4′-*C*-Cyano-2-deoxyguanosine (14a). To a solution of **12a** (70.0 mg, 0.24 mmol) in 50 mM Tris-HCl buffer (pH, 7.50, 13.3 mL) was added adenosine deaminase (0.13 mL, 58.5 units) and stirred for 1 h at 40°C. The precipitate formed was collected by filtaration and recrystallized from water to give **14a** (56.0 mg, 0.19 mmol, 79.2%).

^{1}H-NMR (DMSO-d_6) δ 10.68 (1H, s, NH), 7.93 (1H, s, H-8), 6.53 (2H, s, NH_2), 6.29 (1H, t, H-1′, J = 6.8), 6.27 (1H, d, 3′-OH, J = 4.9), 5.73 (1H, t, 5′-OH, J = 5.8), 4.60 (1H, dd, H-3′, J = 4.9, 10.8), 3.75 (1H, dd, H-5′a, J = 5.8, 11.7), 3.64 (1H, dd, H-5′b, J = 5.9, 11.7), 2.80, 2.40 (each 1H, m, H-2′). FABMS m/z: 293 (MH^+). Anal. Found: C, 41.54; H, 4.37; N, 26.58. Calcd. for $C_{11}H_{12}N_6O_4 \cdot 0.6H_2O$: C, 41.41; H, 4.17; N, 26.34.

N^6-Benzoyl-3′-*O*-*tert*-butyldimethylsilyl-2′-deoxyadenosine (4b). To a solution of **3b** (2.00 g, 3.04 mmol) in DMF (6.00 mL) was added imidazole (0.83 g, 12.2 mmol) and *tert*-butylchlorodimethylsilane (0.92 g, 6.10 mmol) and the solution was stirred at room temperature overnight. After addition of MeOH, the reaction mixture was diluted with AcOEt and washed with water. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure.

The residue was dissolved in $CHCl_3$ (70.0 mL) and added dropwise toluenesulfonic acid hydrate (0.60 g) in MeOH (30.0 mL) at 0°C. After addition, the solution was stirred for 30 min at same temperature. The reaction mixture was neutralized by addition of saturated $NaHCO_3$. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by silica-gel column chromatography (Eluent: AcOEt/*n*-hexane, 3:1) to give **4b** (1.20 g, 2.56 mmol, 84.2%).

^{1}H-NMR ($CDCl_3$) δ 9.02 (1H, s, NH), 8.79, 8.10 (each 1H, s, H-2 and H-8), 8.03–7.52 (5H, m, aromatic), 6.37 (1H, dd, H-1′, J = 5.5, 9.5), 5.78 (1H, dd, 5′-OH, J = 1.5, 11.5), 4.74 (1H, d, H-3′, J = 6.5), 4.17 (1H, s, H-4′), 4.00–3.74 (2H, m, H-5′), 3.07, 2.27 (each 1H, m, H-2′), 0.94 (9H, s, t-Bu), 0.13 (6H, s, Me). FABMS m/z: 470 (MH^+). Anal. Found: C, 58.45; H, 6.56; N, 14.76. Calcd. for $C_{23}H_{31}N_5O_4Si$: C, 58.83; H, 6.65; N, 14.91.

N^6-Benzoyl-3′-*O*-*tert*-butyldimethylsilyl-2′-deoxy-4′-*C*-hydroxymethyladenosine (5b). The solution of **4b** (2.55 g, 5.43 mmol) in toluene (10.0 mL) and DMSO (15.0 mL) was suspended 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (3.12 g, 16.3 mmol). The mixture was added pyridine (0.41 mL) and trifluoroacetic acid (0.21 mL) and stirred for 2 h at room temperature. The reaction mixture was diluted with AcOEt and washed with water. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure.

The residue was dissolved in dioxane (15.0 mL) and added 37% aqueous formaldehyde solution (2.86 mL) and 2 N sodium hydroxide (2.86 mL). After stirring for 1 h at room temperature, the reaction mixture was neutralized by addition of AcOH, diluted with AcOEt and washed with water. The organic layer was dried over $MgSO_4$ and evaporated under reduce pressure.

The residue was dissolved in EtOH (25.0 mL) and added $NaBH_4$ (0.21 g, 5.55 mmol) at 0°C. After stirring for 30 min, the reaction mixture was neutralized by addition of AcOH. The mixture was diluted with AcOEt and washed with water. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by silica-gel column chromatography (Eluent: AcOEt/*n*-hexane, 3:1–4:1–5:1) to give **5b** (1.68 g, 3.36 mmol, 61.9%).

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^{1}H-NMR ($CDCl_3$) δ 9.03 (1H, s, NH), 8.79, 8.08 (each 1H, s, H-2 and H-8), 8.03–7.52 (5H, m, aromatic), 6.45 (1H, dd, H-1′, *J* = 6.0, 9.0), 5.61 (1H, dd, OH, *J* = 2.0, 11.0), 4.94 (1H, dd, H-3′, *J* = 1.5, 6.0), 3.87–3.66 (4H, m, H-5′ and H-6′), 3.24 (1H, m, H-2′a), 2.68 (1H, dd, OH, *J* = 4.0, 9.0), 2.37 (1H, m, H-2′b), 0.96 (9H, s, *tert*-Bu), 0.19, 0.18 (each 3H, s, Me). FABMS m/z: 500 (MH^+). Anal. Found: C, 55.41; H, 6.50; N, 13.32. Calcd. for $C_{24}H_{33}N_5O_5Si \cdot 1.1H_2O$: C, 60.63; H, 7.15; N, 11.46.

N^6-Benzoyl-3′-*O*-*tert*-butyldimethylsilyl-2′-deoxy-4′-*C*-dimethoxytrityloxymethyladenosine (6b). To a solution of **5b** (0.84 g, 1.68 mmol) in CH_2Cl_2 (17.0 mL) was added triethylamine (0.47 mL, 3.37 mmol) and dimethoxytrityl chloride (0.85 g, 2.51 mmol) and stirred for 30 min at 0°C. After addition of MeOH, the reaction mixture was diluted with $CHCl_3$ and washed with saturated $NaHCO_3$. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by silica-gel column chromatography (Eluent: *n*-hexane/AcOEt, 2:1–1:1) to give **6b** (0.91 g, 1.13 mmol, 67.3%).

^{1}H-NMR ($CDCl_3$) δ 9.17 (1H, s, NH), 8.96, 8.24 (each 1H, s, H-2 and H-8), 8.23–6.94 (18H, m, aromatic), 6.41 (1H, dd, H-1′, *J* = 6.0, 8.5), 5.44 (1H, dd, 5′-OH, *J* = 2.5, 11.0), 4.84 (1H, dd, H-3′, *J* = 1.5, 5.5), 4.43 (1H, dd, H-5′a, *J* = 3.0, 12.5), 3.92 (6H, s, OMe), 3.78 (1H, t, H-5′b, *J* = 12.0), 3.67 (1H, d, H-6′a, *J* = 11.0), 3.30 (1H, m, H-2′a), 3.20 (1H, d, H-6′b, *J* = 11.0), 2.39 (1H, m, H-2′b), 0.90 (9H, s, *tert*-Bu), 0.13, 0.11, (each 3H, s, Me). FABMS m/z: 802 (MH^+). Anal. Found: C, 67.02; H, 6.52; N, 8.55. Calcd. for $C_{45}H_{51}N_5O_7Si$: C, 67.39; H, 6.41; N, 8.73.

N^6-Benzoyl-3′,5′-di-*O*-*tert*-butyldimethylsilyl-2′-deoxy-4′-*C*-hydroxymethyladenosine (7b). To a solution of **6b** (1.61 g, 2.01 mmol) in DMF (8.00 mL) was added imidazole (0.41 g, 6.02 mmol) and *tert*-butylchlorodimethylsilane (0.45 g, 2.99 mmol) and stirred at room temperature overnight. After addition of MeOH, the reaction mixture was diluted with AcOEt and washed with water. The organic layer was dried over $MgSO_4$ and evaporated.

The residue was dissolved to $CHCl_3$ (70.0 mL) and added dropwise 1% TsOH hydrate in MeOH (30.0 mL) at 0°C. After addition, the solution was stirred for 30 min at same temperature. The reaction mixture was neutralized by addition of saturated $NaHCO_3$. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by silica-gel column chromatography (Eluent: *n*-hexane/AcOEt, 3:1) to give **7b** (1.00 g, 1.63mmol, 81.1%).

^{1}H-NMR ($CDCl_3$) δ 9.04 (1H, s, NH), 8.81, 8.28 (each 1H, s, H-2 and H-8), 8.04–7.52 (5H, m, aromatic), 6.52 (1H, t, H-1′, *J* = 6.5), 5.78, 4.88 (1H, dd, H-3′, *J* = 4.5, 6.5), 3.89–3.73 (4H, m, H-5′ and H-6′), 3.04, 2.58 (each 1H, m, H-2′), 2.48 (1H, dd, 6′-OH, *J* = 5.5, 8.5) 0.95, 0.89 (each 9H, s, *tert*-Bu), 0.16, 0.06 (each 6H, s, Me). FABMS m/z: 614 (MH^+). Anal. Found: C, 58.33; H, 7.80; N, 11.14. Calcd. for $C_{30}H_{47}N5O5Si2$: C, 58.69; H, 7.72; N, 11.41.

N^6-Benzoyl-3′,5′-di-*O*-*tert*-butyldimethylsilyl-4′-*C*-cyano-2′-deoxyadenosine (10b). The solution of **7b** (1.00 g, 1.63 mmol) in toluene (3.00 mL) and DMSO (6.00 mL) was suspended 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.94 g, 4.90 mmol). Pyridine (0.13 mL) and trifluoroacetic acid (62.8 μL) were added and stirred for 1 h at room temperature. The reaction mixture was diluted with

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AcOEt and washed with water. The organic layer was dried over $MgSO_4$ and evaporated to give crude aldehyde **8b**.

To a solution of crude aldehyde **8b** in pyridine (10.0 mL) was added hydroxylamine hydrochloride (0.17 g, 2.45 mmol) and stirred for 30 min at room temperature. The reaction mixture was evaporated and the residue was partitioned between AcOEt and water. The organic layer was dried over $MgSO_4$ and evaporated to give crude oxime **9b**.

To a solution of crude oxime **9b** in CH_2Cl_2 (10.0 mL) was added triethylamine (0.45 mL, 3.23 mmol) and methanesulfonyl chloride (0.19 mL, 2.45 mmol) at 0°C and stirred for 30 min at same temperature. The reaction mixture was diluted with $CHCl_3$ and washed with saturated $NaHCO_3$. The organic layer was dried over $MgSO_4$ and evaporated. The residue was purified by silica-gel column chromatography (Eluent: $CHCl_3$/MeOH, 100:1) to give **10b** (0.87 g, 1.41 mmol, 87.7%).

^{1}H-NMR ($CDCl_3$) δ 9.10 (1H, s, NH), 8.79, 8.14 (each 1H, s, H-2 and H-8), 8.04–7.52 (5H, m, aromatic), 6.52 (1H, dd, H-1′, J = 6.0, 6.5), 5.02 (1H, t, H-3′, J = 6.0), 4.05, 3.89 (each 1H, d, H-5′, J = 11), 3.22, 2.62 (each 1H, m, H-2′), 0.98, 0.87 (each 9H, s, *tert*-Bu), 0.21, 0.19, 0.08, 0.03 (each 3H, s, Me). FABMS m/z: 609 (MH^+). Anal. Found: C, 58.64; H, 7.38; N, 13.60. Calcd. for $C_{30}H_{44}N_6O_4Si_2 \cdot 0.2H_2O$: C, 58.83; H, 7.31; N, 13.72.

4′-*C*-Cyano-2′-deoxyadenosine (12b). The mixture of **10b** (0.70 g, 1.15 mmol) in MeOH (10.5 mL) and conc. NH_4OH (3.50 mL) was stirred at room temperature overnight. Precipitate formed was collected by filtration to give crude debenzoylated product **11b** (0.50 g).

To a solution of crude **11b** (0.50 g) in THF (7.80 mL) was added tetra-*n*-butylammonium fluoride (1 M solution of THF, 2.18 mL, 2.18 mmol) and stirred for 15 min at room temperature. The reaction mixture was evaporated and the residue was purified by silica-gel column chromatography (Eluent: $CHCl_3$/MeOH, 20:1–10:1). The residue was recrystallized from water to give **12b** (0.21 g, 0.76 mmol, 66.1% from **10b**).

^{1}H-NMR (DMSO-d_6) δ 8.34 (1H, s, H-8), 8.16 (1H, s, H-2), 7.36 (2H, s, NH_2), 6.52 (1H, t, H-1′, J = 6.5), 6.32 (1H, d, 3′-OH, J = 5.0), 5.83 (1H, t, 5′-OH, J = 6.0), 4.74 (1H, dd, H-3′, J = 5.5, 11.5), 3.82 (1H, dd, H-5′a, J = 5.0, 11.5), 3.66 (1H, dd, H-5′b, J = 6.0, 12), 3.01, 2.47 (each 1H, m, H-2′). FABMS m/z: 277 (MH^+). Anal. Found: C, 45.90; H, 4.42; N, 29.12. Calcd. for $C_{11}H_{12}N_6O_3 \cdot 0.6H_2O$: C, 46.02; H, 4.63; N, 29.28.

4′-*C*-Cyano-2′-deoxyinosine (14b). To a solution of **12b** (0.15 g, 0.54 mmol) in Tris-HCl buffer (pH, 7.50, 30.0 mL) was added adenosine deaminase (0.30 mL, 135 units) and stirred for 2 h at 40°C. The reaction mixture was concentrated and purified reverse phase column chromatography (0–2.5% EtOH). The residue was recrystalized from water to give **14b** (90.0 mg, 0.32 mmol, 59.3%).

^{1}H-NMR (DMSO-d_6) δ 12.46 (1H, s, NH), 8.32 (1H, s, H-8), 8.10 (1H, s, H-2), 6.49 (1H, t, H-1′, J = 6.5), 6.36 (1H, d, 3′-OH, J = 5.0), 5.76 (1H, t, 5′-OH, J = 6.0), 4.69 (1H, dd, H-3′, J = 5.5, 11.5), 3.78 (1H, dd, H-5′a, J = 6.0, 12.0), 3.66 (1H, dd, H-5′b, J = 6.0, 12.0), 2.92, 2.49 (each 1H, m, H-2′). FABMS m/z: 278 (MH^+). Anal. Found: C, 44.91; H, 4.06; N, 24.06. Calcd. for $C_{11}H_{11}N_5O_4 \cdot 1H_2O$: C, 44.75; H, 4.44; N, 23.72.

9-(3,5-Di-*O*-*tert*-Butyldimethylsilyl-2-*deoxy*-4-C-ethynyl-*ribo*-pentofuranosyl)-2,6-dibenzamidopurine (16a). The solution of **7a** (2.00 g, 2.73 mmol) in toluene

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(6.00 mL) and DMSO (12.0 mL) was suspended 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (1.57 g, 8.19 mmol). Pyridine (0.22 mL) and trifluoroacetic acid (0.11 mL) were added and stirred for 90 min at room temperature. The reaction mixture was diluted with AcOEt and washed with water. The organic layer was dried over $MgSO_4$ and evaporated to give crude aldehyde **8a**.

To a suspension of bromomethyltriphenylphosphonium bromide (2.40 g, 5.50 mmol) in THF (32.0 mL) was added potassium *tert*-butoxide (0.93 g, 8.29 mmol) at −40°C and stirred for 90 min at same temperature to prepare bromomethylene triphenylphosphorane. The solution was added crude aldehyde **8a** in THF (32.0 mL) and stirred for 2 h at −40°C. The reaction mixture was added saturated NH_4Cl and stirred. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by silica-gel column chromatography (Eluent: *n*-hexane/AcOEt, 3:1–2:1–1:1) to give crude bromoethene **15a** (1.96 g).

To a solution of crude **15a** (1.96 g) in THF (50.0 mL) was added potassium *tert*-butoxide (0.91 g, 8.11 mmol) at −40°C and the solution was stirred for 1 h at same temperature. After addition of saturated NH_4Cl, the mixture was stirred at room temperature. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by silica-gel column chromatography (Eluent: *n*-hexane/AcOEt, 2:1) to give **16a** (1.21 g, 1.66 mmol, 60.8% from **7a**).

^{1}H-NMR ($CDCl_3$) δ 9.38, 9.37 (each 1H, s, NH), 8.19 (1H, s, H-8), 8.04–7.49 (10H, m, aromatic), 6.54 (1H, dd, H-1′, J = 4.5, 7.0), 4.84 (1H, t, H-3′, J = 6.5), 4.03, 3.83 (each 1H, d, H-5′, J = 11.0), 2.86, 2.66 (each 1H, m, H-2′), 2.55 (1H, s, ethynyl), 0.94, 0.90 (each 9H, s, *tert*-Bu), 0.14, 0.13, 0.08, 0.06 (each 3H, s, Me). FABMS m/z: 727 (MH^+). Anal. Found: C, 61.91; H, 6.94; N, 11.21. Calcd. for $C_{38}H_{50}N_6O_5Si_2 \cdot 0.5H_2O$: C, 62.01; H, 6.98; N, 11.42.

9-(2-Deoxy-4-*C*-ethynyl-*ribo*-pentofuranosyl)-2,6-diaminopurine (18a). To a solution of **16a** (1.24 g, 1.71 mmol) in THF (30.0 mL) was added tetra-*n*-butylammonium fluoride (1 M solution of THF, 4.30 mL, 4.30 mmol) and stirred for 30 min at room temperature. The reaction mixture was evaporated and the residue was purified by silica-gel column chromatography (Eluent: $CHCl_3$/MeOH, 20:1) to give **17a** (0.70 g, 1.40 mmol).

The mixture of **17a** (0.65 g, 1.30 mmol) in MeOH (15.0 mL) and 40% $MeNH_2$ aqueous solution (30.0 mL) was stirred at room temperature overnight. The reaction mixture was evaporated and the residue was purified by silica-gel column chromatography (Eluent: $CHCl_3$/MeOH, 10:1). The residue was recrystallized from water to give **18a** (0.33 g, 1.14 mmol, 87.7%), whose structure was confirmed by comparing spectroscopic data of **18a** with these of previously reported 9-(2-deoxy-4-*C*-ethynyl-*ribo*-pentofuranosyl)-2,6-diaminopurine.[6]

***N*^{6}-Benzoyl-3′,5′-di-*O*-*tert*-butyldimethylsilyl-4′-*C*-ethynyl-2′-deoxyadenosine (16b).** The solution of **7b** (1.80 g, 2.93 mmol) in toluene (6.00 mL) and DMSO (12.0 mL) was suspended 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.69 g, 8.82 mmol). Pyridine (0.24 mL) and trifluoroacetic acid (0.12 mL) were added and stirred for 3 h at room temperature. The reaction mixture was diluted with AcOEt and washed with water. The organic layer was dried over $MgSO_4$ and evaporated to give crude aldehyde **8b**.

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To a suspension of bromomethyltriphenylphosphonium bromide (2.56 g, 5.87 mmol) in THF (35.0 mL) was added potassium *tert*-butoxide (1.00 g, 8.91 mmol) at −40°C and stirred for 2 h at same temperature to prepare bromomethylene triphenylphosphorane. The solution was added crude aldehyde **8b** in THF (35.0 mL) and stirred for 2 h at −40°C. The reaction mixture was added saturated NH_4Cl and stirred. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by silica-gel column chromatography (Eluent: *n*-hexane/AcOEt, 3:1−2:1−1:1) to give crude bromoethene **15b**.

To a solution of crude **15b** in THF (70.0 mL) was added potassium *tert*-butoxide (1.00 g, 8.91 mmol) at −40°C and the solution was stirred for 2 h at same temperature. After addition of saturated NH_4Cl, the mixture was stirred at room temperature. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by silica-gel column chromatography (Eluent: *n*-hexane/AcOEt, 2:1−1:1) to give **16b** (1.21 g, 1.99 mmol, 67.9% from **7b**).

^{1}H-NMR ($CDCl_3$) δ 8.99 (1H, s, NHBz), 8.81, 8.30 (each 1H, s, H-2 and H-8), 8.04−7.51 (5H, m, aromatic), 6.54 (1H, dd, H-1′, J = 4.9, 7.3), 4.83 (1H, t, H-3′, J = 6.8), 3.97 (1H, d, H-5′a, J = 11.2), 3.81 (1H, d, H-5′b, J = 11.2), 2.79, 2.68 (each 1H, m, H-2′), 2.57 (1H, s, ethynyl), 0.94, 0.89 (each 9H, s, *tert*-Bu), 0.14, 0.13, 0.08, 0.04 (each, 3H, s, Me). FABMS m/z: 608 (MH^+). Anal. Found: C, 60.25; H, 7.50; N, 11.10. Calcd. for $C_{31}H_{45}N_5O_4Si_2 \cdot 0.5H_2O$: C, 60.36; H, 7.52; N, 11.35.

2′-Deoxy-4′-*C*-ethynyladenosine (18b). To a solution of **16b** (0.118 g, 0.235 mmol) in THF (3.2 mL) was added tetra-*n*-butylammonium fluoride (1 M solution of THF, 0.7 mL, 0.7 mmol) and stirred for 30 min at room temperature. The reaction mixture was evaporated and the residue was purified by silica-gel column chromatography (Eluent: $CHCl_3$/MeOH, 20:1) to give crude **17b** (0.122 g).

The mixture of **17b** (0.122 g) in MeOH (2.1 mL) and conc. NH_4OH (0.7 mL) was stirred at room temperature overnight. The reaction mixture was evaporated and the residue was purified by silica-gel column chromatography (Eluent: $CHCl_3$/MeOH, 20:1−10:1). The residue was recrystallized from water to give **18b** (0.057 g, 0.207 mmol, 88.1% from **16b**), whose structure was confirmed by comparing spectroscopic data of **18b** with these of previously reported 2′-deoxy-4′-*C*-ethynyladenosine.[6]

Antiviral Evaluation

Antiviral Agents. 3′-Azido-3′-deoxythymidine (AZT or zidovudine), 2′,3′-dideoxyinosine (ddI or didanosine), and 2′,3′-dideoxycytidine (ddC or zalcitabine) were purchased from Sigma (St. Louis, MO). (-)-2′,3′-Dideoxy-3′-thiacytidine (3TC or lamivudine) was a kind gift from Dr. R. F. Schinazi (Atlanta, GA). A series of 4′-position substituted nucleosides were designed and synthesized as described by us.

Determination of Drug Susceptibility of HIV-1. The inhibitory effects of test compounds on HIV-1 replication were monitored by the inhibition of virally induced cytopathicity in MT-4 cells. Briefly, MT-4 cells were suspended at 10^5 cells/mL and exposed to HIV-1_{LAI} at 100 50% tissue culture infectious doses ($TCID_{50}$s). Immediately after viral exposure, the cell suspension (10^4 cells in 100 μL) was brought into each well of a 96-well flat microtiter culture plate (Costar, Cambridge,

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Mass.) containing various concentrations of test compounds. After incubation for 5 days, the number of viable cells was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method as previously described.[13,14]

The sensitivity of infectious clones to various RTIs was determined in the multinuclear activation of the galactosidase indicator (MAGI) assay,[17] with some modifications using the viral preparations titrated as previously described.[18] Briefly, target cells (HeLa CD4-LTR/β-gal; 104/well) were plated in 96-well flat microtiter culture plates. On the following day, the medium was aspirated and the cells were inoculated with HIV-1 clones (70 MAGI units/well, which gave 70 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of drug in fresh medium. Forty-eight hours after viral exposure, all blue cells in each well were counted. The cytotoxicity of the compound was determined by the MTT method as previously described.[13] All experiments were performed in triplicate.

Preliminary Toxicity Test for Mice. Six-week-old, random-bred, Swiss albino ICR male mice, (Jcl:ICR) were purchased from Clea Japan. The drugs were dissolved in saline and administered once to the mice either orally (*p.o.*) or intravenously (*i.v.*). The mice were observed twice daily for 7 days for their death.

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Novel patterns of nevirapine resistance-associated mutations of human immunodeficiency virus type 1 in treatment-naïve patients

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Abstract

Several reports have recently shown that drug-resistant human immunodeficiency virus type 1 (HIV-1) is often isolated from treatment-naïve patients. We phenotypically analyzed HIV-1 strains isolated from 44 treatment-naïve individuals and found two strains highly resistant (69- and >310-fold) against nevirapine (NVP). Direct sequencing showed these two isolates had a novel mutation, K238S, in reverse transcriptase (RT), but did not have any reported NVP resistance-associated mutation.

A 48-h culture in the presence of NVP, however, selected HIV-1 carrying NVP resistance-associated mutations, V106A, V108I, or both, suggesting that minor viral populations of these two isolates had harbored these mutations. Replication kinetic studies of recombinant HIV-1 clones suggested that K238S conferred a significant resistance against NVP, especially when accompanied with V106A (530-fold) or V108I (56-fold). Our study identified a novel NVP resistance-associated mutation, K238S, which could be persistently detected by genotypic assay longer than V106A and V108I during off-treatment period.

Keywords: Human immunodeficiency virus type 1; K238S; Drug-naïve; Nevirapine-resistant

Introduction

The emergence of human immunodeficiency virus type 1 (HIV-1) variants resistant to antiretroviral agents is one of the most common causes for therapeutic failure in infected individuals. Fortunately, the availability of drug-resistance testing has substantially improved the ability of clinicians to deal knowledgeably with drug-resistant HIV-1 variants. Various guidelines for therapy of HIV-1 infection recommend that each individual therapy should be optimized by choosing efficient agents based on the results of drug-resistance testing. Numerous studies have recently reported that significant proportion of newly diagnosed HIV-1 infection cases are infected with viral strains resistant to at least one antiretroviral agent, justifying drug-resistance testing even in treatment-naïve patients (Briones et al., 2001; Duwe et al., 2001; Grant et al., 2002; Little et al., 2002; Salomon et al., 2000; Simon et al., 2002). However, it is important to recognize that the ability to detect resistant viruses may decrease as a function of time from initial infection because there is generally a shift to wild type over time during off-treatment (Devereux et al., 1999; Miller et al., 2000; Verhofstede et al., 1999). The usefulness of phenotypic assay could be enhanced if the virus is propagated in the presence of drug, because such a condition could propagate resistant viral strains that might have been outgrown by wild-type viruses in the absence of drug pressure. In this study, we screened 44 clinical strains isolated from newly infected individuals by using phenotypic assay and identified two highly nevirapine (NVP)-resistant isolates that could not be detected by genotypic

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assay. Phylogenetic analyses showed these two isolates were genetically closely related and probably originated from a single patient. Furthermore, we identified a novel NVP resistance-associated mutation, K238S, in these isolates and determined its role in viral replication and resistance using newly generated recombinant HIV-1 clones.

Results

NVP-resistant HIV-1 from treatment-naïve patients

From January 2000 through December 2001, a total of 44 patients, who had had clinical evidence of recent HIV-1 infection (seroconversion or increased bands of western blotting against HIV-1 antigens), visited our clinic and all of them gave written informed consent to the participation in this study. To determine the prevalence of drug resistance in recent HIV-1 infection, susceptibility of HIV-1 isolates derived from these patients to six antiretroviral agents including zidovudine (AZT), lamivudine (3TC), stavudine (d4T), abacavir (ABC), NVP, and efavirenz (EFV) was examined phenotypically by using MAGIC-5 cells (CCR5 and CD4 expressing HeLa-LTR-β-gal cells) (Hachiya et al., 2001). As expected, most of such isolates were sensitive to all tested antiretroviral agents. However, two isolates (4.5%; isolated from Cases 33 and 23) showed significantly greater resistance (69-fold and more than 310-fold compared with NL4-3) to NVP, though their resistance to EFV was not high (Fig. 1, Table 1).

To delineate the mechanism of the NVP resistance in the above two isolates, direct sequencing of HIV-1 reverse transcriptase (RT) gene from plasma samples was performed. Both plasma-derived sequences did not have any non-nucleoside RT inhibitor (NNRTI) resistance-associated mutations listed in resistance table of the International AIDS Society (Johnson et al., 2003) but had six-amino-acid substitutions including V35I, R83K, I135T, T200K, R211K, and K238S, compared with HXBII sequences, and the plasma-derived sequence in Case 23 had a mixture of K102 and K102R (Table 2). We postulated that there might be a minor viral population harboring NNRTI resistance-associated mutations in both plasma samples, which could not be detected by direct sequencing. To detect such a minor NVP-resistant viral population, the clinical isolates were propagated in MAGIC-5 cells in the presence of NVP at various concentrations and the RT gene of HIV-1 obtained from a 48-h culture supernatant was analyzed. In the HIV-1 isolated from Case 33, V108I, known as a NVP resistance-associated mutation, emerged in the presence of NVP (1 μM) although it could not be detected in direct sequencing of plasma (Table 2). In the HIV-1 isolated from Case 23, V106A, and V108I emerged in the presence of NVP (1 and 10 μM). Interestingly, clonal sequencing analysis of HIV-1 culture (NVP 1 μM) isolated from Case 23 revealed that K102R and V106A always coexisted on the same molecules and that V108I did not coexist with K102R and V106A (Table 2). All clones analyzed had V35I, R83K, I135T, T200K, R211K, and K238S. Four of these mutations (V35I, R83K, I135T, and R211K) are known as polymorphic mutations and were frequently observed in HIV-1 isolated from treatment-naïve individuals in our cohort (72%, 18%, 27%, and 36%, respectively, $n = 11$). T200K and K238S had not been reported previously and might be associated with NVP resistance.

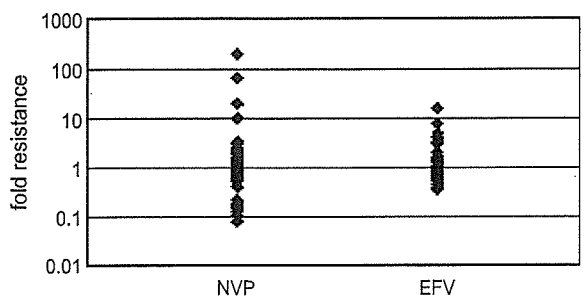

Fig. 1. Results of drug resistance assay using MAGIC-5 cells in treatment-naïve individuals. Fold resistances compared with NL4-3 are shown.

Phylogenetic analysis of clinical HIV-1 isolates

Because the clinical HIV-1 isolates derived from Cases 33 and 23 had unusually high resistance against NVP and had exactly the same amino acid substitutions (V35I, R83K, I135T, T200K, R211K, and K238S), we suspected that these two patients had acquired HIV-1 infection from a single infected patient. Then, we phylogenetically analyzed the direct sequences of *env* region and RT gene of the two HIV-1 isolates derived from Cases 33 and 23, and then compared their sequences with four clinical isolates derived from other patients (Cases 2, 3, 26, and 29) and five referential unrelated strains [U63632, HXB2CG, OY1, RF, and simian immunodeficiency virus (SIV)]. In both phylogenetic trees using the sequences of the *env* region and RT gene, Cases 33- and 23-derived sequences formed a tight cluster separated from other sequences by the tree branch with high bootstrap probabilities (99% and 100%) (Figs. 2A and B), suggesting that the isolates from Cases 33 and 23 were genetically closely related and probably originated from a single source. It was noteworthy that the *env* sequences derived from Cases 33 and 23 were closely related but not identical, which excludes the possibility of contamination during the procedures of viral culture and PCR.

Three-dimensional positions of mutations and NVP-binding pocket in HIV-1 RT

To delineate the positional relationship between NVP-binding pocket and the mutations in RT described above, a structural model of HIV-1 RT complexed with NVP was

Table 1
Results of phenotypic drug resistance testing in two untreated patients

HIV-1	IC_{50}[a] [μM] (fold resistance[b])					
	AZT	3TC	d4T	ABC	NVP	EFV
Case 33	0.04 ± 0.03 (1.2)	0.61 ± 0.20 (1.8)	1.7 ± 0.10 (0.77)	3.6 ± 0.87 (2.3)	2.2 ± 0.29 (69)	0.011 ± 0.003 (4.2)
Case 23	0.12 ± 0.03 (3.5)	0.81 ± 0.15 (2.4)	3.2 ± 0.95 (1.5)	3.3 ± 0.87 (2.1)	>10 (>310)	0.023 ± 0.00002 (8.8)

Data are mean ± SD. Numbers in parentheses represent fold resistance.
[a] Phenotypic drug resistance assay was performed using MAGIC-5 cells.
[b] Fold resistance was calculated by dividing IC_{50} of clinical isolate by that of NL4-3 (AZT, 0.034 μM; 3TC, 0.34 μM; d4T, 2.2 μM; ABC, 1.6 μM; NVP, 0.032 μM; EFV, 0.0026 μM).

prepared based on the published crystal structures (Fig. 3) (Ren et al., 2000). The Val residues at 106 and 108 codons of RT (V106 and V108), the site of NNRTI resistance-associated mutations, V106A and V108I, respectively, were in close contact with NVP, packed in the hydrophobic pocket of the palm domain. Five residues, V35, R83, I135, T200, and R211, were located distantly from the hydrophobic pocket. Two residues, K102 and K238, were located outside the frame of hydrophobic pocket and the substitutions of these amino acids, such as K102R and K238S, were thought to produce some structural changes of the pocket and might be associated with NNRTI resistance.

Role of K102R and K238S in HIV-1 replication and resistance against NVP

Because K238S has not been reported previously and K102 and K238 were located on the frame of NVP-binding pocket, we chose to construct seven recombinant HIV-1 strains, HIV-1_{K102R}, HIV-1_{V106A}, HIV-1_{V108I}, HIV-1_{K238S}, HIV-$1_{V106A/K238S}$, HIV-$1_{V108I/K238S}$, and HIV-$1_{K102R/V106A/K238S}$, to delineate the effects of K102R and K238S on viral susceptibility against NVP. K102R did not confer significant resistance against NVP and EFV (Table 3), while V106A and V108I, both of which were known as NVP resistance-associated mutations, conferred 97- and 3.8-fold resistance against NVP, respectively. However, these mutations did not confer significant resistance against EFV. K238S conferred a significant resistance against NVP as did V108I. Interestingly, the combination of V106A and K238S (V106A/K238S) conferred surprisingly high-level resistance (530-fold) against NVP, and the combination of V108I and K238S (V108I/K238S) also conferred high resistance (56-fold) only against NVP but not against EFV (Table 3). Furthermore, the triple combination of K102R, V106A, and K238S (K102R/V106A/K238S), which were found in 15 of 21 clones derived from Case 23 (Table 2), also conferred high resistance (340-fold) against NVP, although its effect on viral resistance against EFV was not significant (Table 3). These data suggest that K238S is strongly associated with NVP resistance in the combination with V106A or V108I.

We next analyzed the effects of K102R and K238S on viral replication kinetics in the absence or presence of NVP. The addition of K238S to HIV-1WT (HIV-1_{K238S}) reduced the replication of HIV-1_{WT} in the absence of NVP, and the addition of V108I to HIV-1_{K238S} (generating HIV-$1_{V108I/K238S}$) further reduced the viral replication (Fig. 4A). On the other hand, in the presence of NVP (1 μM), K238S made HIV-1 replication-competent (HIV-1_{WT} could not replicate), and HIV-$1_{V108I/K238S}$ had facilitated viral replication (Fig. 4B). The combination of V106A to K238S (HIV-$1_{V106A/K238S}$) severely compromised viral replication and the addition of K102R (HIV-$1_{K102R/V106A/K238S}$) did

Table 2
Sequences of HIV-1 RT-coding region of clinical samples and isolates

	Sequenced sample	Amino acid residue														
		35	83	100[a]	102	103[a]	106[a]	108[a]	135	181[a]	188[a]	190[a]	200	211	230[a]	238
HXBII		V	R	L	K	K	V	V	I	Y	Y	G	T	R	M	K
Case 33	plasma	I	K	–	–	–	–	–	T	–	–	–	K	K	–	S
	1 μM[b]	I	K	–	–	–	–	V/I	T	–	–	–	K	K	–	S
Case 23	plasma	I	K	–	K/R	–	–	–	T	–	–	–	K	K	–	S
	1 μM[b]	I	K	–	K/R	–	V/A	V/I	T	–	–	–	K	K	–	S
	10 μM[b]	I	K	–	K/R	–	A	V/I	T	–	–	–	K	K	–	S
	Number of clones															
Isolate from	15	I	K	–	R	–	A	–	T	–	–	–	K	K	–	S
Case 23	3	I	K	–	–	–	–	–	T	–	–	–	K	K	–	S
(1 μMb)	2	I	K	–	–	–	–	I	T	–	–	–	K	K	–	S
	1	I	K	–	–	R	–	–	T	–	–	–	K	K	–	S

Amino acids identical to HXBII (top column) are indicated with dashes.
[a] Reported residue associated with NVP resistance (Johnson et al., 2003).
[b] Cultured HIV-1 isolate in the presence of NVP at the indicated concentrations.

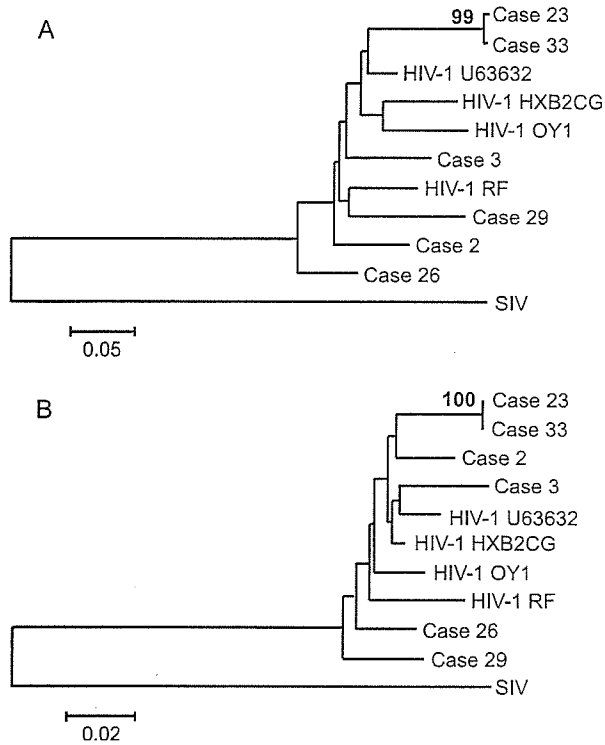

Fig. 2. Phylogenetic analysis of *env* sequences (A) and RT genes (B) of six clinical isolates and five referential strains. Bootstrap probabilities of important tree branches are shown as percentages.

not seem to rescue its replicability in the absence of drugs (Fig. 4C). In the presence of NVP (1 μM), the combination of V106A and K238S (HIV-$1_{V106A/K238S}$) rendered HIV-1

Table 3
Drug resistance of recombinant HIV-1s

Recombinant HIV-1	IC_{50}[a] [μM] (fold resistance[b])	
	NVP	EFV
K102R	0.047 ± 0.02 (1.5)	0.002 ± 0.001 (0.77)
V106A	3.1 ± 0.36 (97)	0.002 ± 0.0003 (0.77)
V108I	0.12 ± 0.03 (3.8)	0.001 ± 0.0001 (0.38)
K238S	0.14 ± 0.04 (4.4)	0.002 ± 0.0003 (0.77)
V106A/K238S	17 ± 4.6 (530)	0.004 ± 0.001 (1.5)
V108I/K238S	1.8 ± 0.66 (56)	0.001 ± 0.0001 (0.38)
K102R/V106A/K238S	11 ± 3.1 (340)	0.002 ± 0.0001 (0.77)

Data are mean ± SD. Numbers in parentheses represent fold resistance.
[a] Phenotypic drug resistance assay was performed using MAGIC-5 cells.
[b] Fold resistance was calculated by dividing IC_{50} of clinical isolate by that of NL4-3 (NVP; 0.032 μM, EFV; 0.0026 μM).

replicable, and the addition of K102R to V106A/K238S (HIV-$1_{K102R/V106A/K238S}$) did not significantly alter the replication (Fig. 4D). These data showed that V108I/K238S and V106A/K238S can confer viral replicability in the presence of NVP, although the role of K102R when combined with V106A/K238S remained to be determined.

To further define the significance of each mutation, the viral replicability was compared among molecular infectious HIV-1 clones described above in the presence and absence of NVP using competitive HIV-1 replication assay (CHRA) (Kosalaraksa et al., 1999). Two infectious HIV-1 clones to be compared for their fitness were mixed and used to infect H9 cells, and their population changes through passages were determined by the relative peak height on sequencing electrogram. In the absence of NVP, HIV-1_{WT} readily outgrew HIV-

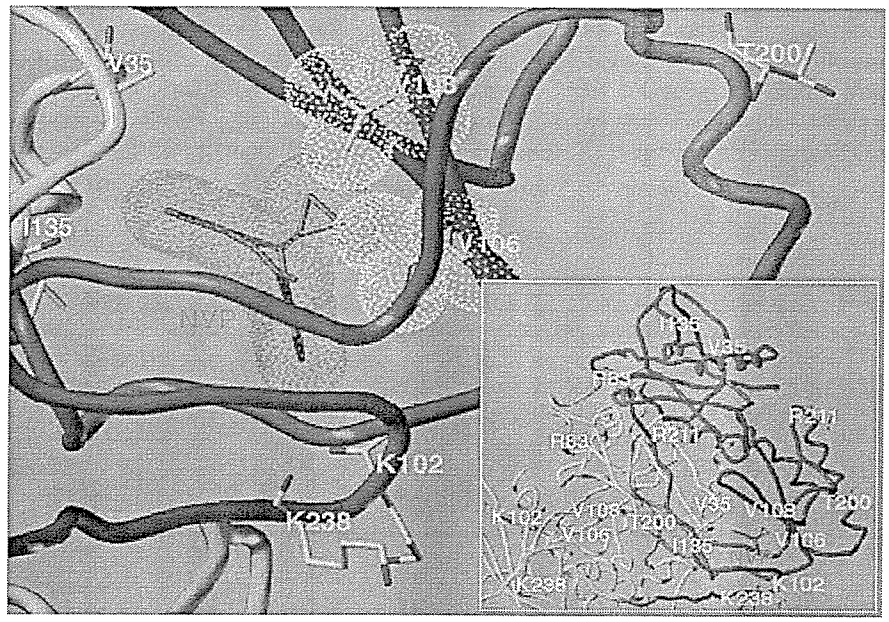

Fig. 3. The structure of the HIV-1 RT complexed with NVP. A view of the RT complexed with NVP, generated using SYBYL 6.7 and the coordinate set 1FKP.pdb obtained from the Protein Data Bank (Ren et al., 2000). The fingers, palm, thumb, and connection subdomains of the p66 subunit are colored blue, red, green, and yellow, respectively. The p51 subunit is colored white. Residues of the p66 subunit are yellow-colored, while those of the p51 subunit are white-colored. The van der Waals volumes of side chain of V106 and V108 (white) are shown to interact with NVP (orange).

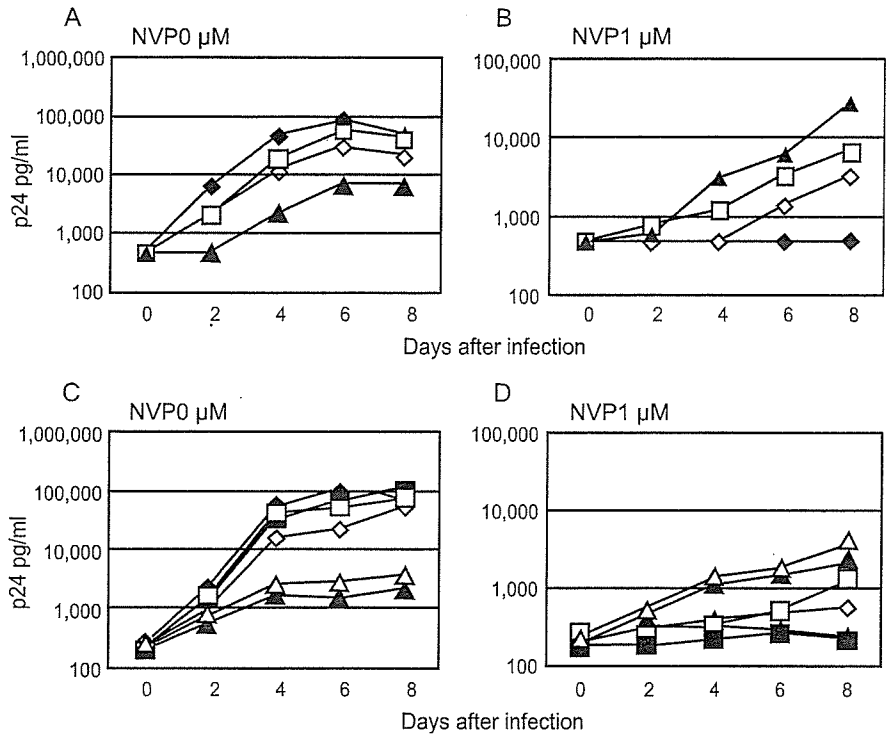

Fig. 4. Replication kinetics of recombinant HIV-1s. Graphs A and B show replication kinetics of HIV-1_{WT} (solid diamonds), HIV-1_{V108I} (open squares), HIV-1_{K238S} (open diamonds), and HIV-$1_{V108I/K238S}$ (solid triangles) in the absence and presence of NVP (1 μM), respectively. Graphs C and D show replication kinetics of HIV-1_{WT} (solid diamonds), HIV-1_{K102R} (solid squares), HIV-1_{V106A} (open squares), HIV-1_{K238S} (open diamonds), HIV-$1_{V106A/K238S}$ (solid triangles), and HIV-$1_{K102R/V106A/K238S}$ (open triangles) in the absence and presence of NVP (1 μM), respectively. The results shown are representative of three independent experiments.

1_{K238S} (Fig. 5A). In the presence of NVP (0.1 μM), however, HIV-1_{K238S} predominated over HIV-1_{WT} (Fig. 5B), suggesting that K238S compromised the viral replication in the absence of NVP but conferred resistance against NVP. Next, we analyzed the effect of V106A and V108I on HIV-1_{K238S} replication. In comparison between HIV-1_{K238S} and HIV-$1_{V106A/K238S}$, HIV-1_{K238S} predominated over HIV-$1V_{106A/K238S}$ in the absence of NVP (Fig. 5C), but was outgrown by HIV-$1_{V106A/K238S}$ in the presence of NVP (1 μM) (Fig. 5D). In comparison between HIV-1_{K238S} and HIV-$1_{V108I/K238S}$, HIV-1_{K238S} predominated over HIV-$1_{V108I/K238S}$ in the absence of NVP (Fig. 5E), but was outgrown by HIV-$1_{V108I/K238S}$ in the presence of NVP (1 μM) (Fig. 5F). Taken together, these data suggest that each of V106A and V108I compromised the viral replication in the absence of NVP but conferred resistance against NVP. Finally, we analyzed the effect of K102R on replication of HIV-1_{WT} and HIV-$1_{V106A/K238S}$. HIV-1_{K102R} was slowly overcome by HIV-1_{WT} in the absence of NVP (Fig. 5G). In comparison between HIV-$1_{V106A/K238S}$ and HIV-$1_{K102R/V106A/K238S}$, HIV-$1_{K102R/V106A/K238S}$ predominated over HIV-$1_{V106A/K238S}$ both in the absence (Fig. 5H) and presence (1 μM) (Fig. 5I) of NVP. Considered together, these results suggest that K102R compromised the replication of HIV-1_{WT} but compensated the replication ability of HIV-$1_{V106A/K238S}$ regardless of the presence of NVP.

Discussion

Several studies reported the recent spread of drug-resistant HIV-1 in the developed countries and among treatment-naïve individuals as well, which has been a menace for clinicians. Therefore, for certain treatment-naïve patients, such as cases of acute or recent HIV-1 infection, drug-resistance assay is recommended (Hirsch et al., 2003). However, resistant HIV-1 can be replaced with wild-type HIV-1 rapidly in plasma in the absence of treatment, and it may be difficult to detect resistant HIV-1 in the treatment-naïve patients even if they harbored resistant HIV-1. In this regard, phenotypic assay may be superior to genotypic assay using direct sequencing, because in phenotypic assay, resistant viruses could be propagated during culture in the presence of antiretroviral agents, while direct sequencing can only detect relatively major viral population in plasma. In fact, in the present study, the phenotypic assay using MAGIC-5 cells detected two highly NVP-resistant isolates in treatment-naïve patients, in whom direct sequencing failed to detect the presence of minor population of V106A and V108I.

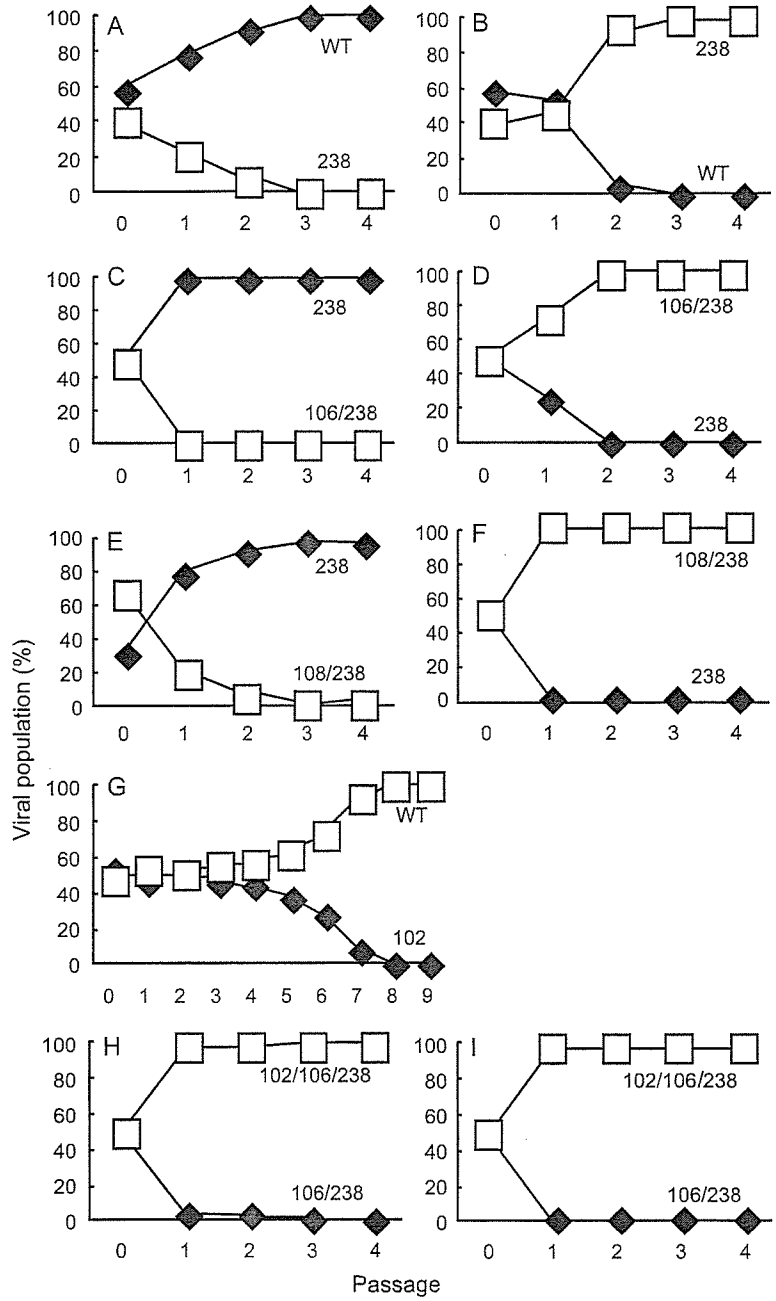

Fig. 5. Competitive HIV-1 replication assay for recombinant HIV-1s. Two infectious HIV-1 clones to be compared for their fitness were mixed and used to infect H9 cells in the absence (A, C, E, G, and H) and presence of NVP (B: 0.1 μM; D, F, and I: 1 μM). The cell-free supernatant was transferred to fresh H9 cells every 7 days. High-molecular-weight DNAs extracted from infected cells at the end of each passage were subjected to nucleotide sequencing, and proportion of Lys and Ser at position 238 (Graphs A and B), those of Val and Ala at position 106 (Graphs C and D), those of Val and Ile at position 108 (Graphs E and F), and those of Lys and Arg at position 102 (Graphs G–I) were determined.

The use of NVP has markedly increased worldwide because a single formulation of NVP combined with d4T and 3TC is a generic drug widely used in resource-limited situations (Cohen, 2003; Penzak et al., 2003). The use of NVP in pregnant women is also one of the main strategies to prevent mother-to-child transmission of HIV-1 (Guay et al., 1999; Marseille et al., 1999). Therefore, understanding of the mechanism of NVP resistance development is urgently needed.

In the present study, we identified a novel mutation, K238S, which was associated with a high multitude of NVP resistance when it was coupled with V106A or V108I. Interestingly, direct sequencing identified only K238S in the two patients infected with highly NVP-resistant HIV-1.

These results showed that V106A and V108I were reverting to their wild-type V106 and V108, respectively, in major viral populations in the plasma. Furthermore, the results indicate that K238S can remain longer than V106A and V108I in the absence of NVP treatment, partly because at least two nucleotide substitutions are required for K238S while V106A and V108I can occur with only a single nucleotide substitution.

M230 is located on the opposite side of Q151 in relation to the incoming dNTP, forming the primer grip of RT and contacting the primer strand (Huang et al., 1998; Sarafianos et al., 1999), and several mutations around this site were reported to occur in patients during NNRTI treatment. Parkin et al. (2000) observed the emergence of F227L and M230L during NVP-containing combination therapy and it was associated with the loss of NVP susceptibility of their clinical isolates. Demeter et al. (2000) reported that P236L occurred in subjects receiving delavirdine monotherapy. With regard to K238, Demeter et al. (1998) observed the emergence of K238T in one patient treated with atevirdine (an NNRTI) and AZT. Considering these reports, the region containing M230 and K238 is important for drug susceptibility and there may be more resistance-associated mutations around these sites.

Phylogenetic analysis showed a close relation between the isolates from Cases 33 and 23 (Fig. 2), suggesting that the two patients had acquired HIV-1 infection from the same source, although they did not know each other and interviewing them could not identify the source patient. This source patient presumably had been under antiretroviral treatment including NVP, which had failed to suppress his viral load because of the sets of mutations, K102R/V106A/K238S and V108I/K238S. It remains unknown whether K238S had existed as a polymorphism before the introduction of antiretroviral treatment, or it had emerged during NVP treatment in the source patient. Considering that a single dose of NVP to prevent mother-to-child transmission can induce resistant strains (Morris et al., 2003; Sullivan, 2003), some polymorphic mutations in treatment-naïve population could be associated with NVP resistance.

Materials and methods

Reagents and cells

AZT, d4T, and ABC were purchased from Sigma Co. (Tokyo, Japan). 3TC, NVP, and EFV were generously provided by Nippon Glaxo-Smithkline (Tokyo, Japan), Boehringer Ingelheim Pharmaceutics Inc. (Ridgefield, CT), and Merck Co. Inc. (Rahway, NJ), respectively.

H9 cells and COS-7 cells were grown in RPMI 1640 and Dulbecco's modified eagle medium (DMEM), respectively, supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). MAGIC-5 cells (CCR5 and CD4 expressing HeLa-LTR-β-gal cells) (Hachiya et al., 2001, 2003) were grown in DMEM supplemented with 10% FCS, hygromycin B (100 μg/ml) (Invitrogen Co., Carlsbad, CA), and blasticidin (1 μg/ml) (Funakoshi, Tokyo). Peripheral blood mononuclear cells (PBMCs) obtained from healthy donors were stimulated by phytohemagglutinin (PHA) in RPMI 1640-based medium containing interleukin-2 (20 U/ml) (R & D Systems, Minneapolis, MN) for 2 days before HIV-1 exposure.

Patients and clinical isolates

Clinical strains were isolated from fresh plasma of the study participants attending the outpatient clinic of the AIDS Clinical Center, International Medical Center of Japan, by using MAGIC-5 cells as described previously (Hachiya et al., 2001). The obtained isolates were stored at −80 °C until use and the infectivity was measured as blue cell-forming unit (BFU) of MAGIC-5 cells (Hachiya et al., 2001). The Institutional Review Board approved this study (IMCJ-H13-80) and a written informed consent was obtained from all the participants.

Case 33 was a 33-year-old homosexual man. He suffered from high fever and severe headache, probably symptoms of primary HIV-1 infection, in April 2000. On May 15, Western blotting showed faint bands against HIV-1 gp160, p68, p55, p25, and p18 antigens. One week after, these bands became bold and new bands against HIV-1 gp110, p40, and p34 antigens appeared. The CD4 count was 401/μl and HIV-1 viral load was 2.0×10^5/ml.

Case 23 was a 23-year-old homosexual man. He also suffered from severe headache and was hospitalized in January, 2001. Western blotting showed one faint band against HIV-1 gp160 antigen. In April, 2001, the band became bold and new bands against gp110, p68, p55, p52, gp41, p40, p34, p25, and p18 antigens appeared. The CD4 count was 210/μl and HIV-1 viral load was 7.1×10^4/ml.

Sequence analyses of HIV-1 RT and V3 region

Viral RNA was extracted from plasma and clinical isolates with Smi-test nucleic acid extraction kit (Genome Science, Fukushima, Japan). The HIV-1 RT gene was amplified by RT-PCR using One Step RNA PCR Kit (Takara Shuzo, Otsu, Japan). For plasma-derived RNA, nested PCR was conducted subsequently to amplify enough DNA for direct sequencing. The sequences of primer sets for the first PCR (T1 and T4) and the second PCR (T12 and T15) were as follows (Gatanaga et al., 1999; Hachiya et al., 2001, 2003): T1, 5′-AGGGGGAATTGGAGGTTT (RF positions, 1910 to 1927); T4, 5′-TTCTGTTAGTGCTTTGGTT (RF positions, 2939–2921); T12, 5′-CCAGTAAAATTAAAGCCAG (RF positions, 2091–2109); and T15, 5′-TCCCACTAACTTCTGTATGTC (RF positions, 2852–2832). The gp120 V3 domain of several HIV-1 isolates was amplified by RT-

PCR and nested PCR. The sequences of primer sets for the first PCR (ES1 and EA1) and the second PCR (ES2 and EA2) were as follows (Ida et al., 1997): ES1, 5′-AATGTCAGCACAGTACAATGTACAC (RF positions, 6502–6526); EA1, 5′-ACAATTTCTGGGTCCCCTCCTGAGGA (RF positions, 6890–6865); ES2, 5′-ATGGAATTAGGCCAGTAGTG (RF positions, 6527–6546); EA2, 5′-CTCCTAATTTTGTAACTAC (RF positions, 6829–6811). Specific precautions, including physical separation of processing areas, were taken to avoid template and amplified product carryover. Stringent quality control to prevent PCR contamination was employed to protect against cross-contamination of product DNA. Direct sequencing was performed using dye terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster, CA) and model 3700 automated DNA sequencer (Applied Biosystems). Amino acid sequences were deduced with the Genetyx-Win program version 4.1 (Software Development, Tokyo). For some PCR products, molecular cloning was performed with pT7 Blue T-Vector (Novagen, CA) and their sequences were analyzed.

For the phylogenetic analysis, RT gene and V3 sequences were aligned by Clustal-W program with reference sequences from the Los Alamos sequence database. The results of the alignment were then analyzed by the neighbor-joining method and the distance matrixes were generated with Kimura two-parameter model.

Bootstrap resampling (1000 data sets) of the multiple alignments was performed to test the statistical robustness of the tree.

Construction of recombinant HIV-1 clones

Recombinant infectious HIV-1 clones carrying various mutations in RT were constructed with a site-directed mutagenesis. Briefly, the desired mutations were introduced into *Xma*I–*Nhe*I region (759 bp) of pTZNX1, which encoded Gly-15 to Ala-267 of HIV-1 RT (strain BH 10), by the oligonucleotide-based mutagenesis method (Kodama et al., 2001). The *Xma*I–*Nhe*I fragment was inserted into pNL101-based plasmid (a kind gift from Jeang Kuan-The, NIAID/NIH, Bethesda, MD), generating various molecular clones with the desired mutations. Determination of the nucleotide sequences of plasmids confirmed that each clone had the desired mutations but no unintended mutations. Each molecular clone (10 μg/ml as DNA) was transfected into COS-7 cells (4×10^5 cells/100-mm-diameter dish) by Fugene transfection reagent (Roche Diagnosis, Basal, Switzerland). After 48 h, culture supernatants were harvested and stored at −80 °C until use. The infectivity of the viruses was determined as BFU in MAGIC-5 cells.

Drug susceptibility assay with MAGIC-5 cells

HIV-1 drug susceptibility to various RTIs was determined in triplicate by using MAGIC-5 cells as described previously (Hachiya et al., 2001). Briefly, MAGIC-5 cells were infected with adjusted virus stock (300 BFU) in the presence of increasing concentrations of RTIs, cultured for 48 h, fixed, and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Takara Shuzo). The blue-stained cells were counted under a light microscope. Drug concentrations that inhibited 50% of the stained cells of drug-free control (IC_{50}) were determined by referring to the dose–response curve.

Viral replication kinetic assays

PHA-stimulated PBMCs were exposed to each infectious virus preparation (300 BFU) in a final volume of 200 μl for 2 h. Infected cells were then washed and cultured in a volume of 200 μl in the absence or presence of NVP (1 μM). Assays were performed in triplicate and repeated at least three times using independently generated virus preparations. On days 0, 2, 4, 6, and 8 of infection, 100-μl aliquots of culture supernatants were filtered and stocked for measurements of p24 antigen concentration and replaced with equal amount of fresh medium with or without NVP. The concentrations of p24 in the supernatants were determined by chemiluminescence enzyme immunoassay (CLEIA) kit (Fuji-Rebio, Tokyo, Japan).

Competitive HIV-1 replication assay

Freshly prepared H9 cells (3×10^5) were exposed to virus preparations (300 BFU) to be examined for their replication ability and cultured in the presence or absence of NVP as described previously (Gatanaga et al., 2002; Kosalaraksa et al., 1999; Yoshimura et al., 1999). On day 1 in culture, one-third of the infected H9 cells were harvested and washed twice with PBS, and cellular DNA was extracted with Smi-test nucleic acid extraction kit. Purified DNAs were subjected to nested PCR for sequencing HIV-1 RT gene. Every 7 days, the supernatant of the virus culture was transmitted to new uninfected H9 cells, the cells harvested at each passage were subjected to direct DNA sequencing of HIV-1 RT gene, and the viral population change was determined by the relative peak height on sequencing electrogram. The persistence of the original amino acid substitution was confirmed for all infectious clones used in this assay.

Structural analysis of mutations in HIV-1 RT

To examine the interaction of HIV-1 RT with NVP, the SYBYL 6.7 (http://www.tripos.com/, Tripos Associates, St. Louis, MO) on a Silicon Graphics Octane2 workstation was employed. The site of the enzyme ligand complex was constructed based on the previously reported X-ray structure of the covalently trapped catalytic complex of HIV-1 RT with NVP (Protein Data Bank entry 1FKP) (Ren et al., 2000).

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Studies of nonnucleoside HIV-1 reverse transcriptase inhibitors. Part 1: Design and synthesis of thiazolidenebenzenesulfonamides

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Abstract—A random high-throughput screening (HTS) program to discover novel nonnucleoside reverse transcriptase inhibitors (NNRTIs) has been carried out with MT-4 cells against a nevirapine-resistant virus, HIV-$1_{IIIB\text{-}R}$. The primary hit, a thiazolidenebenzenesulfonamide derivative, possessed good activity. A systematic modification program examining various substituents at the 3-, 4-, and 5-positions on the thiazole ring afforded compounds with enhanced anti-HIV-1 and reverse transcriptase (RT) inhibitory activities. These results confirm the important role of the substituents at these positions and the thiazolidenebenzenesulfonamide motif as a valuable lead series for the next generation NNRTIs.

1. Introduction

Acquired immunodeficiency syndrome (AIDS), which is caused by the human immunodeficiency virus type 1 (HIV-1) and results in life-threatening opportunistic infections and malignancies, has become a major worldwide pandemic.[1,2] Three million people had died from AIDS and 40 million people were living with HIV-1 or AIDS at the end of 2003.[3] From the beginning of anti-HIV-1 chemotherapy development, HIV-1 reverse transcriptase (RT) has been one of the main targets, and the majority of drugs used clinically are RT inhibitors. RT inhibitors can be classified into two groups: nucleoside reverse transcriptase inhibitors (NRTIs), which act as chain terminators to block the elongation of the HIV-1 viral DNA strand, and nonnucleoside reverse transcriptase inhibitors (NNRTIs), which directly inhibit RT enzyme by binding to the allosteric site near the polymerase active site.[4]

NRTIs inhibit RT selectively but are considerably toxic to cellular and mitochondrial DNA synthesis.[5] In this regard, NNRTIs are more specific and less toxic than NRTIs because they do not affect the activity of cellular polymerases. On the other hand, during NNRTI monotherapy for HIV-1-infected patients[6] and in vitro culture

Keywords: Thiazolidene; Benzenesulfonamide; Nonnucleoside HIV-1 reverse transcriptase inhibitor.
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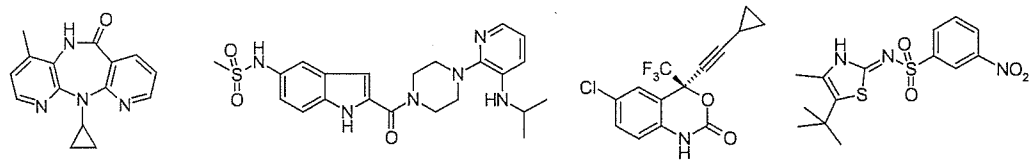


1 (nevirapine) 2 (delavirdine) 3 (efavirenz) 4

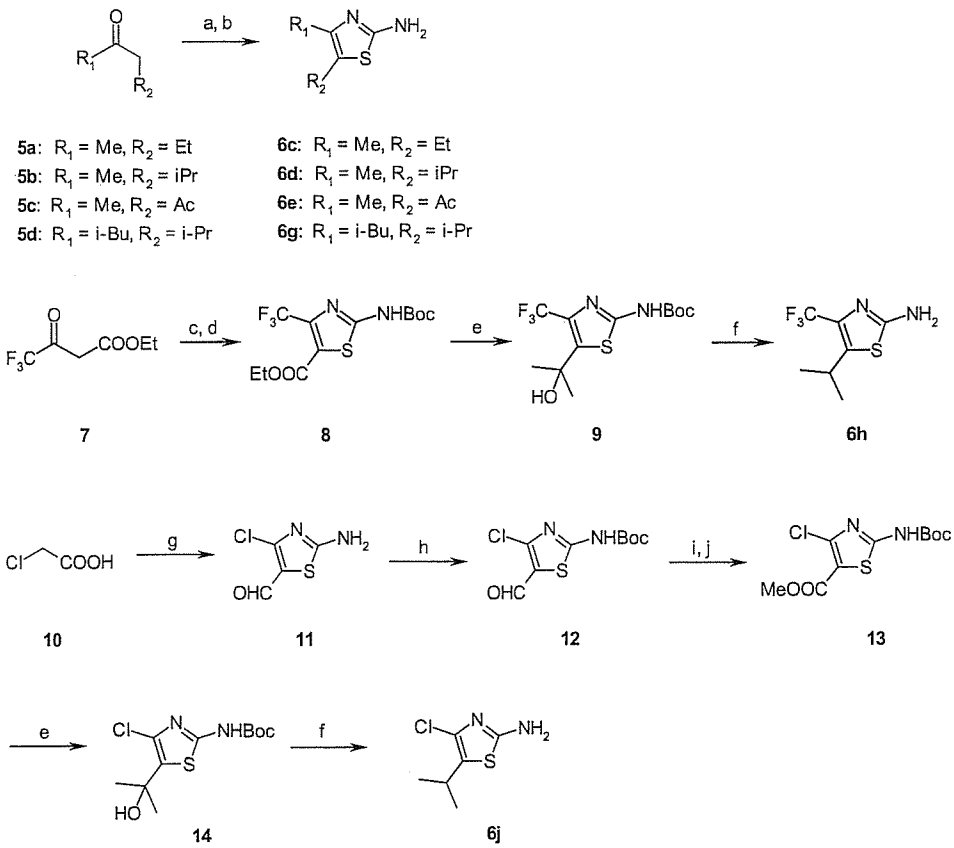
Figure 1. Structures of NNRTIs and lead compound **4**.

experiments,[7] rapid emergence of highly drug resistant viruses is often observed. In the case of nevirapine (**1**), the primary cause of drug resistance is the substitution of tyrosine by cysteine at the 181 position in HIV-1 RT (Y181C).[8] In fact, the Y181C mutant RT is less susceptible to nevirapine in vitro than the wild-type (WT) enzyme.[9] Furthermore, the RT mutation of the lysine at the 103 position to asparagine (K103N) is frequently observed in patients who have failed in the treatment with antiretroviral regimens containing currently available NNRTIs, such as nevirapine (**1**), delavirdine (**2**), and efavirenz (**3**).[10] Therefore, it is still important to find a new lead compound that may be able to overcome the resistance issue of NNRTIs (Fig. 1).

In this paper, we have examined the synthesis and structure–activity relationship (SAR) analysis of thiazolidenebenzenesulfonamide derivatives and found that a novel class of NNRTIs is active against the Y181C and K103N mutants.

2. Chemistry

A series of 4,5-dialkyl-2-aminothiazole derivatives, **6c–e** and **6g**, were synthesized (Scheme 1). The α-bromination of ketones **5a–d** under thermodynamic conditions[11] and subsequent cyclization using thiourea afforded 4,5-dialkyl-2-aminothiazoles **6c–e** and **6g**. 4-Trifluoromethyl-2-



5a: R_1 = Me, R_2 = Et
5b: R_1 = Me, R_2 = iPr
5c: R_1 = Me, R_2 = Ac
5d: R_1 = i-Bu, R_2 = i-Pr

6c: R_1 = Me, R_2 = Et
6d: R_1 = Me, R_2 = iPr
6e: R_1 = Me, R_2 = Ac
6g: R_1 = i-Bu, R_2 = i-Pr

7 8 9 6h

10 11 12 13

14 6j

Scheme 1. Reagents: (a) TMSBr, DMSO/CH_3CN; (b) thiourea/EtOH; (c) thiourea/I_2, *i*-PrOH; (d) Boc_2O, DMAP/THF; (e) MeLi/THF; (f) Et_3SiH/TFA; (g) thiourea, $POCl_3$, $Ca(OH)_2$, NaCl/DMF; (h) Boc_2O, DMAP/dioxane; (i) $NaClO_2$, KH_2PO_4, 2-methyl-2-butene/*t*-BuOH; (j) MeOH, water soluble carbodiimide, HOBt, DMAP/DMF.