Derivatives of Mesoxalic Acid Block Translocation of HIV-1 Reverse Transcriptase*

Received for publication, September 24, 2014, and in revised form, October 27, 2014. Published, JBC Papers in Press, October 29, 2014, DOI 10.1074/jbc.M114.614305

Jean A. Bernatchez1,†, Rajesh Paul2,†, Egor P. Tchesnokov3,∥, Marianne Ngure4,∥, Greg L. Beilhartz5,∥, Albert M. Berghuis6,∥, Rico Lavoie6,∥, Lianhai Li6,∥, Anick Auger6,∥, Roman A. Melnyk6,∥, Jay A. Grobler6,∥, Michael D. Miller6,∥, Daria J. Hazuda6,∥, Sidney M. Hecht6,∥, and Matthias Götte6,∥,‡

From the 1Department of Biochemistry, McGill University, Montreal, Quebec H3G 1Y6, Canada, the 2Biodesign Institute and 3Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona 85287, the 4Department of Microbiology and Immunology, McGill University, Montreal, Quebec H3A 2B4, Canada, the 5Merck Research Laboratories, West Point, Pennsylvania 19486-8000, and the 6Department of Medicine, Division of Experimental Medicine, McGill University, Quebec H3A 1A3, Canada

Background: The 4-chlorophenylhydrazone of mesoxalic acid (CPHM) is a known inhibitor of HIV-1 reverse transcriptase (RT).

Results: We demonstrate that CPHM traps the pre-translocational conformation of the RT-DNA complex.

Conclusion: The data validate this complex as a possible drug target.

Significance: This work can therefore contribute to the development of novel classes of antiretroviral agents.

The reverse transcriptase (RT) of the human immunodeficiency virus type 1 (HIV-1) is a virally encoded RNA- and DNA-dependent polymerase that represents an important target for antiretroviral therapeutics (1–4). RT also possesses a ribonuclease H (RNase H) activity that degrades the RNA of RNA/DNA replication intermediates. Two different classes of approved antiviral drugs target the polymerase active site (5) as follows: nucleoside analog RT inhibitors (NRTIs)6 that compete with natural deoxynucleoside triphosphate (dNTP) pools for binding and incorporation (3, 6) and the chemically diverse class of non-nucleoside analog RT inhibitors that bind to a hydrophobic pocket 10 Å away from the active site (7, 8).

Phosphonoformic acid (PFA, foscarnet) (compound 1) (Fig. 1) targets HIV-1 RT and other viral DNA polymerases and shows a broad spectrum of antiviral activities against HIV and several members of the herpesviridae, including herpes simplex virus types 1 and 2 (HSV1 and HSV2) and the human cytomegalovirus (HCMV) (9). However, toxic side effects limit its clinical utility. PFA is currently only approved as a component in second line regimens to treat infection with HCMV (10). Despite such unfavorable properties as a drug, the mechanism of action is unique and could potentially be exploited in efforts to design novel classes of RT inhibitors. PFA interferes with the translocation of RT between two distinct cycles of nucleotide incorporation (11–13). Catalytic incorporation of nucleotides by RT occurs stepwise (14, 15). Following binding of the primer-template (P/T), the incoming deoxynucleoside triphosphate (dNTP) binds to the nucleotide-binding site (N-site). The chemical step is preceded by a conformational change in the flexible “fingers” subdomain that traps the nucleotide substrate in a closed conformation. Inorganic pyrophosphate (PPi) is generated concomitantly with formation of a new phosphodiester bond (16, 17). Following the release of PPi, RT has to translocate to shift the newly synthesized primer terminus to

* This work was supported in part by grants from the Canadian Institutes of Health Research, the Quebec Consortium for Drug Discovery, and Merck (to M. G.).

† Recipient of a doctoral training scholarship from the Fonds de la Recherche en Santé du Québec and a Chemical Biology Training Program Scholarship from the Canadian Institutes of Health Research.

‡ Recipient of a career award from the Fonds de la Recherche en Santé du Québec. To whom correspondence should be addressed: Dept. of Medical Microbiology and Immunology, University of Alberta, 6-020 Katz Group Centre, Edmonton, Alberta T6G 2E1, Canada. Tel.: 780-492-2309; Fax: 780-492-7521; E-mail: gotte@ualberta.ca.

§ The abbreviations used are: NRTI, nucleoside analog reverse transcriptase inhibitor; PFA, phosphonoformic acid; CPHM, 4-chlorophenyl hydrazone of mesoxalic acid; HCMV, human cytomegalovirus; PPT, polyuridine tract; P/T, primer-template; SAR, structure-activity relationship.
the priming site (P-site). Several lines of evidence suggest that the polymerase establishes a dynamic equilibrium between pre- and post-translocational conformations. Site-specific footprinting experiments revealed that the sequence of the nucleic acid substrate can influence this equilibrium (18). Certain sequences show an equal distribution, although other sequences show a bias toward pre- or post-translocation. Increasing concentrations of the next nucleotide substrate can convert a population of predominantly pre-translocated complexes into a population of predominantly post-translocated complexes (11, 19). Conversely, PFA traps the pre-translocated complex of RT, which prevents binding of the next dNTP substrate (11).

It is currently unknown whether this type of inhibition is limited to small molecules that are structurally highly related to PFA or whether more complex compounds that provide specificity to HIV-1 RT are also capable of blocking RT translocation through a similar mechanism of action. The 4-chlorophenylhydrazone of mesoxalic acid (CPHM) (compound 2) (Fig. 1) is a potential candidate in this context. This compound was originally identified as a strand transfer inhibitor of HIV-1 RT (20, 21). CPHM was shown to inhibit the RT-associated RNase H activity under assay conditions that allow multiple turnovers. Moreover, at high concentrations, CPHM delays the second of two consecutive nucleotide incorporation events. These findings pointed to a defect in RT translocation; however, the underlying mechanism remained elusive. Here, we demonstrate that CPHM, like PFA, binds to the polymerase active site and traps the pre-translocated complex. These findings demonstrate that the pre-translocated RT complex can be targeted by structurally diverse compounds, which provide novel opportunities in the development of antiretroviral drugs.

**EXPERIMENTAL PROCEDURES**

**Enzymes, Nucleic Acids, and Small Molecules—**Heterodimeric p66/p51 reverse transcriptase was expressed and purified as described previously (22). Wild-type HCMV polymerase (UL54) was derived from recombinant viruses generated by overlapping cosmids (23). The UL54 coding sequence was derived from recombinant viruses generated by structurally diverse compounds, which provide novel opportunities in the development of antiretroviral drugs.

**Experimental Procedures**

**Enzymes, Nucleic Acids, and Small Molecules—**Heterodimeric p66/p51 reverse transcriptase was expressed and purified as described previously (22). Wild-type HCMV polymerase (UL54) was derived from recombinant viruses generated by overlapping cosmids (23). The UL54 coding sequence was kindly provided by Dr. Guy Boivin (Laval University). The UL54 coding sequence was cloned into pPR-IBA1 (IBA) using the Bsal site to generate pPR-IBA1/UL54. This construct facilitates protein purification through Strep-tag affinity chromatography (IBA). D542A substitution was introduced to remove the 3‘-5’ exonuclease activity. The amino acid substitution was introduced with PfuUltra DNA polymerase (Stratagene) according to the manufacturer’s recommendations. The HCMV polymerase UL54 was expressed in insect cells using the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer’s recommendations. UL54 was purified using Strep-tag affinity chromatography (IBA) according to the manufacturer’s recommendations. Oligo(deoxy)ribose nucleotides were synthesized and purchased from Integrated DNA Technologies. Sequences are provided in the figures. Phosphonoforamic acid was purchased from Sigma. CPHM and derivatives were provided by Dr. Sidney Hecht (Arizona State University) and Merck (West Point).

**DNA Synthesis—**To monitor the incorporation of multiple nucleotides by only HIV-1 RT, a 3-fold molar excess of PPT-57 DNA template was hybridized to 50 nM 5’-radiolabeled PPT DNA primer. The hybrid was heat-annealed in a buffer containing 50 mM Tris-HCl, pH 7.8, and 50 mM NaCl and then incubated with 500 nM RT in a buffer containing 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 0.3 mM EDTA, and 2 μM each of dATP, dTTP, dGTP, and dCTP. The samples were treated with either no inhibitor, 20 μM PFA, or 200 μM CPHM. DNA synthesis was initiated by the addition of 6 mM MgCl₂ at 37 °C. The reaction was stopped with formamide-loading dye containing xylene cyanol and bromphenol blue. Samples were resolved on a 15% denaturing polyacrylamide gel followed by phosphorimaging (Amersham Biosciences). To monitor the incorporation of multiple nucleotides by both HIV-1 RT and HCMV UL54, 100 nM DNA/DNA template-primer hybrid T1/P1 was preincubated for 10 min at 37 °C with a given DNA polymerase (30 nM HIV-1 RT or 4 nM HCMV UL54) in a buffer containing 25 mM Tris-HCl, pH 8, 50 mM NaCl, 0.5 mM dithiothreitol (DTT), 0.2 mg/ml bovine serum albumin, 5% glycerol, and 10 μM dNTP mix in the presence or absence of increasing concentrations of PFA or CPHM. Nucleotide incorporation was initiated by the addition of MgCl₂ to a final concentration of 10 mM, and the reactions were allowed to proceed for 30 min. The reactions were stopped by the addition of 3 reaction volumes of formamide containing traces of bromphenol blue and xylene cyanol. Samples were then subjected to 15% denaturing PAGE followed by phosphorimaging.

**Electrophoretic Mobility Shift Assay (EMSA)—**The formation of ternary complexes was monitored with a 5’-radiolabeled primer. The labeled primer was annealed to a 5-fold molar excess of the template. The DNA hybrid (50 nM) was then incubated with 500 nM WT HIV-1 RT. Increasing concentrations of PFA and CPHM were added to each sample and incubated for 10 min at room temperature. The complexes were subsequently challenged with 3 μg/μl heparin, followed by incubation for 1 h at room temperature. The samples were resolved on 6% nondenaturing polyacrylamide gels, analyzed with a phosphorimager (Amersham Biosciences), and quantified with Quantity One and ImageQuant software.

**Site-specific Footprinting—**Site-specific footprints with Fe²⁺ were monitored on 5’-end-labeled DNA templates. The template (100 nM) was hybridized with the complementary primer (300 nM) as described above. The hybridization was conducted in a buffer containing 20 mM sodium cacodylate, pH 7, and 20 mM NaCl. The duplex was incubated with 750 nM HIV-1 RT in a buffer containing 120 mM sodium cacodylate, pH 7, 20 mM NaCl, and 6 mM MgCl₂, in a final volume of 50 μl. Prior to the treatment with Fe²⁺, complexes were preincubated at 37 °C for 10 min with increasing concentrations of PFA or CPHM. Treatment with Fe³⁺ was performed as described previously (18).

**HIV-1 Replication Assays—**Multiple cycle replication assays were performed with HIV isolate R8 in MT-4 human T lymphoid cells as described (24).

**Time Course of RNase H Activity—**For steady-state (multiple turnover) RNase H reactions, 3’-radiolabeled RNA template (PBS-52r) was heat-annealed to a 3-fold excess of DNA primer (PBS-22Dpol). The RNA/DNA hybrid (150 nM) was incubated with 25 nM RT in a buffer of 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 500 μM EDTA, and 6 mM MgCl₂, in the absence or pres-
Inhibition of HIV-1 RT Translocation

ence of CPHM (100 μM) or PFA (100 μM). Reactions in the absence of inhibitor contained 0.1% dimethyl sulfoxide (DMSO). The reaction was allowed to proceed at 37 °C and stopped by the addition of 100% formamide containing traces of xylene cyanol and bromphenol blue. RNA fragments were resolved on a 12% denaturing polyacrylamide gel and visualized by phosphorimaging. Pre-steady-state (single turnover) RNase H reactions were conducted using a Kin-Tek RQF-3 rapid quench-flow apparatus. RNA/DNA hybrids were prepared as described above to a final concentration of 100 nM and incubated with 1000 nM RT in a buffer containing 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 500 μM EDTA and 6 mM MgCl₂, in the presence or absence of CPHM or PFA (50 μM). Primary and subsequent secondary RNase H cleavage events were monitored under the same conditions with 5'-radiolabeled chimeric RNA-DNA (PBS14r8d), heat-annealed to a 3-fold excess of DNA primer (PBS-22D). Increasing concentrations of CPHM or the RNase H inhibitor β-thujaplicinol (0, 0.8, 1.6, 3.2, 6.4, 12.5, 25, 50, and 100 μM) were added to the reaction mixture to assess RNase H inhibition.

Molecular Modeling—Molecular models were generated using the crystal structure of the post-translocated ternary complex HIV-1 RT/chain-terminated hybrid/dTTP (Protein Data Bank code 1RTD) (25). The p66 subunit of HIV-1 RT, the chain-terminated hybrid, the two magnesium ions located at the polymerase active site, and dTTP were retained, and all other components were removed from the Protein Data Bank file. The β- and γ-phosphates of dTTP, as well as one of the oxygen atoms of the α-phosphate, were removed to generate a pseudo-pre-translocated complex. The structures of PFA and CPHM were downloaded from PubChem and were energy-minimized for 100 cycles using Chimera (26). Protein and small molecular structures were prepared using AutoDockTools 1.5.4 (27); all hydrogens were added, and Gasteiger partial charges were assigned to the protein and the small molecules. Nonpolar hydrogens were merged with carbon atoms, and AD4 atom types were assigned. Protonation states for CPHM and PFA were assigned using AutoDock Tools 1.5.4.. Both nitrogen atoms in the hydrazone moiety were partially protonated during the simulation. The protein and the inhibitors were saved as PDBqt files. A charge of +2 was assigned to the magnesium ions. The grid box, of size 63 × 63 × 63, was centered at x = 48.666, y = 24.911, and z = 41.55. The spacing in the grid box was 0.375 Å. PFA and CPHM were docked to the polymerase active site using a Lamarckian genetic algorithm. The docking algorithm parameters included 50 genetic algorithm runs, a randomized initial population of 150 inhibitor conformations, a maximum number of energy evaluations of 2,500,000, a maximum number of generations of 27,000, a rate of gene mutation of 0.02, and a rate of crossover of 0.8. Docking poses were scored using the AutoDock (27) empirical free energy function. Results that displayed a position root-mean-squared deviation of less than 0.5 Å were grouped into clusters; the lowest energy pose for each inhibitor was selected from the collection of docked poses of the highest ranked cluster.

RESULTS

Assessment of Antiviral Activity of CPHM—Although CPHM was shown to affect strand transfer reactions of HIV-1 RT, its potential as an antiviral agent remained to be assessed. We found that CPHM did not inhibit HIV replication in multiple cycle infectivity assays at a concentration up to 50 μM nor did it exhibit any cytotoxicity. The control compounds efavirenz and indinavir exhibited IC₉₅ values of 23 and 34 nM when tested side-by-side with CPHM. The absence of antiviral effects could be due to a number of reasons related to restricted cellular uptake, compound stability, or limitations inherent to the particular mechanism of action. Here, we focused on detailed mechanistic studies with the goal to establish CPHM as a specific inhibitor of HIV-1 RT.

CPHM and PFA Induce Identical Pausing Patterns in DNA Synthesis by HIV-1 RT—To investigate the mechanism by which CPHM inhibits RT-mediated DNA synthesis, we initially examined the pattern of inhibition of CPHM and PFA during multiple nucleotide incorporation events (Fig. 1). The P/T system used in this experiment is derived from the PPT of the
HIV-1 genome (28). The pausing patterns associated with CPHM and PFA, respectively, are identical in that inhibition of DNA synthesis occurs predominantly at positions +3 and +16. The two “hot spots” disappear with time, which shows that inhibition is reversible. Previous footprinting experiments revealed that P/T substrates corresponding to positions +3 and +16 shift the translocational equilibrium toward pre-translocation (11). Thus, these findings provide evidence to suggest that the mechanisms of action associated with the two inhibitors are highly related.

**CPHM and PFA Trap the Pre-translocated Complex of HIV-1 RT—** We subsequently used site-specific footprinting experiments to confirm that CPHM and PFA can stabilize the pre-translocated state of RT, thereby shifting the translocational equilibrium of RT at a given position to favor the pre-translocated state. Fe\(^{2+}\) ions generate hydroxyl radicals in solution, which will selectively cleave the template at positions −17 or −18. A major cut at position −17 is indicative for the post-translocated complex, whereas a cut at position −18 corresponds to the pre-translocated complex. Fe\(^{2+}\) footprinting was performed using a primer-template system that biases RT to favor the post-translocated complex (Fig. 2). In the presence of increasing concentrations of either CPHM or PFA, the translocational equilibrium of WT RT is shifted to the pre-translocated complex, as shown by the change in the pattern of DNA cleavage products. These results are consistent with the RT pausing pattern and support the notion that CPHM and PFA act as inhibitors of the pre-translocated complex.

**Binding of CPHM Requires Catalytic Metal Ions—** Despite possible differences in binding of the two compounds, their negatively charged carboxylate and/or phosphonate moieties may interact with the bound divalent metal ions at the polymerase active site. Binary RT-P/T complexes are unstable when challenged with an enzyme trap, such as heparin. However, the complex can be stabilized in the presence of PFA, provided that P/T sequence shows a bias toward pre-translocation (11). Here, we utilized a PPT-derived substrate that mimics inhibition at position +3 and monitored complex formation through use of EMSA (Fig. 3). The EMSA was performed in the presence and absence of MgCl\(_2\) to establish whether or not catalytic metal ions are required for inhibitor binding. The data show that complex formation increases in the presence of increasing concentrations of either CPHM or PFA, provided that MgCl\(_2\) was added to the reaction. Hence, binding of both CPHM and PFA depends on the presence of Mg\(^{2+}\), suggesting that CPHM, like PFA, also binds to the polymerase active site.
In an attempt to provide more detailed information on the binding site, we studied the effects of selected mutant enzymes on ternary complex formation monitored through EMSA (Table 1). In these studies we included mutants with changes of the three catalytic aspartic acid residues (D110N, D185N, and D186N). We also included two mutant enzymes with amino acid changes K65R and E89K that are known to confer resistance to PFA (29–31). Lys-65 is located in the vicinity of the phosphate moiety of an incoming nucleotide, whereas Glu-89 is found in close proximity to the template (25). Thus, both mutations contribute to the PFA-resistant phenotype through different mechanisms. K65R can potentially affect binding of PFA, whereas E89K was shown to disturb the translocational equilibrium of HIV-1 RT thereby diminishing access to the pre-translocated complex (11). Although D110N and D186N show subtle effects on complex formation, the D185N mutant shows

Inhibition of HIV-1 RT Translocation

| Enzyme/condition | Inhibitor | $K_d$ (app)$^{a}$ | FC$^{b}$ |
|------------------|-----------|-------------------|---------|
| WT/* MgCl$_2$    | PFA       | 0.064 ± 0.013     | 1       |
| WT/* − MgCl$_2$  | PFA       | >200              | >50     |
| D110N            | PFA       | 0.10 ± 0.019      | 1.6     |
| D185N            | PFA       | >200              | >50     |
| D186N            | PFA       | 0.20 ± 0.037      | 3.1     |
| K65R             | PFA       | 0.46 ± 0.12       | 7.2     |
| E89K             | PFA       | >200              | >50     |
| WT/* MgCl$_2$    | CPHM      | 3.2 ± 0.64        | 1       |
| WT/* − MgCl$_2$  | CPHM      | >200              | >50     |
| D110N            | CPHM      | 7.1 ± 0.90        | 2.2     |
| D185N            | CPHM      | >200              | >50     |
| D186N            | CPHM      | 6.0 ± 1.0         | 1.9     |
| K65R             | CPHM      | 1.1 ± 0.25        | 0.34    |
| E89K             | CPHM      | >200              | >50     |

$^{a}$ Each reported apparent $K_d$ value is the result of three separate experiments, ± S.E.
$^{b}$ The fold change (FC) in $K_d$ value is obtained by dividing the $K_d$ for a given inhibitor and specific enzyme/condition by the $K_d$ for WT RT for that same inhibitor.

![FIGURE 4. Effect of PFA and CPHM on primary RNase H cleavages. A, inhibition of RNase H activity under steady-state conditions. Time course reaction (0–32 min) was in the absence and presence of PFA (100 μM) and CPHM (100 μM). RNase H cleavages at positions −18 and −19 are marked post- and pre-translocation, respectively. B, time course experiments (0.05–30 s) under pre-steady-state conditions in the presence and absence of CPHM (50 μM) and PFA (50 μM). C, graphs of data shown in A and B with no inhibitor (black circle), PFA (open square), and CPHM (black triangle).]
marked increases in \( K_d \) values when compared with WT HIV-1 RT (>200 \( \mu M \) versus 0.06 \( \mu M \)). This suggests that Asp-185 plays a critical role in the binding of both compounds to RT, possibly through its coordination of the \( Mg^{2+} \) ions in the polymerase active site. This is in agreement with previously published data suggesting the importance of Asp-185 in the context of CPHM and PFA activity (32). We then proceeded to examine the effect of the E89K and K65R mutations on CPHM and PFA binding. Ternary complex formation is not observed with the E89K mutation, whereas the K65R shows differential effects that depend on the nature of the compound. The \( K_d \) value for PFA is 7.2-fold higher than WT, and the \( K_d \) value for CPHM is 0.3-fold that of the WT HIV-1 RT value. Thus, K65R decreases binding of PFA and increases binding of CPHM.

**Effect of CPHM on the RNase H Activity of HIV-1 RT**—We have previously shown that PFA decreases RNase H degradation indirectly by stabilizing the RT-P/T complex, which diminishes the turnover of the reaction (33). Here, we included CPHM in these studies, and we measured RNase H activity under both multiple and single turnover conditions (Fig. 4). In the absence of inhibitor, two major cleavage fragments are produced by RNase H degradation of a 3'-radiolabeled template, corresponding to the post-translocated state of RT (−18) and the pre-translocated state of RT (−19) (33). Inhibition of RNase H activity with either PFA or CPHM is solely observed under...
Inhibition of HIV-1 RT Translocation

Multiple turnover conditions, i.e., steady-state conditions. However, no inhibitory effects are seen when CPHM or PFA are added to a preformed RT-P/T complex under single turnover conditions. The single cleavage product at position 19 is indicative for the pre-translocated state. In agreement with our previously published data on PFA, we propose that binding of either CPHM or PFA to the pre-translocated complex of RT diminishes the turnover. However, there is no evidence to suggest that these compounds can act as bona fide RNase H active site inhibitors.

To address this question in greater detail, we used a chimeric DNA-RNA/DNA primer-template substrate that allowed us to monitor primary and secondary RNase H cleavages. The latter were shown to be sensitive to inhibition by the RNase H active site inhibitor β-thujaplicinol (32). In this experiment, primary RNase H cleavage is seen 1 bp downstream of the DNA-RNA junction, followed by downstream secondary cleavages. The addition of CPHM in a dose-dependent manner did not affect RNase H degradation (Fig. 5), which provides strong evidence to show that this compound is indeed not a bona fide RNase H inhibitor. In contrast, secondary cleavages were efficiently inhibited in the presence of β-thujaplicinol, as described previously (32).

**CPHM Does Not Inhibit the UL54 Polymerase of HCMV**—To investigate the specificity of CPHM for HIV-1 RT, we performed DNA synthesis inhibition assays using both HIV-1 RT and the UL54 polymerase of HCMV (Fig. 6). Inhibition of DNA synthesis by PFA is evident with both polymerases; this lack of specificity is characteristic of PFA that inhibits several viral DNA polymerases. However, although CPHM inhibits DNA synthesis by HIV-1 RT, no inhibition of DNA synthesis by the HCMV UL54 enzyme is seen. This suggests that structural differences in the architecture of the polymerase active sites of these two enzymes can determine specificity for CPHM.

**Structure-Activity Relationship Analysis of CPHM Derivatives Revealed Important Chemical Motifs for Inhibitor Activity**—In attempts to further characterize the specific binding site for the inhibitor, we synthesized a panel of derivatives of CPHM and tested their abilities to inhibit DNA synthesis by HIV-1 RT (Table 2 and Fig. 7). Representative CPHM derivatives were tested in the gel-based assay (compounds 3–6) (Fig. 7) to assess the importance of the dicarboxylate anchor motif and the aromatic ring of the putative specificity domain. To further probe the necessity of having a dicarboxylate moiety for inhibitory activity, we initially tested derivatives, which contained di-acetyl (compound 3) (Fig. 6), mono-ethyl ester, mono-carboxylate (compound 29) (Table 2), and di-ethyl ester (compound 30) (Table 2) functionalities. None of these compounds displayed inhibition of DNA synthesis, confirming that the dicarboxylate moiety of CPHM is essential for inhibitor activity. In addition, we investigated the effect of replacing the chloro-group in the para-position of the phenyl ring (compounds 4 and 6–15) (Table 2) (compounds 4 and 6) (Fig. 6). Of the compounds tested from Table 2, only the p-bromo and p-methyl derivatives retained comparable activity to CPHM; the others were less active or inactive. Compounds 4 and 6 from Fig. 6 caused very weak pausing at position 16 when compared with CPHM. Substitution at position R1 (compound 28)
abolishes inhibitor activity. These data demonstrate that the chlorophenylhydrazone moiety is a requirement for inhibitor activity and that even the removal of the chlorine from the inhibitor results in a dramatic decrease in compound activity. We subsequently derivatized the phenyl ring of CPHM with methyl groups to probe the binding site of the inhibitor. The \(m\)-methyl (compound 5) (Table 2) (compound 5) (Fig. 7) and \(o\)-methyl (compound 24) (Table 2) derivatives of CPHM were tested for DNA synthesis inhibition. Although compound 24 showed weaker inhibitory activity than CPHM, compound 5 showed stronger activity than the parent compound, as evidenced by stronger pausing at positions +3 and +16 (compound 5) (Fig. 7B). This suggests that even subtle changes to the phenyl ring can influence the inhibitory activity of the compound. Finally, we examined \(p\)-cyano (compound 9) (Table 2) and \(p\)-nitro (compound 6) (Table 2) (compound 6) (Fig. 7) derivatives of CPHM to establish the requirement of a chloro-group in the \(para\)-position for inhibitor activity. Both of these compounds had less activity in our assays, suggesting that although derivatization at the \(para\)-position is essential for compound activity, unfavorable interactions may arise when larger groups are introduced at this position.

**In Silico Docking Revealed Differences in CPHM and PFA Binding**—To gain structural insight into the binding modes of CPHM and PFA to HIV-1 RT, we constructed molecular models to illustrate stabilization of the pre-translocated complex of RT (Fig. 8). Using the crystal structure of the ternary complex composed of a chain-terminated primer-template and a bound dTTP (Protein Data Bank code 1RTD), we generated a pseudo pre-translocated complex by removing the \(\beta\)- and \(\gamma\)-phos-
Inhibition of HIV-1 RT Translocation

**FIGURE 8. Molecular models of PFA and CPHM binding to the polymerase active site of HIV-1 RT.** The different binding orientations of docked PFA and CPHM are highlighted by their proximity to the two active site magnesium ions (red spheres) and Lys-65. The “primer terminus” of the pseudo pre-translocated complex generated for the docking simulation (see under “Experimental Procedures”) is shown with an arrow.

molecules are likewise under pre-clinical investigation. PFA is the only known non-nucleosidic compound that was shown to trap the post-translocated complex (39–46). These compounds interact with the Mg$^{2+}$ ion distal from the primer terminus through their carboxylates. This may explain why Asp-185 is essential for binding of both CPHM and PFA.

**DISCUSSION**

RT translocation is an underexplored target. Like the natural dNTP substrate, NRTIs bind to the nucleotide-binding site that is accessible in the post-translocated state. The translocation equilibrium is re-established following NRTI incorporation and may show a bias toward post-translocational states, depending on the structure of the inhibitor. Recent studies have shown that the investigational NRTI 4’-ethynyl-2-fluoro-2’-deoxyadenosine causes a shift toward pre-translocation (34). Although this compound contains a 3’-hydroxyl group, DNA synthesis is terminated because the next nucleotide substrate has no access to its designated binding site. 4’-Ethynyl-2-fluoro-2’-deoxyadenosine is therefore dubbed a translocation-defective RT inhibitor (TDRTI) (35–38). Nucleotide-competent RT inhibitors, such as INDOPY-1, can reversibly bind to and trap the post-translocated complex (39–46). These compounds are likewise under pre-clinical investigation. PFA is the only known non-nucleosidic compound that was shown to trap the pre-translocated complex of HIV-1 RT. Our data suggest that binding of the inhibitor is Mg$^{2+}$-dependent. Mutations at the polymerase active site result in the loss of inhibitor binding. D185N shows the strongest effect in this regard. In addition, we have shown that the compound cannot stabilize a ternary complex when the enzyme is situated at sequence positions that show a bias toward post-translocation. The E89K mutation, which results in resistance to the pyrophosphate analog PFA by diminishing the population of pre-translocated complexes, is in our enzymatic assays also less sensitive to CPHM; chemical footprinting and RNase H mapping experiments confirm that both PFA and CPHM can trap the pre-translocated complex of HIV-1 RT.

It is also important to note that CPHM does not directly target the RNase H activity of HIV-1 RT. Rather, inhibition of RNase H-mediated RNA cleavage is exclusively seen under steady-state conditions, because binding of PFA (33) or CPHM (this study) to the polymerase active site facilitates formation of a stable ternary complex, which in turn diminishes the turnover of the reaction. Under single turnover conditions, this inhibitory effect is not observed, and the RNase H cleavage pattern demonstrates that the pre-translocated complex is trapped. Together, these data provide compelling evidence to suggest that the inhibitor binds to the polymerase active site.

In *silico* docking studies are in good agreement with the experimental data. An important observation is the differential effect of the K65R mutation on sensitivity to PFA and CPHM, respectively. Although sensitivity to PFA is reduced in this mutational context, CPHM shows hypersusceptibility. This difference can be rationalized if one considers different binding modes of the two compounds. In our model, binding of PFA appears to be stabilized by Lys-65 through an electrostatic interaction between the negatively charged phosphate of PFA and the positively charged ε-amino group of the lysine side chain. The K65R mutation would result in a steric clash between the guanidinium of arginine and the phosphate of PFA. Conversely, CPHM does not appear to be located in the vicinity of Lys-65. The guanidinium of K65R would be too far away from CPHM to clash with the compound, and it may even stabilize binding of CPHM to the polymerase active site.

Interestingly, mutations at active site residues Asp-110 and Asp-186 did not result in dramatic losses of binding for PFA and CPHM, although mutation of Asp-185 precluded inhibitor binding in both cases. This may be accounted for by the spatial orientation of Asp-185 with respect to the binding orientations of PFA and CPHM to the polymerase active site. The plane in which the divalent metal ions are coordinated may be essential for the proper binding of PFA and CPHM to the enzyme. The D185N mutation might cause a complete loss of metal stabilization in the active site, which would in turn result in the loss of PFA and CPHM binding.

This study opens the possibility for developing novel inhibitors of related chemical classes. Indeed, the selective inhibition of HIV-1 RT by CPHM points to structural differences in the polymerase active sites of HIV-1 RT and HCMV UL54. CPHM hypersusceptibility in the context of the K65R mutation suggests that pre-translocational inhibitors can be designed to escape resistance pathways that are associated with NRTIs. Although our SAR studies have shown a number of chemical
constituents of CPHM, which are essential for inhibition of RT, we have demonstrated that derivatization of the phenyl ring may be an avenue to investigate for improved inhibitor potency. The absence of antiviral effects of CPHM could be due to the inability of the compound to permeate the cell membrane as it contains two negative charges, although this has not been directly evaluated in this study. A prodrug approach is likely required to address this problem. Further detailed SAR studies will be undertaken to dissect the chemical constituents required for inhibitor activity, as well as testing of activity-optimized compounds for toxicity and antiviral effects in cell culture.

Acknowledgments—We thank Dinesh Medpelli and Suzanne McCormick for excellent technical assistance.

REFERENCES

1. Das, K., and Arnold, E. (2013) HIV-1 reverse transcriptase and antiviral drug resistance. Part 1. Curr. Opin. Virol. 3, 111–118
2. Le Grice, S. F. (2012) Human immunodeficiency virus reverse transcriptase: 25 years of research, drug discovery, and promise. J. Biol. Chem. 287, 40850–40857
3. Götte, M. (2006) Effects of nucleotides and nucleotide analog inhibitors of HIV-1 reverse transcriptase in a ratchet model of polymerase translocation. Curr. Pharm. Des. 12, 1867–1877
4. De Clercq, E. (2005) Emerging anti-HIV drugs. Expert Opin. Emerg. Drugs 10, 241–273
5. Sarafianos, S. G., Marchand, B., Das, K., Himmel, D. M., Parniak, M. A., Hughes, S. H., and Arnold, E. (2009) Structure and function of HIV-1 reverse transcriptase: molecular mechanisms of polymerization and inhibition. J. Mol. Biol. 385, 693–713
6. Chowers, M., Gottesman, B. S., Leibovici, L., Schapiro, J. M., and Paul, M. (2010) Nucleoside reverse transcriptase inhibitors in combination therapy for HIV patients: systematic review and meta-analysis. Eur. J. Clin. Microbiol. Infect. Dis. 29, 779–786
7. Spence, R. A., Anderson, K. S., and Johnson, K. A. (1996) HIV-1 reverse transcriptase resistance to nonnucleoside inhibitors. Biochemistry 35, 1054–1063
8. Zhan, P., Chen, X., Li, D., Fang, Z., De Clercq, E., and Liu, X. (2013) HIV-1 NNRTIs: Structural diversity, pharmacophore similarity, and implications for drug design. Med. Res. Rev. 33, Suppl. 1, E1–E72
9. Oberg, B. (1982) Antiviral effects of phosphonoformate (PFA, fosarnet sodium). Pharmacol. Ther. 19, 387–415
10. Torres-Madriz, G., and Boucher, H. W. (2008) Immunocompromised hosts: perspectives in the treatment and prophylaxis of cytomegalovirus disease in solid-organ transplant recipients. Clin. Infect. Dis. 47, 702–711
11. Marchand, B., Tchesnokov, E. P., and Götte, M. (2007) The pyrophosphate analogue fosarnet traps the pre-translocational state of HIV-1 reverse transcriptase in a Brownian ratchet model of polymerase translocation. J. Biol. Chem. 282, 3357–3346
12. Meyer, P. R., Matsura, S. E., Zonarich, D., Chopra, R. R., Pendarvis, E., Bazmi, H. Z., Mellors, J. W., and Scott, W. A. (2003) Relationship between 3′-azido-3′-deoxythymidine resistance and primer unblocking activity of fosarnet-resistant mutants of human immunodeficiency virus type 1 reverse transcriptase. J. Virol. 77, 6127–6137
13. Crucchaga, C., Ansó, E., Rouzaut, A., and Martínez-Irujo, J. I. (2006) Selective excision of chain-terminating nucleotides by HIV-1 reverse transcriptase with phosphonoformate as substrate. J. Biol. Chem. 281, 27744–27752
14. Sarafianos, S. G., Clark, A. D., Jr., Das, K., Tuske, S., Birktoft, J. I., Iankumaran, P., Ramesha, A. R., Sayer, J. M., Jerina, D. M., Boyer, P. L., Hughes, S. H., and Arnold, E. (2002) Structures of HIV–1 reverse transcriptase with pre- and post-translocation AZTMP-terminated DNA. EMBO J. 21, 6614–6624
15. Sarafianos, S. G., Clark, A. D., Jr., Tuske, S., Squire, C. J., Das, K., Sheng, D., Iankumaran, P., Ramesha, A. R., Kroth, H., Sayer, J. M., Jerina, D. M., Boyer, P. L., Hughes, S. H., and Arnold, E. (2003) Trapping HIV-1 reverse transcriptase before and after translocation on DNA. J. Biol. Chem. 278, 16280–16288
16. Hsieh, J. C., Zinnen, S., and Modrich, P. (1993) Kinetic mechanism of the DNA-dependent DNA polymerase activity of human immunodeficiency virus reverse transcriptase. J. Biol. Chem. 268, 24607–24613
17. Kati, W. M., Johnson, K. A., Jerva, L. F., and Anderson, K. S. (1992) Mechanism and fidelity of HIV reverse transcriptase. J. Biol. Chem. 267, 25988–25997
18. Marchand, B., and Götte, M. (2003) Site-specific footprinting reveals differences in the translocation status of HIV-1 reverse transcriptase. Implications for polymerase translocation and drug resistance. J. Biol. Chem. 278, 35362–35372
19. Marchand, B., and Götte, M. (2004) Impact of the translocational equilibrium of HIV-1 reverse transcriptase on the efficiency of mismatch extensions and the excision of mispaired nucleotides. Int. J. Biochem. Cell Biol. 36, 1823–1835
20. Gabbara, S., Davis, W. R., Hupe, L., Hupe, D., and Peliska, J. A. (1999) Inhibitors of DNA strand transfer reactions catalyzed by HIV-1 reverse transcriptase. Biochemistry 38, 13070–13076
21. Davis, W. R., Tomsho, J., Nikam, S., Cook, E. M., Somand, D., and Peliska, J. A. (2000) Inhibition of HIV-1 reverse transcriptase-catalyzed DNA strand transfer reactions by 4-chlorophenylhydrazide of mesoxalic acid. Biochemistry 39, 14279–14291
22. Le Grice, S. F., and Grüninger-Leitch, F. (1990) Rapid purification of homodimer and heterodimer HIV-1 reverse transcriptase by metal chelate affinity chromatography. Eur. J. Biochem. 187, 307–314
23. Cihlar, T., Fuller, M. D., and Cherrington, J. M. (1998) Characterization of drug resistance–associated mutations in the human cytomegalovirus DNA polymerase gene by using recombinant mutant viruses generated from overlapping DNA fragments. J. Virol. 72, 5927–5936
24. Lai, M. T., Munshi, V., Touch, S., Tynedor, R. M., Tucker, T. J., McKenna, P. M., Williams, T. M., DiStefano, D. J., Hazuda, D. J., and Miller, M. D. (2009) Antiviral activity of MK-4969, a novel nonnucleoside reverse transcriptase inhibitor. Antimicrob. Agents Chemother. 53, 2424–2431
25. Huang, H., Chopra, R., Verdone, G. L., and Harrison, S. C. (1998) Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science 282, 1669–1675
26. Petersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612
27. Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., and Olson, A. J. (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J. Comput. Chem. 30, 2785–2791
28. Götte, M., Kameoka, M., McEllan, L., Cellai, L., and Wainberg, M. A. (2001) Analysis of efficiency and fidelity of HIV-1 –1’ strand DNA synthesis reveals a novel rate-limiting step during retroviral reverse transcription. J. Biol. Chem. 276, 6711–6719
29. Hammond, J. L., Koontz, D. L., Bazmi, H. Z., Beadle, J. R., Hostetler, S. E., Kini, G. D., Aldern, K. A., Richman, D. D., Hostetler, K. Y., and Mellors, J. W. (2001) Alkylglycerol prodrugs of phosphonoformate are potent in vitro inhibitors of nucleoside-resistant human immunodeficiency virus type 1 and select for resistance mutations that suppress zidovudine resistance. Antimicrob. Agents Chemother. 45, 1621–1628
30. Sluis-Cremer, N., Arion, D., Kaushik, N., Lim, H., and Parniak, M. A. (2000) Mutational analysis of Lys65 of HIV-1 reverse transcriptase. Biochem. J. 348, 77–82
31. Tachedjian, G., Hooker, D. J., Gurusinhe, A. D., Bazmi, H., Deacon, N. J., Mellors, J., Birch, C., and Mills, I. (1995) Characterisation of fosarnet-resistant strains of human immunodeficiency virus type 1. Virology 212, 58–68
32. Shaw-Reid, C. A., Munshi, V., Graham, P., Wolfe, A., Witmer, M., Danzeisen, R., Olsen, D. B., Carroll, S. S., Embrey, M., Wai, J. S., Miller, M. D., Cole, J. L., and Hazuda, D. J. (2003) Inhibition of HIV-1 ribonucleoside H by
Inhibition of HIV-1 RT Translocation

a novel diketo acid, 4-[[5-(benzoylarnino)thien-2-yl]-2,4-dioxobutanoic acid. J. Biol. Chem. 278, 2777–2780

33. Beilhardt, G. L., Wendeler, M., Baichoo, N., Rausch, J., Le Grice, S., and Götte, M. (2009) HIV-1 reverse transcriptase can simultaneously engage its DNA/RNA substrate at both DNA polymerase and RNase H active sites: implications for RNase H inhibition. J. Mol. Biol. 388, 462–474

34. Michailidis, E., Marchand, B., Kodama, E. N., Singh, K., Matsuoka, M., Kirby, K. A., Ryan, E. M., Ryan, E. M., Hachiya, A., Kirby, K. A., Marchand, B., Leslie, E. W. (2009) Mechanism of inhibition of HIV-1 reverse transcriptase by 4’-ethynyl-2-fluoro-2’-deoxyadenosine triphosphate, a translocation-defective reverse transcriptase inhibitor. J. Biol. Chem. 284, 35681–35691

35. Kawamoto, A., Kodama, E., Sarafianos, S. G., Sakagami, Y., Kohgo, S., Kitano, K., Ashida, N., Iwai, Y., Hayakawa, H., Nakata, H., Mitsuya, H., Arnold, E., and Matsuoka, M. (2008) 2’-Deoxy-4’-C-ethyl-2-halo-adenosine active against drug-resistant human immunodeficiency virus type 1 variants. Int. J. Biochem. Cell Biol. 40, 2410–2420

36. Kirby, K. A., Singh, K., Michailidis, E., Marchand, B., Kodama, E. N., Ashida, N., Mitsuya, H., Parniak, M. A., and Sarafianos, S. G. (2011) The sugar ring conformation of 4’-ethynyl-2-fluoro-2’-deoxyadenosine and its recognition by the polymerase active site of HIV reverse transcriptase. Cell. Mol. Biol. 57, 40–46

37. Michailidis, E., Singh, K., Ryan, E. M., Hachiya, A., Ong, Y. T., Kirby, K. A., Marchand, B., Kodama, E. N., Mitsuya, H., Parniak, M. A., and Sarafianos, S. G. (2012) Effect of translocation defective reverse transcriptase inhibitors on the activity of N348I, a connection subdomain drug resistant HIV-1 reverse transcriptase mutant. Cell. Mol. Biol. 58, 187–195

38. Michailidis, E., Ryan, E. M., Hachiya, A., Kirby, K. A., Marchand, B., Leslie, M. D., Huber, A. D., Ong, Y. T., Jackson, J. C., Singh, K., Kodama, E. N., Mitsuya, H., Parniak, M. A., and Sarafianos, S. G. (2013) Hypersusceptibility mechanism of Tenofovir-resistant HIV to EFdA. Retrovirology 10, 65

39. Jochmans, D., Deval, J., Kesteleyn, B., Van Marck, H., Bettens, E., De Baere, I., Dehertogh, P., Ivens, T., Van Ginderen, M., Van Schoubroeck, B., Ehteshami, M., Wigerinck, P., Götte, M., and Hertogs, K. (2006) Indolopyridones inhibit human immunodeficiency virus reverse transcriptase with a novel mechanism of action. J. Virol. 80, 12283–12292

40. Zhang, Z., Walker, M., Xu, W., Shim, J. H., Girardet, J. L., Hamatake, R. K., and Hong, Z. (2006) Novel nonnucleoside inhibitors that select nucleoside inhibitor resistance mutations in human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. 50, 2772–2781

41. Ehteshami, M., Scarth, B. J., Tchesnovok, E. P., Dash, C., Le Grice, S. F., Hallenberger, S., Jochmans, D., and Götte, M. (2008) Mutations M184V and Y115F in HIV-1 reverse transcriptase discriminate against “nucleotide-competing reverse transcriptase inhibitors.” J. Biol. Chem. 283, 29904–29911

42. Ehteshami, M., Nijhuis, M., Bernatchez, J. A., Ablenas, C. I., McCormick, S., de Jong, D., Jochmans, D., and Götte, M. (2013) Formation of a quaternary complex of HIV-1 reverse transcriptase with a nucleotide-competing inhibitor and its ATP enhancer. J. Biol. Chem. 288, 17336–17346

43. Rajotte, D., Tremblay, S., Pelletier, A., Salois, P., Bourgon, L., Coulombe, R., Mason, S., Lamorte, L., Sturino, C. F., and Bethell, R. (2013) Identification and characterization of a novel HIV-1 nucleotide-competing reverse transcriptase inhibitor series. Antimicrob. Agents Chemother. 57, 2712–2718

44. Maga, G., Radi, M., Zanoli, S., Manetti, F., Cancio, R., Hübscher, U., Spadari, S., Falciani, C., Terrazas, M., Vilarasa, J., and Botta, M. (2007) Discovery of non-nucleoside inhibitors of HIV-1 reverse transcriptase competing with the nucleotide substrate. Angewandte Chemie 46, 1810–1813

45. Radi, M., Falciani, C., Contemori, L., Petricci, E., Maga, G., Samuele, A., Zanoli, S., Terrazas, M., Castria, M., Togninelli, A., Esté, J. A., Clotet-Codina, I., Armand-Ugón, M., and Botta, M. (2008) A multidisciplinary approach for the identification of novel HIV-1 non-nucleoside reverse transcriptase inhibitors: S-DABOCs and DAVPs. ChemMedChem 3, 573–582

46. Freisz, S., Bec, G., Radi, M., Wolff, P., Crespan, E., Angeli, L., Dumas, P., Maga, G., Botta, M., and Ennifar, E. (2010) Crystal structure of HIV-1 reverse transcriptase bound to a non-nucleoside inhibitor with a novel mechanism of action. Angewandte Chemie 49, 1805–1808

47. Zahn, K. E., Tchesnovok, E. P., Götte, M., and Doublié, S. (2011) Phosphonoformic acid inhibits viral replication by trapping the closed form of the DNA polymerase. J. Biol. Chem. 286, 25246–25255