biochemical analysis supports recent biophysical analyses of NNRTI–RT interactions that indicate mutually exclusive binding.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bio-chem.9b00028. Figures S1 and S2 showing assay results and Table S1 showing determination of K_d for binding RT-P/T aptamer (PDF)

Notes

The author declares no competing financial interest.

Non-nucleoside reverse transcriptase inhibitors (NNRTIs), first approved for use in 1996, have been a hallmark of highly active antiretroviral therapy (HAART). These drugs have relatively low toxicity, high efficacy, and good tolerability.^{1,2} Drawbacks include a low genetic barrier to resistance, ineffectiveness against HIV-2, high cost, and limited availability.³ Second generation FDA approved NNRTIs (including rilpivirine (RPV) and etravirine (ETR)) have improved resistance profiles compared to first generation drugs (nevirapine (NVP), efavirenz (EFV), and delavirdine (DLV)). However, despite clear advancements in dosing, combination therapy regimens, and drug resistance profiles, concerns in these and other areas still remain, highlighting the need for development of new drugs. A better understanding of the efficacy, interactions between drug and drug target, and mechanism of action of the existing antiretroviral drugs is pivotal for designing novel drugs.

All current FDA approved NNRTIs interact with a hydrophobic pocket that is approximately 10 Å from the polymerase active site of HIV-1 reverse transcriptase (RT).^{4–8} Unlike nucleoside reverse transcriptase inhibitors (NRTIs) that compete with normal nucleotides for binding at the nucleotide binding site, biochemical analysis using steady state and presteady state approaches suggests that NNRTIs are classic noncompetitive inhibitors with the binding dramatically slowing the chemical step of nucleotide catalysis.^{9–12} Consistent with this, crystallographic analyses show that NNRTI bound RT is structurally altered with finger and thumb domains locked in a rigid hyperextended conformation, a distorted primer-grip and YMDD loop (involved in metal binding and catalysis) conformation, and a distorted nucleotide binding pocket^{13–16} (for reviews, see refs 17–19).

Although it is well-established that structural alterations induced by NNRTI binding are likely to perturb the chemistry of RT, several findings suggest that the mechanism of inhibition may be more complex and dependent on specific conditions. Some reports indicate NNRTIs affect RT-primer/template (RT-P/T) binding^{11,20} and inhibit binding of dNTPs to the RT-P/T complex,^{21,22} with the level of reduction depending on the nature of the P/T.²³ In contrast, others, using different approaches, found that NNRTIs do not strongly inhibit dNTP binding to RT-P/T complexes, while dNTP incorporation is dramatically inhibited^{9–11} (for further elaboration on dNTP binding affinity measurements in the presence of NNRTIs, see ref 24). Although NRTIs are more effective with higher Mg^{2+} concentrations in vitro, NNRTIs show the opposite trend with higher potency in low Mg^{2+} .²⁵ This suggests the possibility, among others, of a direct competition between divalent cation or divalent cation-dNTP complex binding and NNRTI binding. Consistent with this possibility, analysis of RT-NNRTI complexes by fluorescence anisotropy²² as well as isothermal titration calorimetry (ITC)²¹ found that dNTPs cannot bind to the complex of RT with drug. It should be noted that reports indicating that RT-NNRTI complexes retain low

but detectable catalytic activity^{9–11} are inconsistent with NNRTIs and dNTPs being mutually exclusive.

In this report, we present evidence that NNRTIs and divalent cation–dNTP complexes compete with each other for binding to RT, with binding of one excluding or strongly inhibiting binding of the other. Using a biochemical approach based on Ca^{2+} displacement, the relative binding affinity of various catalytic divalent cation–dNTP complexes to RT was determined. Binding affinity correlated well with susceptibility of the divalent cations to NNRTI inhibition in primer-extension assays with stronger binding complexes (i.e., Mn^{2+} , Zn^{2+} , and Co^{2+}) being more resistant to NNRTI inhibition than weaker ones (Mg^{2+} and Ni^{2+}). The binding of radiolabeled EFV to RT-P/T complexes was also more strongly inhibited by stronger binding divalent cations, and significant binding inhibition occurred only in the presence of dNTPs, suggesting that it is the divalent cation–dNTP complex that competes with the NNRTI for binding. This is consistent with ITC experiments that indicate dNTPs and NVP cannot bind to RT-P/T complexes at the same time.²¹ The NNRTI-resistant K103N RT bound relatively weakly to EFV in our assays, and binding was stimulated by P/T but inhibited by dNTPs. This indicated that K103N behaves essentially as wild-type RT with a significantly weaker binding association with EFV, a finding that is inconsistent with some previous reports.²² Further, this work demonstrates that divalent cation–dNTP complexes that support RT catalysis bind with profoundly different affinities to RT, and the potency of NNRTIs correlates with divalent cation–dNTP binding strength.

MATERIALS AND METHODS

Materials.

T4 polynucleotide kinase (PNK) was from New England Biolabs. Deoxyribonucleotides were obtained from Roche. The phiX174 *HinfI* digest DNA ladder was from Promega. DNA oligonucleotides were from Integrated DNA Technologies. G-25 spin columns were from Harvard Apparatus. Centrifugal filters (Amicon Ultra –0.5 mL 30 000 nominal molecular weight cutoff (Ultracel-30K)) were from Merck Millipore Ltd. NNRTIs, nevirapine (NVP), rilpivirine (RPV), and efavirenz (EFV) were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. After resuspension in DMSO, NNRTIs were aliquoted and stored at -80°C . Radiolabeled EFV (^{14}C , >50 mCi/mmol) was from ViTrax. Radiolabeled ATP ($[\gamma\text{-}^{32}\text{P}]$ 3000 Ci/mmol, 10 mCi/mL), dTTP ($[\alpha\text{-}^{32}\text{P}]$ 3000 Ci/mmol, 10 mCi/mL), dTTP ($[\text{methyl-}^3\text{H}]$ 70–90 Ci (2.59–3.33 TBq)/mmole), and glass fiber DEAE Filtermats were from PerkinElmer. Nitrocellulose filters disks (Protran BA 85, $0.45\ \mu\text{m}$ pore size and 25 mm diameter) were from Whatman. All other chemicals were obtained from Fisher Scientific, VWR, or Sigma.

Preparation of Enzymes.

The plasmid clone for HIV-1 RT (HXB2 strain, NCBI accession number NP_705927) expression was a generous gift from Dr. Michael Parniak,²⁶ and the RT was prepared as described.²⁷ The plasmid clone for HIV-1 RT K103N (HXB2 backbone) was a generous gift from Dr. Stephen Hughes (National Institutes of Health), and the RT was prepared as

described.²⁵ Aliquots were stored at -80°C , and a separate aliquot was used for each experiment.

5' End-Labeling of Primers.

Reactions for labeling various primers were done in a $50\ \mu\text{L}$ volume of the reaction buffer (70 mM Tris-HCl (pH 7.6), 10 mM MgCl_2 , 5 mM dithiothreitol (DTT)) containing 50 pmol of the oligo, $7\ \mu\text{L}$ of $\gamma\text{-}^{32}\text{P}$ ATP, and 10 units of PNK. The reaction mixture was incubated for 60 min at 37°C , and then the PNK was heat inactivated for 15 min at 70°C . The material was then run through a Sephadex G-25 spin column to remove excess radiolabeled nucleotide.

Ca^{2+} Competition Assay.

Assays were performed in a final volume of $30\ \mu\text{L}$ at 37°C in 50 mM Tris-HCl (pH 8), 80 mM KCl, $5\ \mu\text{M}$ $\alpha\text{-}^{32}\text{P}$ dTTP ($\sim 1\ \mu\text{Ci}/\text{reaction}$), $2.5\ \mu\text{g}$ poly-(rA):oligo(dT₂₀) (8:1 w:w), and 0.5 mM of divalent cation (Mg^{2+} , Mn^{2+} , Zn^{2+} , Co^{2+} , or Ni^{2+}). In addition, different concentrations (0, 0.063, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, or 32 mM) of Ca^{2+} were added to reactions. Chloride salts were used for all divalent cations except Zn^{2+} , for which ZnSO_4 was used to prevent precipitation. DTT was omitted from reactions as some of the cations demonstrated precipitation in its presence. Reactions were initiated by the addition of $2\ \mu\text{L}$ of HIV-1 RT (5 nM for Mg^{2+} , Mn^{2+} , and Co^{2+} , or 50 nM for Zn^{2+} and Ni^{2+} , final concentration in reactions) and incubated for 10 min before termination by the addition of $4\ \mu\text{L}$ of 500 mM EDTA (pH 8). Higher enzyme concentrations were used for Zn^{2+} and Ni^{2+} due to low activity. Reactions matched for ionic strength were also performed for Mn^{2+} and Zn^{2+} . For these, the concentration of KCl was progressively decreased as the concentration of CaCl_2 was increased (KCl concentrations of 100, 94, 88, 76, 52, and 4 mM were used with CaCl_2 concentrations of 0, 2, 4, 8, 16, and 32 mM, respectively). Ten microliter aliquots from each reaction were spotted in one square of a DEAE glass fiber Filtermat ($90 \times 120\ \text{mm}$ printed 96 squares) and air-dried. The filter was placed in a dish with 0.5 M NaPO_4 (pH 7) and shaken for 30 min at room temperature. The radioactive liquid was poured off, and a second wash was conducted. Filters were then rinsed in water and dried under a drying light. Dried filters were wrapped in saran wrap and exposed to phosphorimager screens. Exposed screens were developed and quantified using a Fuji FLA7000 or Amersham Typhoon imaging system. For determining 50% inhibitory concentrations (IC_{50}) for Ca^{2+} , data were plotted on a graph of relative (to reactions with no CaCl_2) poly(dT) synthesis (calculated from exposed screens) vs $[\text{CaCl}_2]$. To determine the IC_{50} values, the data were fitted in SigmaPlot with the equation for one site competition with max and min values set at 100 and 0%, respectively.

Nucleic Acid Hybridization.

Primer-template hybrids were prepared by mixing the template and end-labeled DNA primer at the indicated ratio in buffer containing 50 mM Tris-HCl (pH 8) and 80 mM KCl. The mixtures were heated to 65°C for 5 min and then cooled slowly to room temperature.

Polyacrylamide Gel Electrophoresis.

Denaturing polyacrylamide gels (10 and 20% w/v) were prepared and run as described.²⁸

Primer Extension Reactions to Test NNRTI Inhibition.

Radiolabeled primer extension reactions were performed to study the inhibition of extension by NNRTIs with different divalent cations. Radiolabeled primer (15 nM (all values listed are final concentrations in reaction) ³²P 5' end-labeled, sequence: 5'-TTGTTGTCTCTTCCCCAAAC-3', and 22.5 nM template, sequence: 5'-TGGCCTTCCCACAAGGGAAGGCCAGGGAATTTCTTCAGAGCAGACCAGAGCCAACAGCCCCACCAGAAGAGAGCTTCAGGTTTGGGGAAGAGACAACAA-3', hybridized as described above, were preincubated for 15 min at room temperature in 17 μ L of reaction buffer containing 50 mM Tris-HCl (pH 8) and 80 mM KCl, 0.5 mM divalent cation (Mg^{2+} , Mn^{2+} , Zn^{2+} , Co^{2+} , or Ni^{2+} , see above under Ca^{2+} Competition Assay for details), 50 nM HIV-1 RT, and various concentrations of NNRTIs (as indicated in text and figures). All NNRTIs were added in 20% DMSO to reactions such that the final concentration of DMSO in the reactions was 2%. After the preincubation, the reactions were moved to 37 °C, and incubation was continued for 5 min. Reactions were initiated by the addition of 5 μ g of poly(rA):oligo(dT₂₀) (8:1 w:w) and dGTP, dCTP, dTTP, and ddATP (20 μ M final concentration in reactions for dGTP, dCTP, and dTTP and 10 μ M for ddATP) and incubation was continued for 2 min (Mg^{2+} , Mn^{2+} , and Co^{2+}) or 5 min (Zn^{2+} and Ni^{2+}) before termination with 20 μ L of 2 $\times$ gel loading buffer (90% formamide, 10 mM EDTA (pH 8.0), and 0.025% bromophenol blue and xylene cyanol). Samples were run on 20% denaturing polyacrylamide gels and quantified with a phosphorimager as described above. The poly(rA)-oligo(dT₂₀) was used as a "trap" to sequester free RT molecules and those that dissociated from the primer/template. This limited extension to a single round.²⁹ Addition of ddATP terminated extension after the addition of four nucleotides to the primer (see template and primer above). This made quantitation of the level of extension easier.

Determination of Equilibrium Dissociation Constants (K_d) for RT to Aptamer (APT) with Different Divalent Cations.

Values for K_d were determined as described previously using nitrocellulose filter binding assays³⁰ with the following changes: divalent cations (Mg^{2+} , Mn^{2+} , or Ni^{2+} , see above for details) were included at 5 mM; reactions contained 2% DMSO, and the buffer included 50 mM Tris-HCl (pH 8) and 80 mM KCl.

Size Exclusion Assay to Test the Binding of EFV to HIV-1 RT in the Presence of Different Divalent Cations.

Assays were conducted in a final volume of 100 μ L in buffer containing 50 mM Tris-HCl (pH 8), 80 mM KCl, 2% DMSO, and 0 or 5 mM divalent cation (Mg^{2+} , Mn^{2+} , or Ni^{2+} , see above under Ca^{2+} Competition Assay for details). Zn^{2+} and Co^{2+} were omitted from these assays due to sporadic precipitation at the 5 mM concentration being used. Reactions also included 100 nM HIV-1 RT (always added last) and 200 nM radiolabeled ¹⁴C-EFV (~2220 dpm/reaction). EFV was added to reactions as above in 20% DMSO such that the final concentration of DMSO in reactions was 2%. Some reactions included 200 nM of a primer/

template mimicking APT (5'-TAATACmCCmCCCCCTTCGGTGCTTTGCACCGAAGGGGGGG-3', "m" denotes 2'-O-methyl group on the sugar) that binds with high affinity to HIV-1 RT.³⁰ The 3' G residue of the APT was replaced with dideoxy G (ddG) to prevent extension by RT. Some reactions also included various dNTPs at a concentration of 20 μ M as specified in the text and figures. After RT addition, reactions were incubated 20 min at room temperature and then pipetted into 0.5 mL 30 000 kDa MW cutoff filter concentrators. Wash buffer (250 μ L) containing 50 mM Tris-HCl (pH 8), 80 mM KCl, 2% DMSO, and 5 mM of the same divalent cation used in the reaction was then added, and samples were spun in an ultracentrifuge at 14 000 g for 5 min at 4 °C. A second wash was performed with 250 μ L of wash buffer, and the retained material (~40 μ L) was recovered per the manufacturers protocol. This material was counted in a scintillation counter after the addition of 2 mL of scintillation fluid. Samples were generally counted several times until the counts stabilized.

Filter Binding Assay to Measure dNTP Displacement by EFV.

The ability of EFV to displace ³H-dTTP from RT-APT complexes in the presence of Mg²⁺ or Mn²⁺ was measured using nitrocellulose filter binding assays. This approach could not be used to measure displacement of ¹⁴C-EFV from these complexes as EFV bound tightly to the filters, while ³H-dTTP can be removed with a wash step. Assays were conducted in a final volume of 20 μ L in buffer containing 50 mM Tris-HCl (pH 8), 80 mM KCl, 2% DMSO, 1 μ M ³H-dTTP (~500 000 dpm/reaction), and 0 or 5 mM divalent cation (Mg²⁺ or Mn²⁺). When added, the final concentrations of RT and APT in reactions were 100 and 200 nM, respectively. EFV was included at 0, 100, 200, 400, 800 nM and 1.6 or 100 μ M for Mg²⁺ and 0, 1.6, 3.2, 6.4, 12.8, 25.6, or 100 μ M for Mn²⁺. Components were mixed, and RT was added last. Incubation at room temperature was then continued for 20 min. Samples were filtered through 25 mm nitrocellulose filters with vacuum. Two milliliters of a buffer corresponding to the reaction buffer without RT, EFV, or APT was then applied for a wash step. Filters were then counted in a scintillation counter as described above. For experiments performed with HIV-1 K103N RT different concentrations of EFV were used for reactions with Mg²⁺: 0 and 800 nM and 1.6, 3.2, 6.4, 12.8, and 100 μ M.

RESULTS

Calcium Displacement Can Be Used to Evaluate the Relative Affinity of Divalent Cation-dNTP Complexes for HIV RT.

Several divalent cations can stimulate nucleotide catalysis with HIV RT.³¹ The level of activity in primer extension assays varies greatly with the natural cation Mg²⁺ having high activity and Zn²⁺ showing extremely low but measurable activity.³² In contrast, Ca²⁺ is known to bind strongly to RT at the putative divalent cation binding sites but does not support catalysis. To test the relative binding affinity of divalent cation-dNTP complexes, the activity of RT with Mg²⁺, Mn²⁺, Zn²⁺, Co²⁺, and Ni²⁺ was tested on dT primed poly(rA) at a concentration of 0.5 mM divalent cation. Ca²⁺ was titrated into reactions to displace the complexes with other divalent cations. Displacement would result in lower activity as Ca²⁺ bound RT molecules cannot catalyze incorporation. The results of an experiment with the different cations are shown in Figure 1A, which shows a phosphorimager scan of a DEAE

filter (see Materials and Methods). Quantification showed that in the absence of Ca^{2+} , Mg^{2+} produced greater activity than other divalent cations with Co^{2+} and Mn^{2+} , showing about less activity, respectively. Both Ni^{2+} and Zn^{2+} had considerably less activity (greater than 10-fold reduced with the same amount of RT), and the assays shown were performed with 50 nM RT rather than the 5 nM RT used for other divalent cations. Despite these discrepancies, it was clear that Mn^{2+} and Zn^{2+} were much harder to displace than other divalent cations. In Figure IB, a graph of relative activity (relative to the no Ca^{2+} reaction for each divalent cation) vs Ca^{2+} concentration is shown. The graph illustrates the strong resistance of Mn^{2+} and Zn^{2+} to displacement compared to others. In the Figure IB insert, it is clear that Co^{2+} is more resistant than Mg^{2+} and Mn^{2+} , which are comparable. Fitting of the data allowed the calculation of an IC_{50} value for Ca^{2+} for each divalent cation (Table 1). The results show that Mg^{2+} and Ni^{2+} are the weakest binding divalent cations. About 7 times more Ca^{2+} (compared to reactions with Mg^{2+}) was required to inhibit Co^{2+} reactions by 50%. The amount of Ca^{2+} in this case was 0.25 mM, which was $1/2$ the concentration of Co^{2+} in the reactions. This implies that Co^{2+} -dTTP complexes bind RT approximately V_2 as tightly as Ca^{2+} -dTTP complexes. Both Mn^{2+} and Zn^{2+} bound much more tightly. Binding measurements with Mn^{2+} and Zn^{2+} required much higher concentrations of Ca^{2+} such that there was a significant effect on the overall ionic strength of the reactions (e.g., with 32 mM CaCl_2 , the ionic strength is more than twice what it was at 0 mM), while inhibitory Ca^{2+} concentrations with Mg^{2+} , Ni^{2+} , and Co^{2+} had negligible effects (e.g., 0.25 mM CaCl_2 increased the ionic strength in reactions by about 1%). Changes in ionic strength with Mn^{2+} and Zn^{2+} could have affected enzyme activity. To test this, a second analysis was conducted in which the ionic strength in all reactions was equalized by reducing the concentration of KCl as the concentration of CaCl_2 was increased (see Materials and Methods). Because divalent and monovalent cations are known to interact differently with proteins and nucleic acids, removing KCl to compensate for CaCl_2 , although correcting for ionic strength, may not mimic other interactions. It was not possible to use a divalent cation for ionic strength correction due to possible interactions with dNTPs and RT. Values for IC_{50} in Table 1 for reactions with Mn^{2+} and Zn^{2+} compensated for ionic strength showed modest differences compared to noncompensated reactions. Overall, the results indicate that the relative RT binding strength of the divalent cation-dTTP binding complexes was $\text{Mn}^{2+} \# \text{Zn}^{2+} \gg \text{Co}^{2+} > \text{Mg}^{2+} \approx \text{Ni}^{2+}$.

Inhibition of RT Primer Extension by EFV, RPV, and NVP is Dependent on the Divalent Cation Used with Stronger Binding Divalent Cations Showing Greater Resistance to the NNRTIs.

The effect of NNRTIs and different divalent cations on RT primer extension was examined using a 20-nucleotide radiolabeled DNA primer bound to a 100 nucleotide DNA template (Figures 2A–E). Extension was performed with 0.5 mM of each divalent cation (which corresponds to the approximate concentration of free Mg^{2+} in lymphocytes and other cell types^{33–38}) and 20 μM each of dCTP, dGTP, and dTTP and 10 μM ddATP. This limited extension to four nucleotides as the fourth base downstream from the primer 3' terminus was a T on the template, which would lead to the incorporation of ddA. This approach was taken to make it easier to quantify primer extension by limiting extension to a discrete position on the gel that is a short but easily resolved distance above the radiolabeled primer.

In addition, the reactions were initiated by adding a nucleotide mix along with a poly(rA)-oligo(dT) “trap” to RT-P/T complexes that was preformed in the presence of the appropriate divalent cation and NNRTI. The trap was used to limit RT extension to a single round by sequestering RT molecules that are unbound or dissociate from the P/T. Therefore, the assay measures the ability of RT to bind to and extend the primer in the presence of NNRTIs and various divalent cations. In Figure 2B, results with EFV (0–400 nM) are shown. Again, due to low activity, reactions with Zn^{2+} and Ni^{2+} were carried out for 5 min after nucleotide addition, while those with other divalent cations were run for 2 min. Because all reactions were carried out with excess enzyme over P/T (50/15 nM primer), most of the primer was extended in the reactions without EFV except for reactions with Zn^{2+} , which showed lower extension even in the absence of EFV. Addition of even 100 nM EFV strongly inhibited Mg^{2+} and Ni^{2+} reactions and to a lesser extent Co^{2+} (see Figure 2C for a graphical analysis of the data). In contrast, both Mn^{2+} and Zn^{2+} were much more resistant with both retaining greater than 50% extension relative to the no EFV reactions at 200 nM EFV where both Mg^{2+} and Ni^{2+} were at ~10%. Experiments with Mg^{2+} were also conducted with a higher Mg^{2+} concentration (6 mM) that is known to be more optimal for RT activity^{39–41} (Figure S1). The higher Mg^{2+} concentration resulted in a modestly greater resistance to EFV inhibition but still could not compare to the resistance observed with Mn^{2+} and Zn^{2+} . This is consistent with previous results showing that NNRTIs are more effective in low Mg^{2+} .²⁵ Experiments were also conducted at concentrations that corresponded to the determined optimum for each divalent cation in primer extension reactions (approximately 1.6, 0.15, 0.15, 0.07, and 2.6 mM free divalent cation concentration for Mg^{2+} , Mn^{2+} , Zn^{2+} , Co^{2+} , and Ni^{2+} , respectively, as determined previously using a heteropolymeric RNA template⁴² for all except Ni^{2+}). Similar trends were observed in these assays compared to those with 0.5 mM of each divalent cation (Figure S2). The most notable differences were the improved level of extension with Zn^{2+} and greater sensitivity to EFV with Co^{2+} . The latter may have resulted from the very low (0.07 mM) optimum for this divalent cation.

Experiments were also conducted with NNRTIs RPV and NVP. Concentrations of each were chosen such that strong inhibition was observed in the presence of Mg^{2+} . For RPV, 50, 100, 150, and 200 nM drug was used, which is ½ the level of EFV that was used above. Even at 50 nM where the concentrations of RPV was equal to the concentration of RT, reactions with Mg^{2+} were almost completely inhibited (Figure 2D). Unlike EFV where Ni^{2+} and Mg^{2+} showed comparable sensitivity (Figures 2B and 2C), reactions with Ni^{2+} were more resistant to RPV compared to Mg^{2+} . Once again, Co^{2+} showed even more resistance than Ni^{2+} , and Mn^{2+} and Zn^{2+} were highly resistant at the concentrations employed. A similar trend was observed with NVP (Figure 2E); however, in this case, a much higher concentration of drug (10, 20, 40, and 80 μM) was required to observe inhibition, consistent with the lower affinity of NVP for RT.⁴³ Also, the sensitivities of both Mg^{2+} and Ni^{2+} to NVP were similar, as was observed with EFV. Overall, divalent cations that complexed more strongly with RT (see Figure 1) were more resistant to all the NNRTIs, although there were some subtle differences between the various drugs.

Binding Complex Measurements Indicate That EFV is Displaced Most Strongly by the Divalent Cation-dNTP Complex When RT is Bound to P/T.

The above results were consistent with, among other possibilities, binding competition between NNRTIs and divalent cations or divalent cation–dNTP complexes. However, the assays rely on analysis of downstream nucleotide addition and cannot directly test binding competition. We took advantage of the stable binding between NNRTIs and RT⁴⁴ to directly evaluate the effect of various divalent cations and P/T on NNRTI binding to RT.

Radiolabeled ¹⁴C-EFV was used for the assays along with a primer/template mimicking APT that binds HIV RT with low pM affinity.³⁰ The tight binding of the APT assured that a stable long-lived complex is formed with RT. A ddG residue was placed at the 3' end of the APT to prevent nucleotide addition (Table S1 and Figure 3A). Although the aptamer is not a typical double stranded primer/template and has a short 15 nucleotide duplex region, the DNA duplex tracks essentially identically with a canonical primer/template in the RT-aptamer crystal structure.^{30,45} It also makes contacts with RT (based on hydrogen–deuterium exchange (HDX) reactions) that mimic those of a “weak” binding control of the same basic structure and shows comparable nucleotide extension kinetics when compared to a canonical primer/template in presteady state assays (manuscript in preparation). Therefore, we expect that the results obtained with the APT would be consistent with a typical primer/template.

The K_d values for RT–APT complexes indicated highly stable low pM binding in the presence of all the divalent cations used (Table S1). RT bound most tightly in the presence of Mn^{2+} , while the highest K_d was with Mg^{2+} followed by Ni^{2+} . Zn^{2+} and Co^{2+} were not tested as sporadic precipitation occurred under the conditions of the assay with these divalent cations (note that 5 mM divalent cation was used vs 0.5 mM in the primer extension assays described above). Binding results were consistent with previous results showing that RT binds DNA–DNA P/T constructs more tightly in the presence of Mn^{2+} (and Zn^{2+}) vs Mg^{2+} .

³² Assays to measure the binding of radiolabeled EFV to RT were performed in 100 μ L volume with 0.1 μ M HIV-RT and 0.2 μ M radiolabeled EFV in the presence or absence of 5 mM divalent cation, 0.2 μ M APT, and 20 μ M dTTP (the next correct template-directed nucleotide on the APT). The high amounts of RT, APT, and EFV were needed because of the low specific activity of EFV, a consequence of the ¹⁴C label. The experiments were also performed with a higher concentration of divalent cation (5 mM compared 0.5 mM for the previously discussed experiments). This was done to “saturate” whatever effect the divalent cations were having on EFV binding as it was not feasible to perform these experiments with several concentrations of each divalent cation. After a 20 min incubation, samples were run on 30 000 MW cutoff membrane filters and washed to remove unbound EFV. Retained material was then counted in a scintillation counter. Figure 3A shows the results from EFV binding assays using various conditions. All values are relative to EFV binding in the absence of divalent cation (Mg^{2+} , Mn^{2+} , or Ni^{2+}). The addition of divalent cation alone did not have a significant effect on binding of EFV to RT (consistent with previous results⁴⁶), and adding dTTP with divalent cation did not significantly change the results when compared to divalent cation alone. The addition of APT in the absence of nucleotide led to a small but insignificant decrease in EFV binding with Mg^{2+} and Mn^{2+} . Interestingly, binding with Ni^{2+} declined significantly. The reason for this observation was unclear; however, it could be related to differential interactions of divalent cations with nucleic acid or alternative sites on RT.^{47,48} Binding was strongly reduced for all divalent cations when both APT and

dTTP were included. Most notable was the drop to about 40% and less than 20% binding for Mg^{2+} and Mn^{2+} , respectively. Additional experiments with these two divalent cations indicated that the observed EFV binding inhibition occurred only if the nucleotide used was the next correct template-directed nucleotide (dTTP in this case) in the case of Mg^{2+} (Figure 3B). Note that complexes of dideoxy-terminated P/T, RT, and the next correct template-directed nucleotide have been referred to as “dead-end” complexes because they are highly stable⁴⁹ (see Discussion). For Mn^{2+} , the next correct nucleotide (dTTP) produced the greatest inhibition of EFV binding while dATP also reduced binding. Although it is not clear why this occurred, the second template directed nucleotides on the APT was dATP. Overall, the results indicate that the divalent cation in complex with a nucleotide is a strong competitor for EFV binding in the presence of P/T.

Consistent with Previous Reports Using Other Techniques, EFV Can Displace dNTPs from RT-P/T Complexes.

Others have shown that NNRTIs displace dNTPs from RT or RT-P/T complex.^{21,22} We took advantage of the strong binding of the “dead-end” complexes described above, which allowed radiolabeled dTTP bound to RT to be retained on nitrocellulose filters, to determine if EFV could displace dTTP from the RT-P/T complex. Note that a lower concentration of dTTP was used in these reactions than in the ¹⁴C-EFV displacement assays above (1 vs 20 μ M). Therefore, the assays cannot be directly compared with respect to the concentration of one component required to displace the other. Results in Figure 4 show that dTTP was displaced by EFV in a concentration-dependent manner for both Mg^{2+} and Mn^{2+} . In the absence of APT, dTTP did not bind tight enough to RT to be retained on the filters at significant levels (-APT reactions, Figure 4A and 4B). However, dTTP was retained on the filters when both RT and APT were included in the reactions, presumably due to the formation of a dead-end complex (-EFV reactions, Figures 4A and 4B). Consistent with other results described above, Mn^{2+} -dTTP complexes (Figure 4B) were more resistant to displacement by EFV than Mg^{2+} -dTTP complexes (Figure 4A), and several-fold higher concentrations of EFV were required for displacement. Overall, the results reinforce the idea that stronger binding divalent cation—dNTP complexes are more resistant to NNRTIs, and divalent cation—dNTP complexes and NNRTIs can exclude each other's binding to RT-P/T.

HIV RT K103N, Which Is Resistant to Many NNRTIs, Binds More Weakly to EFV but Also Demonstrates Mutually Exclusive Binding with Divalent Cation-dNTP Complexes.

HIV-1 NNRTI resistance develops through mutations in the NNRTI binding pocket. The Lys103Asn (K103N) mutant is 10–100-fold more resistant than wild-type HIV-1 to most NNRTIs, including nevirapine and efavirenz.^{50–53} We tested the ability of K103N to bind ¹⁴C-EFV in the size exclusion assay shown in Figure 3 in the presence of Mn^{2+} or Mg^{2+} . In both cases, EFV was able to bind to K103N but at much lower levels than wild-type RT (Figure 5). Adding the P/T aptamer enhanced binding, but it was still significantly lower than wild-type. Adding dTTP to form the dead-end complex described above displaced EFV from K103N with either divalent cation. This implies that K103N, like wild-type, demonstrates mutually exclusive binding of divalent cation complexes and EFV. Further, in the dNTP displacement assay shown in Figure 4, EFV displaced H³-dTTP from the enzyme P/T complex (Figure 6). Consistent with the lower affinity of EFV for K103N, several-fold

more EFV was required to displace dTTP in the presence of Mg^{2+} (compare Figure 4A with 6A), while only partial displacement occurred with the strong binding Mn^{2+} -dTTP complexes (Figure 6B). Overall, the results support a mechanism based on weak binding of EFV to K103N while the enzyme retains the mutually exclusive phenotype.

DISCUSSION

In this report, results are presented that indicate NNRTIs compete for binding to RT with divalent cation-dNTP complexes. Clearly, they do not compete for the same binding site as structural analysis shows the binding sites are unique (see introductory section). More likely, binding of one changes RT's shape, locking it in a conformation that excludes binding of the other, i.e. the binding is mutually exclusive. This biochemical analysis is in general agreement with biophysical,²² thermodynamic,²¹ and computational⁵⁴ analyses, while previous biochemical presteady state kinetic experiments did not reveal mutually exclusive binding.⁹⁻¹¹

Some conclusions in this report are based on the Ca^{2+} -displacement experiments (Figure 1) that allow for an assessment of the relative binding of various divalent cations to RT. Those experiments indicate that Mn^{2+} and Zn^{2+} bind much more tightly to RT than Mg^{2+} , Ni^{2+} , and to a lesser extent Ca^{2+} and Co^{2+} . Because the experiments indirectly measure binding affinity by downstream nucleotide addition, they probably assess only the binding of the two putative divalent cations involved in nucleotide catalysis (the possible involvement of a third divalent cation in catalysis is beyond the scope of this report^{55,56}), referred to as the "A and B site" divalent cation.^{57,58} Although it is possible that divalent cations bound elsewhere could affect this step by allosterically inhibiting or enhancing substrate binding or catalysis, solution calorimetric studies indicate that 3 Mn^{2+} or 2 Mg^{2+} ions can bind purified RT in the absence of nucleotide or P/T.⁵⁹ The authors indicate that one metal binds in the polymerase site, while 2 Mn^{2+} but only 1 Mg^{2+} can bind near the RNase H site, with the Mg^{2+} accessing one of two mutually exclusive sites at any given time. This suggests that specific divalent cations may bind at alternative positions that could influence binding or catalysis. Related to this, binding of Mg^{2+} at the RNase H site is known to stabilize P/T binding.⁶⁰ Interestingly, a recent report used calorimetric analysis to measure the binding of Mg^{2+} , Mn^{2+} , and Zn^{2+} to the isolated RNase H domain of HIV RT.⁶¹ Binding to Mn^{2+} and Zn^{2+} was 40 and 400 times tighter, respectively, than to Mg^{2+} . Complexes of RT and P/T formed in the presence of Mn^{2+} and Zn^{2+} are also much more stable than those formed with Mg^{2+} .³² Results presented here indicate that Mn^{2+} and Zn^{2+} ions involved (either directly or indirectly) in polymerase catalysis are also bound much more tightly to RT than the natural divalent cation Mg^{2+} . This seems to follow the general trend of previous work and supports the idea that sites designed to bind Mg^{2+} for catalysis may also bind Mn^{2+} and Zn^{2+} with very high affinity. Despite differences in binding affinity and perhaps different binding stoichiometry in the RNase H site, Mn^{2+} , Zn^{2+} , and Co^{2+} can all carry out catalysis at fidelity levels similar to Mg^{2+} when concentrations optimized for extension rate are used.⁴² This suggests that there is little if any distortion of the primer/template positioning during extension.

For Mn^{2+} and the more physiologically relevant Mg^{2+} , it appeared that potent displacement of the NNRTI required a bound divalent cation-nucleotide complex. The dideoxy terminated

P/T APT that was used binds very strongly to RT³⁰ (and Table S1), but did not significantly inhibit NNRTI binding in the presence of Mg²⁺ or Mn²⁺ (Figure 3A). A small but statistically insignificant decrease in binding was consistently observed. This may suggest that there is some decrease in binding that may be measurable with a more precise assay. Presumably, the P/T in the presence of the next correct nucleotide assembles a “dead end complex” that cannot undergo catalysis due to the dideoxy at the 3' terminus. The complex includes RT, divalent cation(s), nucleotide, and P/T. Such complexes are known to be very stable.⁴⁹ It appears that the trapping of the divalent cation–nucleotide in this complex is what leads to the displacement of the NNRTI with Mg²⁺ and Mn²⁺, as RT-P/T complexes in the absence of nucleotide and presence of divalent cation bound NNRTIs (Figure 3A). This hypothesis is supported by other experiments showing that nucleotides not corresponding to the next correct template-directed nucleotide did not inhibit EFV binding with Mg²⁺ and Mn²⁺ (Figure 3, dATP was an exception with Mn²⁺ and is addressed in the Results). These presumably cannot form a stable dead end complex.⁴⁹

One alternative to mutually exclusive binding is that NNRTIs and divalent cation–dNTP complexes do not affect, or at least do not strongly affect, each other's binding, but NNRTIs dramatically decrease catalysis even when the complexes are bound. Our results and those from others (see introductory section^{21,22}) suggest that this scenario, with respect to divalent cation–dNTP binding, is unlikely. First, the concentration of NNRTIs required for inhibition correlated with the relative strength of binding of the divalent cation used (Figures 1 and 2). Taken alone, this does not prove binding competition as divalent cation–nucleotide complexes that bind more strongly might be expected to bind better to the NNRTI–RT complex, and perhaps stronger binding can enhance catalysis by this complex. However, direct binding experiments showed that divalent cation–nucleotide complexes displaced EFV from RT in the presence of P/T (Figures 3A and 3B). Other combinations, including divalent cation or P/T alone or divalent cation and nucleotide, did not significantly alter the binding of RT to EFV with Mg²⁺ and Mn²⁺. Further, and consistent with previous results,^{21,22} EFV was also able to displace dTTP from the RT-P/T complex, indicating that the NNRTI and dNTP exclude each other's binding (Figure 4). It is important to note that the ¹⁴C-EFV size-exclusion assay employed included two 5 min wash steps to remove unbound radiolabeled EFV (see Materials and Methods). Therefore, it can only measure stable complexes between RT and EFV and is unlikely to be highly quantitative. The nitrocellulose filter binding approach used to test ³H-dTTP binding to RT-P/T did not include long wash steps and is probably a more quantitative assay; however, stable binding of EFV to nitrocellulose filters in the absence of RT precluded its use for ¹⁴C-EFV tests.

Another possibility is that NNRTIs and divalent cation–dNTP complexes strongly reduce but do not eliminate each other's binding. If this were the case, catalysis could be observed, especially with high nucleotide concentrations, providing that RT-NNRTI complexes retain limited catalytic activity. This is, in a sense, a variation of the mutually exclusive hypothesis modulated by an incomplete inhibition of binding by the competitors. Our data neither supports nor eliminates this possibility as assays used here are not sensitive enough to determine whether there are rare RT molecules bound to NNRTI and divalent cation–nucleotide complexes simultaneously, and if such complexes can undergo catalysis. Although the structural distortions and rigidity imposed by NNRTIs (see introductory

section) would seem to be inconsistent with RT catalysis, some results suggest that NNRTI–RT complexes may be more flexible,⁶² a property that could potentially allow catalysis. Previous results using transient and presteady state kinetic analysis indicated that RT was capable of limited nucleotide catalysis in the presence of high concentrations of NNRTIs.^{9,10,19,20} This suggests that NNRTI–RT complexes can bind nucleotides and even carry out catalysis, albeit at a severely reduced rate. One possibility is that at equilibrium, a small subset of NNRTI–RT complexes is present in a catalytically competent form not observed in crystal structures. Another consideration is that most mechanistic experiments with NNRTIs were performed with single or a limited set of drugs. Although all approved NNRTIs bind the same site on RT, their structures and binding affinities vary considerably.⁴⁶ It is possible that this could have subtle effects on the results, although it seems unlikely to completely change the mechanism of inhibition.

Results with HIV-1 K103N indicated that the enzyme binds relatively poorly to EFV, and the binding of EFV displaces divalent cation–dNTP complexes similar to what was observed with wild-type RT (Figures 5 and 6). Essentially, K103N displays mutually exclusive competitive binding with divalent cation–dNTP complexes, but the competitive advantage of EFV has been severely weakened. Again, these assays do not rule out the possibility that some fraction of K103N RTs can bind EFV and dNTPs at the same time and that these complexes could be competent for catalysis. The mechanism of inhibition for K103N is unclear with some reports using surface plasmon resonance (SPR)^{43,63} and structure-based computer simulations,⁶⁴ indicating significantly lower binding of NNRTIs, including EFV, to K103N RT. Mutational analysis and also other studies using NMR⁶⁵ and fluorescence anisotropy²² found no difference in the binding of EFV to K103N vs wild-type RT. The latter study also found that EFV binding to K103N RT caused only a modest threefold decrease in the K_d for binding of nucleotide to the complex.²² Our assays (Figures 4 and 6) indicate EFV displaces nucleotide from both wild-type and K103N RTs with high concentrations, reducing nucleotide binding to undetectable levels in the presence of Mg^{2+} . The reason for the inconsistencies in these experiments and ours is unclear. Notable difference between the experiments include the different techniques used for measurements and the use of chemically modified enzyme in some of the earlier studies,⁶⁵ although the modifications did not appear to significantly affect enzyme structure or activity. Further analysis of the mechanism of drug resistance for K103N and other NNRTI resistant RTs should help clarify these inconsistencies.

In conclusion, we used a novel biochemical approach to show that NNRTIs inhibit RT by excluding or strongly decreasing binding to nucleotides, which in turn can inhibit NNRTI binding via a competitive mutually exclusive mechanism. The common K103N NNRTI drug-resistant mutant retains this phenotype while demonstrating weakened EFV binding. Inhibition may also occur by other modes (e.g., structural alterations and increased enzyme rigidity that are inconsistent with catalysis (see introductory section)); these would be relevant, especially for reducing catalysis if the mutually exclusive mechanism does not completely abrogate nucleotide binding to RT–NNRTI complexes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

HIV	human immunodeficiency virus
RT	reverse transcriptase
RNase H	ribonuclease H
PNK	T4 polynucleotide kinase
NRTI	nucleoside reverse transcriptase inhibitor
NNRTI	non-nucleoside reverse transcriptase inhibitor
NcRTI	nucleotide-competing reverse transcriptase inhibitor
EFV	efavirenz
NVP	nevirapine
RPV	rilpivirine
P/T	primer/template
DMSO	dimethyl sulfoxide
DTT	dithio-threitol
ITC	isothermal titration calorimetry

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Figure 1.

(A and B) Displacement of other divalent cations by Ca²⁺ results in loss of RT activity and can be used to estimate the binding strength of divalent cation complexes to RT. (A) A DEAE Filtermat blot is shown. Assays included 0.5 mM of the divalent cation shown on the left and increasing concentrations of CaCl₂ as indicated. Extension of oligo(dT)₂₀ primer on poly(rA) template in the presence of ³²P-dTTP was carried out with HIV-1 RT as described under Methods. Due to lower activity, assays with ZnSO₄ and NiCl₂ included 50 nM RT while those with other divalent cations included 5 nM. Assays corrected for ionic strength were also performed for Mn²⁺ and Zn²⁺ as described under Methods. (B) Plot from the filter shown in Figure 1A shows relative poly(dT) synthesis with different divalent cations and Ca²⁺ concentrations. All values are relative to the “0” Ca²⁺ assay for a given divalent cation. The insert graph shows an expanded view of values between 0 and 0.5 mM Ca²⁺. This experiment was repeated several times with the ranges of CaCl₂ used adjusted to appropriate levels for a given divalent cation in order to determine 50% inhibition values (IC₅₀) for Ca²⁺ (see Table 1).

Figure 2.

(A–E) Tighter binding divalent cations are more resistant to NNRTI inhibition. (A) A schematic diagram of the assay is shown. The 20 nucleotide primer was 5′ end-labeled with ³²P. (B) Extension on the 100 nucleotide template was carried out in the presence of 0.5 mM divalent cation (as indicated), 3 dNTPs, and ddATP to stop extension after 4 additions. EFV concentrations were 0, 100, 200, 300, or 400 nM, while HIV RT was 50 nM. After a 20 min preincubation, reactions were initiated by adding nucleotides and a poly(rA)-oligo(dT₂₀) trap (see Materials and Methods). Material was resolved on a 20% denaturing gel and exposed to an imager screen to produce the image above. -E, no enzyme added; C, trap control with RT mixed with poly(rA)-oligo(dT₂₀) trap prior to addition to reaction and incubated for the same time as other reactions. (C) A graph of relative extension vs [EFV] is shown for various divalent cations. All values are relative to the level of extension in the absence of EFV (lane 0 on the gel shown in panel B). Points were from an average of three experiments, and error bars represent standard deviations. (D) Extension reaction carried out as described above using RPV instead of EFV. RPV concentrations were 0, 50, 100, 150, and 200 nM. (E) Extension reaction carried out as described above using NVP instead of EFV. NVP concentrations were 0, 100, 200, 400, and 800 μM.

Figure 3.

(A and B) Divalent cation–dNTP complexes can inhibit the binding of EFV to an RT-P/T complex. (A) Reactions included 100 nM RT and 200 nM ¹⁴C-EFV and 5 mM divalent cation (MgCl₂, MnCl₂, or NiCl₂) in the presence or absence of dTTP (20 μM) and/or the P/T mimicking APT (200 nM). The structure of the APT is shown on the right. After a 20 min incubation the reaction was filtered over a 30 000 MW cutoff filter that retained RT and RT-bound components including ¹⁴C-EFV while removing unbound ¹⁴C-EFV (see Materials and Methods for details). Retained material was counted in a scintillation counter. (B) Reactions included 100 nM RT and 200 nM ¹⁴C-EFV and 5 mM divalent cation (MgCl₂ or MnCl₂) in the presence of the indicated nucleotide (20 μM) and P/T mimicking APT (200 nM). Assays were carried out as described for panel A above. Values for these assays are averages from at least three independent experiments, and error bars represent standard deviations. All values are relative to a control reaction that included RT and EFV but no divalent cation.

Figure 4.

(A and B) EFV can inhibit the binding of divalent cation–dNTP complexes to an RT-P/T complex. Reactions included 100 nM RT, 5 mM divalent cation (panel A, MgCl₂; panel B, MnCl₂), 1 μM ³H-dTTP, and P/T mimicking APT (200 nM) (except for the -APT reaction). EFV at the indicated concentration was also included. The structure of the APT is shown in Figure 3A. After a 20 min incubation, the reaction was applied to a nitrocellulose filter that retained RT and RT-bound components, while unbound radiolabeled dTTP was removed (see Materials and Methods for details). Filters were air-dried, and retained material was counted in a scintillation counter. Values for these assays are averages from at least three independent experiments, and error bars represent standard deviations. All values are relative to the reactions in the absence of EFV.

Figure 5.

EFV binds more poorly to RT K103N–P/T complex than the same complexes with wild-type RT. Reactions were conducted essentially as described in Figure 3A using wild-type (WT) RT. All results are relative to the binding of ¹⁴C-EFV WT RT.

Figure 6.

(A and B) A higher concentration of EFV is required to inhibit the binding of divalent cation–dNTP complexes to RT K103N–P/T complex compared to the same complexes with wild-type RT. Assays were conducted as described in Figure 4, but RT K103N was used instead of wild-type RT, and different concentrations of EFV (as indicated) were employed.

Table 1.**Ca²⁺ Competition Assay**

divalent cation^a	50% Ca²⁺ inhibition concentration (mM)^b
Mg ²⁺	0.033 ± 0.015 (1)
Ni ²⁺	0.044 ± 0.012 (1.3)
Co ²⁺	0.24 ± 0.07 (7.2)
Mn ²⁺	7.9 ± 0.2 (239)
Zn ²⁺	16.0 ± 0.9 (484)
^c Mn ²⁺	10.7 ± 4.6 (324)
^c Zn ²⁺	8.1 ± 0.1 (245)

^a Assay was with poly(rA)-oligo (dT), 80 mM KCl, and 5 μ M TTP. Divalent cations (MgCl₂, NiCl₂, CoCl₂, MnCl₂, or ZnSO₄) were at 0.5 mM, and CaCl₂ was increased over a range of 0–32 mM.

^b Concentration of CaCl₂ required to decrease synthesis by 50% was calculated by fitting curves to an equation for exponential decay. Numbers in parentheses are the fold increase in the amount of CaCl₂ for 50% inhibition compared to MgCl₂. Values are averages from a minimum of three experiments ± standard deviation.

^c Assays were equalized for ionic strength as described in the Materials and Methods.