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Differential Inhibition of Polymerase and Strand-Transfer Activities of HIV-1 Reverse Transcriptase

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Abstract—A new class of inhibitors of HIV-1 reverse transcriptase obtained by the systematic structural simplification of epicatechin and epigallocatechin gallates are also shown here to inhibit DNA-strand-transfer, a process critical to the completion of the HIV-1-RT reproduction and to recombination-associated mutation of the virus. Up to 80-fold selectivity for DNA-strand-transfer inhibition over polymerase inhibition was observed for a defined subset of these agents. Such specific DNA-strand-transfer inhibitors may have important therapeutic potential.

The ease with which HIV overcomes the immune system is thought to be due primarily to its extraordinary mutation rate, which is presumed to facilitate production of escape mutants.[1] Ultimately, the genetic variability of the virus exceeds that of the host's immune system. The exact way in which the immune system and the virus interact is, however, complex, and the course of viral replication and mutation also depends on the genetic capacity of the host immune system.[2,3] The capacity of HIV to mutate during replication is thought to be due to two primary mechanisms: (A) the high error rate associated with the lack of exonuclease excision of mismatched bases during the course of polymerization, and (B) recombination-associated mutation mediated through a DNA-strand-transfer process that involves DNA–RNA intermediates in the HIV-1-RT reaction.[4–8] The high error rate of purified HIV-1-RT alone may not accurately reflect the level of genetic variation in a natural infection.[9] It has been suggested that DNA-strand-transfer associated recombination contributes to the prodigious rate of HIV genome mutation during active infection.[4,5]

Recently, it has been shown that base misincorporation during reverse transcription promotes DNA-strand-transfer.[10] Base misincorporations provide strong pause sites in polymerization. DNA-strand-transfer is enhanced at those sites.[11] Incorporation of non-template-encoded nucleotides was reported to occur at the 3′ end of the polymer during DNA-strand-transfer leading to the hypermutation of the HIV-1 genome.[12–14] Furthermore, breaks in the RNA strands have also been shown to cause polymerase pausing and induction of DNA-strand-transfer associated point mutations.[15,16]

A few specific inhibitors of the DNA-strand-transfer reaction have been reported recently. One of these agents, actinomycin D, has been shown to interfere with translocation of the DNA intermediate to the acceptor template.[17] A second class, exemplified by the chlorophenylhydrazone derivative of mesoxalic acid inhibited the RNase H activity of reverse transcriptase.[18,19] Recently, we described the irreversible inhibition of DNA-strand-transfer by a novel group of inhibitors thought to act through a quinone methide inactivation process.[20] Discovery of new DNA-strand-transfer inhibitors, while providing new tools for studying the mechanism of the process, could also be used in new anti-HIV therapeutic strategies.

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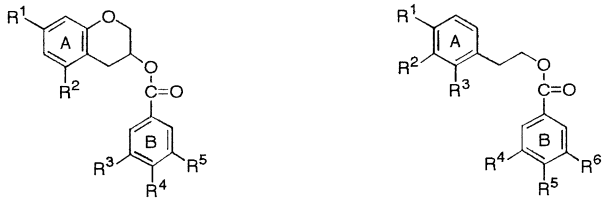
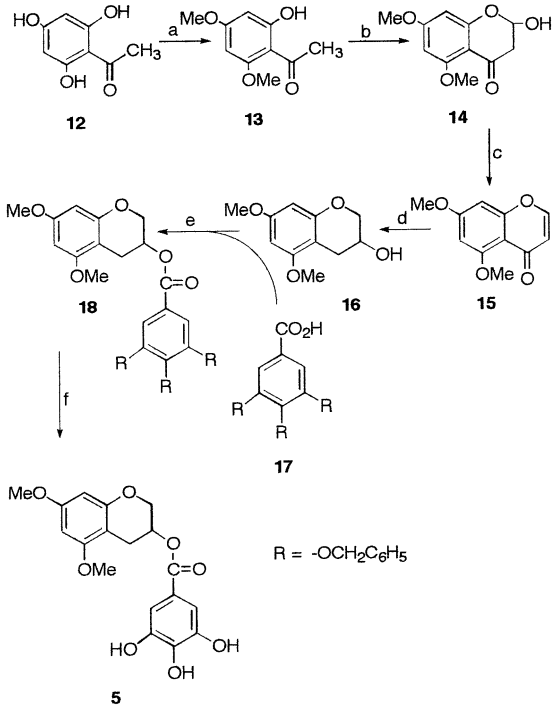


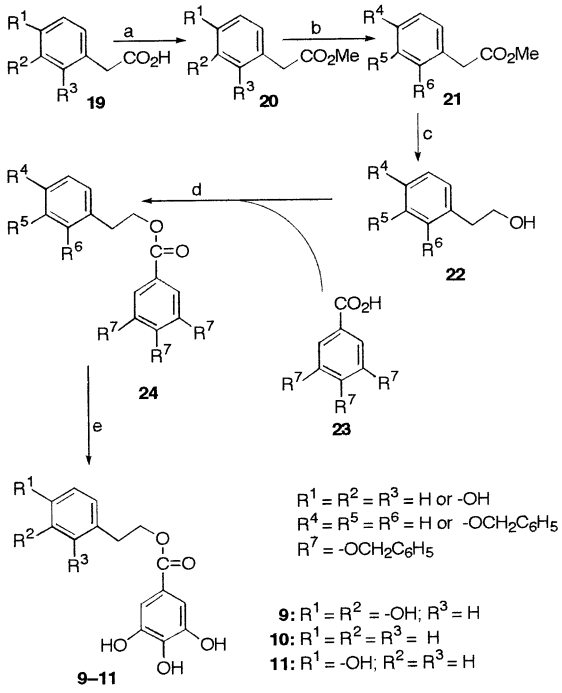
1: $R^1 = R^2 = R^3 = R^4 = R^5 = -OH$
2: $R^1 = H$; $R^2 = R^3 = R^4 = R^5 = -OH$
3: $R^1 = R^3 = R^4 = R^5 = -OH$; $R^2 = H$
4: $R^1 = R^2 = H$; $R^3 = R^4 = R^5 = -OH$
5: $R^1 = R^2 = -OMe$; $R^3 = R^4 = R^5 = -OH$
6: $R^1 = R^2 = -OH$; $R^3 = R^4 = R^5 = H$
7: $R^1 = R^2 = R^3 = R^4 = -OH$; $R^5 = H$
8: $R^1 = R^2 = R^3 = R^5 = -OH$; $R^4 = H$
9: $R^1 = R^2 = R^4 = R^5 = R^6 = -OH$; $R^3 = H$
10: $R^1 = R^2 = R^3 = H$; $R^4 = R^5 = R^6 = -OH$
11: $R^1 = R^4 = R^5 = R^6 = -OH$; $R^2 = R^3 = H$

Figure 1. Structures of inhibitor molecules synthesized.

We recently reported a new class of inhibitors of HIV-1-RT obtained by the systematic simplification of the structures of epicatechin and epigallocatechin gallates.[21,22] These compounds inhibited the native as well as the A17 double mutant (K103N Y181C) forms of the enzyme. By further structural optimization we have defined some of the structural requirements for a unique DNA-strand-transfer inhibitor where we observed up to 80-fold selectivity for DNA-strand-transfer inhibition versus polymerase inhibition. We synthesized compounds **1**–**11** (Fig. 1) and studied and compared their polymerase and DNA-strand-transfer inhibitory activities. The syntheses of the compounds **1**–**4** and **6**–**8** were described previously.[22] Compound **5** was synthesized as shown in Scheme 1. The starting material **13** was obtained by the selective *O*-methylation of **12** with diazomethane. Compound **13** was then reacted with ethyl formate and sodium to obtain the hemiacetal **14**, acid-catalyzed dehydration of which gave **15**.[23] Diborane reduction of **15** gave the chromanol **16**,[24,25] which was esterified with **17** in the presence of DCC and DMAP in CH_2Cl_2 to obtain **18**.[26] The benzyl protecting groups were removed by hydrogenolysis to obtain **5**. Compounds **9**–**11** were prepared by esterifying 2-phenylethyl alcohol derivatives **22** with 3,4,5-tribenzyloxybenzoic acid **23**, followed by the debenzylation of the protecting groups by hydrogenlysis (Scheme 2). All compounds were characterized by ^1H NMR and ^{13}C NMR spectroscopy and by elemental analysis.[27]



Scheme 1. Synthesis of compound **5**. Reagents and conditions: (a) CH_2N_2, ether; (b) HCO_2Et, Na; (c) H^+ ($-H_2O$); (d) BH_3, $NaOH/H_2O$; (e) DCC, DMAP, CH_2Cl_2; (f) $H_2/Pd/C$.



Scheme 2. Synthesis of compounds **9**–**11**. Reagents and conditions: (a) $SOCl_2/MeOH$; (b) benzyl chloride, K_2CO_3, DMF; (c) $LiBH_4$, THF; (d) DCC, DMAP, CH_2Cl_2; (e) Pd/C, H_2.

The HIV-1-RT polymerization assay was carried out as described earlier.[22] The purified, wild-type and A17 mutant HIV-1-RT enzymes used in both polymerase and DNA-strand-transfer assays were p66/51 heterodimers and were also prepared as described earlier.[22] We used the procedure of Peliska and Benkovic converted into a Scintillation Proximity Assay (SPA) based on biotin/streptavidine bead capture, to facilitate the rapid study of a large number of samples (Fig. 2).[12,17,18] The oligonucleotides used were prepared by Pfizer Global Research and Development (Ann Arbor, MI) and purified as described elsewhere.[18] Their sequences were: DNA primer, 5′ Biotin-GCATCTGGGGCTCG-CAAATTTG-3′; RNA template, 5′-AGGUGAGUGA-GAUGAUAACAAAUUUGCGAGCCCCAGAUGC-3′; DNA acceptor template, 5′-CCCCCCCCCCCC-CCAGGTGAGTGAGATGATAACA-3′. The 22-oligomeric DNA primer containing biotin at its 5′-end rapidly gave an immediate 40-oligomeric DNA product in reverse transcription on the 40-oligomeric RNA

template. In the presence of the 35-oligomeric DNA acceptor designed to hybridize with the terminus of the 40-oligomer DNA, DNA-strand-transfer and extension to a 55-oligomer occurred at a slower pace. The final extension to a 55-oligomer resulted in incorporation of labeled nucleotide into the product which had biotin attached. DNA-strand-transfer is rate-limiting in the overall process. The rate of DNA-strand-transfer is measured as the rate of labeled nucleotide incorporation. On addition of streptavidin SPA beads, which also contained a scintillant, the biotin tagged product bound to the beads. The incorporated tritiated nucleotides stimulated the scintillant to produce a signal which was generated only after successful reverse transcription, coupled RNase H hydrolysis of initial template RNA, DNA-strand-transfer and final extension. The reactions were performed in 96-well plates. The reaction mixture (final volume 100 μL) contained 9.6 nM biotinylated DNA primer, 32 nM RNA primary template, 96 nM DNA acceptor template, dATP, dTTP, and dCTP (each at 600 nM), 500 nM [^{3}H]dGTP (specific activity = ~10 Ci/mmol), reaction buffer [50 mM Tris–HCl (pH 8.0), 80 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5% w/v Nonident P-40], and varying concentrations of the test compounds dissolved in 2 μL of DMSO. Reaction was initiated by the addition of 10 μL of 100 nM enzyme solution (10 nM final concentration). After incubation at 37 °C for 1 h, the reaction was quenched by the addition of 40 μL of 0.5 M EDTA (pH 8.0). SPA beads (Amersham RPNQ 0006, 0.2 mg in 20 μL of 1×Dulbecco's phosphate buffered saline) were added and the mixture was incubated at 37 °C for 10 min and after adding 150 μL of 1×TBE buffer, the radioactivity was counted in a 96-well scintillation counter.

Table 1 shows the IC_{50}s for polymerization and DNA-strand-transfer processes of both wild-type and A17 mutant enzyme for the inhibitors reported. Earlier compounds of the series (**1**–**3**), with all three hydroxyl groups on ring B (gallic acid moiety) as well as at least one hydroxyl group on ring A (chromanol moiety) were found to inhibit HIV-1-RT polymerization as well as the DNA-strand-transfer process. Inhibition of DNA-strand-transfer was of the same order of magnitude as polymerase inhibition for most of these compounds. However, the observed variation in IC_{50}s for polymerase and DNA-strand-transfer in some of these compounds suggested that inhibitors could be separately directed to the two sites. Substantial separation of polymerase and DNA-strand-transfer inhibition was achieved with compounds **4** and **5** for both wild-type and mutant enzyme A17. It was evident that the presence of polar hydroxyl groups on the aromatic ring of the chromanol moiety enhanced polymerase inhibition. Complete removal of these hydroxyl groups or their conversion to less polar methyl ether functions resulted in a 10- to 80-fold selectivity for DNA-strand-transfer inhibition over polymerase inhibition. Removal of one

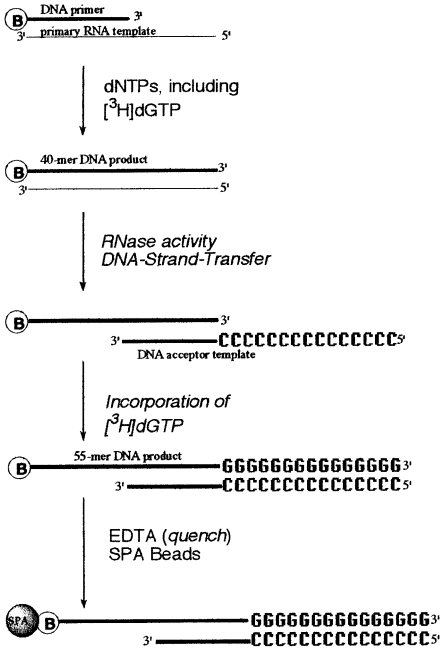


Figure 2. DNA-strand-transfer assay. A 22-mer DNA primer biotinylated at 5′ end and annealed to a 40-mer primary RNA template was used. The 5′-end of the RNA template lacked cytosine to avoid incorporation of [^{3}H]dGPT in pre-strand-transfer polymerization. The 3′-end sequence of the acceptor template is designed to hybridize with the 3′-end of the 40-mer DNA and has a polycytosine sequence at its 5′-end. Post strand-transfer polymerization involves incorporation of [^{3}H]dGTP. The reaction was quenched with EDTA and the DNA products were captured with streptavidin-coated SPA beads containing a scintillant. [^{3}H]dGTP incorporated in to DNA stimulates the scintillant to produce a signal, while [^{3}H]dGTP not physically associated with the beads produces no signal.

Table 1. IC_{50} values for polymerization and strand transfer inhibition

Compd	IC_{50} (μM) polymerization		IC_{50} (μM) strand transfer	
	Wild-type	A17 double mutant	Wild-type	A17 double mutant
1	3.8±0.4	2.4±1.2	6.2±0.8	5.5±0.4
2	45.5±9.7	64.7±15.0	8.7±0.9	ND
3	36.3±7.7	30.8±8.4	7.6±0.6	ND
4	>100	>100	6.1±1.7	2.6±0.9
5	72.8±9.6	>100	0.9±0.2	6.9±1.5
6	>100	>100	>100	>100
7	37.2±10.8	>100	>100	>100
8	96.3±25.9	ND	ND	ND
9	10.7±1.7	6.3±1.5	0.6±0.1	10.5±1.9
10	72.9±25.4	ND	6.9±0.9	ND
11	7.9±0.9	ND	4.5±0.4	ND

ND, not determined.

or more of the hydroxyl groups on the gallic acid moiety (**6**–**8**) led to loss of polymerase as well as DNA-strand-transfer inhibitory activity.

We previously reported that analogues in which the tether between the two aromatic rings is acyclic retained polymerase inhibitory activity.[22] This condition prevailed for DNA-strand-transfer inhibitory activity as well, as exemplified by compound **10**. Compounds **9** and **11**, with one or more hydroxyl groups in the aromatic ring A, were active in both assays and were not able to differentiate between the two processes. It is of interest that the DNA-strand-transfer inhibition differs by 8- to 18-fold for the mutant versus the wild-type HIV-1-RT for some of these selective DNA-strand-transfer inhibitors. However, it is difficult to assess the basis for this difference until more is known about the site of the enzyme to which these agents are directed. Further experiments to identify the enzyme sites that mediate the DNA-strand-transfer process, and to determine the selectivity (toxicity/antiviral activity) of the DNA-strand-transfer agents by cell culture studies are planned.

Acknowledgements

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27. Physical data for test compounds: For compounds **1**–**4** and **6**–**9** see ref 22.
3,4-Dihydro-5,7-dimethoxy-2*H*-1-benzopyran-3-yl 3,4,5-trihydroxybenzoate (**5**). Overall yield 6%; mp 205 °C; ^{1}H NMR (300 MHz, acetone-d_6) 2.76 (dd, 1H, J = 17.06, 1.8 Hz), 2.93 (dd, 1H, J = 17.06, 4.93 Hz), 3.74 (s, 3H), 3.78 (s, 3H), 4.16 (d, 1H, J = 12.33 Hz), 4.27 (m, 1H), 5.37 (brm, 1H), 6.03 (d, 1H, J = 2.29 Hz), 6.13 (d, 1H, J = 2.29 Hz), 7.05 (s, 2H); ^{13}C NMR (acetone-d_6) 25.4 (C-4), 55.51 (–OCH_3), 55.79 (–OCH_3), 66.34 (C-3), 67.40 (C-2), 92.21, 94.26 (ArCH), 101.26 (ArC), 109.88 (ArCH), 121.64, 138.99, 146.03, 156.16, 159.79, 160.65 (ArC), 166.26 (C=O). Anal. calcd for $C_{18}H_{18}O_8$ (+ $1H_2O$) C, 56.84; H, 5.3. Found: C, 56.55; H, 5.13.
2-Phenylethyl 3,4,5-trihydroxybenzoate (**10**). Overall yield 65%; mp 81–82 °C; ^{1}H NMR (300 MHz, MeOH-d_4) 3.02 (t, 2H, J = 6.74 Hz), 4.41 (t, 2H, J = 6.74 Hz), 7.02 (s, 2H), 7.22 (m, 1H), 7.28 (m, 4H); ^{13}C NMR (75.4 MHz, MeOH-d_4) 36.36 (C-2), 66.58 (C-1), 110.20 (ArCH), 121.72 (ArC), 127.66, 129.66, 130.13 (ArCH), 139.69, 139.93, 146.62 (ArC), 168.58 (C=O). Anal. calcd for $C_{15}H_{14}O_5$ (+ $0.25H_2O$): C, 64.28; H, 5.27. Found: C, 64.13; 5.19.
2-(4-hydroxyphenyl)ethyl 3,4,5-trihydroxybenzoate (**11**). Overall yield 51%; mp 197–198 °C; ^{1}H NMR (300 MHz, acetone-d_6) 2.93 (t, 2H, J = 6.89 Hz), 4.35 (t, 2H, J = 6.89 Hz), 6.78 (m, 2H), 7.11 (s, 2H), 7.13 (m, 2H); ^{13}C NMR (75.4 MHz, acetone-d_6) 37.76 (C-2), 68.85 (C-1), 112.59, 118.86 (ArCH), 124.78, 132.67 (ArC), 133.56 (ArCH), 141.45, 148.78, 159.61 (ArC), 169.41 (C=O). Anal. calcd for $C_{15}H_{14}O_6$ (+ $0.2H_2O$) C, 61.12; H, 4.96. Found: C, 61.46; H 4.88.