ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1995, p. 1888–1891 Vol. 39, No. 8
0066-4804/95/$04.00+0

K65R Mutation of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Encodes Cross-Resistance to 9-(2-Phosphonylmethoxyethyl)adenine

ZHENGXIAN GU,[1] HORACIO SALOMON,[1] JULIE M. CHERRINGTON,[2]* ANDREW S. MULATO,[2] MING S. CHEN,[2] ROBERT YARCHOAN,[3] ANDREA FOLI,[3] KRISTINA M. SOGOCIO,[3] AND MARK A. WAINBERG[1]

McGill University AIDS Centre, Jewish General Hospital, Montreal, Quebec, Canada H3T 1E2[1]; Gilead Sciences, Inc., Foster City, California 94404[2]; and Medicine Branch, National Cancer Institute, Bethesda, Maryland 20892[3]

Received 12 April 1995/Returned for modification 15 May 1995/Accepted 6 June 1995

Cloned variants of human immunodeficiency virus type 1 that contain the K65R mutation in reverse transcriptase have previously been shown to display approximately 10- to 30-fold resistance against 2′,3′-dideoxycytidine, 2′,3′-dideoxyinosine, and 2′,3′-dideoxy-3′-thiacytidine. On the basis of tissue culture studies with both primary T cells and established cell lines, we now report that the K65R mutation confers approximately 12- to 15-fold resistance to 9-(2-phosphonylmethoxyethyl)adenine (PMEA). Likewise, a chain termination system revealed that mutated recombinant K65R reverse transcriptase displays resistance to PMEA diphosphate, the active metabolite of PMEA, in cell-free enzyme assays. Parallel studies have shown that the M184V mutation in reverse transcriptase, associated with high-level resistance against the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine, does not confer resistance to PMEA in tissue culture. Viruses and enzymes that included both the K65R and M184V mutations were resistant to PMEA and PMEA diphosphate, respectively, but only to the extent conferred by the K65R mutation alone.

The reverse transcriptase (RT) enzyme of retroviruses plays a key role in the viral life cycle and is therefore an important target for inhibition by antiviral drugs (16). However, the error-prone nature of this enzyme has resulted in a number of mutations in the RT open reading frame that are associated with resistance to these compounds (1, 13, 15, 17, 19, 22). Drug-resistant variants of human immunodeficiency virus (HIV) have been isolated from patients undergoing prolonged therapy with antiviral drugs as well as through tissue culture selection in the presence of increasing concentrations of these antiviral compounds (1, 5, 7, 14, 20, 23). A series of five distinct mutations is associated with resistance against 3′-azido-3′-deoxythymidine (AZT) (13, 15). A K65R mutation is associated with resistance against 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyinosine (ddI), and the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine (3TC) (6, 8, 9, 26). An L74V mutation also confers resistance to ddI and ddC (22). Finally, an M184V substitution is associated with high-level resistance to 3TC and lower levels of resistance to ddI and ddC (9, 10).

9-(2-Phosphonylmethoxyethyl)adenine (PMEA) is active

TABLE 1. Sensitivities of HIV-1 variants to antiviral drugs[a]

Virus strain or clinical isolate	Culture	EC_{50} (μM) of[b]:				
		AZT	PMEA	3TC	ddC	ddI
Strains						
HXB2D	MT-2	0.1 ± 0.07	10 ± 1.6	1.0 ± 0.01	2.0 ± 0.5	4.0 ± 0.5
HXB2D(K65R)	MT-2	0.1 ± 0.03	>120 ± 0	40 ± 3.5	>30 ± 0	20 ± 1.1
HXB2D	CBMC	0.01 ± 0.002	0.22 ± 0.03	1.1 ± 0.1	0.8 ± 0.1	6.4 ± 0.4
HXB2D(K65R)	CBMC	0.01 ± 0.001	3.5 ± 0.5	20.6 ± 3.2	10.3 ± 1.2	22.7 ± 0.9
HXB2D(M184V)	CBMC	0.005 ± 0.001	0.3 ± 0.05	740 ± 101	2.2 ± 0.3	15.5 ± 1.9
HXB2D(K65RM184V)	CBMC	0.01 ± 0.0005	5.01 ± 0.7	825 ± 63	2.9 ± 0.4	18.5 ± 2.2
Clinical isolates[c]						
1 (AZT^r)	CBMC	1.25 ± 0.2	0.17 ± 0.02	ND[d]	0.4 ± 0.06	9.5 ± 1.8
2 (AZT^r)	CBMC	0.73 ± 0.1	0.46 ± 0.1	ND	0.8 ± 0.1	7.0 ± 1.4
3 (ddC^r)	CBMC	0.08 ± 0.01	6.5 ± 1.3	ND	9.3 ± 2.0	32.6 ± 5.9
4 (ddC^r)	CBMC	0.04 ± 0.01	8.2 ± 1.9	ND	12.6 ± 2.3	29.4 ± 2.8

[a] Results for CBMCs were calculated on the basis of p24 antigen levels in culture fluids. Results for MT-2 cells were calculated on the basis of XTT assays.
[b] EC_{50}, 50% effective concentration. Values represent the means ± standard deviations of four different experiments.
[c] Clinical isolates 1 and 2 were derived from patients who had received AZT monotherapy for 12 and 16 months, respectively. Clinical isolates 3 and 4 were derived from patients who had received ddC monotherapy for 6 and 8 months, respectively.
[d] ND, not done.

* Corresponding author. Mailing address: Gilead Sciences, Inc., 353 Lakeside Dr., Foster City, CA 94404. Phone: (415) 573-4837. Fax: (415) 573-4890.

against several herpesviruses (3, 4) and retroviruses (18) and has shown anti-HIV activity in clinical trials (11). This compound is an acyclic adenine derivative; its active intracellular form is PMEA diphosphate (PMEApp), a competitive inhibitor of HIV type 1 (HIV-1) RT with regard to dATP substrate (2). HIV resistance to PMEA has occurred in tissue culture protocols, and a K65R mutation has been observed to develop in HIV cultured in the presence of this drug (21a, 24). In addition, PMEA is a derivative of deoxyadenosine, suggesting the possibility of cross-resistance with ddI and/or ddC. For these reasons, we explored whether either the K65R or M184V mutation confers resistance to PMEA both in tissue culture and in a cell-free assay that employs recombinant HIV-1 RT to generate minus (−) strong-stop DNA.

(Portions of this work were performed by Z. Gu in partial fulfillment of the requirements for a Ph.D. degree, Faculty of Graduate Studies and Research, McGill University.)

Tissue culture studies. The construction of recombinant HIV variants that contain either the K65R or M184V substitution or both of these substitutions has been previously described (8, 9). Wild-type (WT) cloned HXB2D infectious HIV was kindly supplied by R. C. Gallo, National Institutes of Health, Bethesda, Md. The viruses relevant to this study are named HXB2D(K65R), HXB2D(M184V), and HXB2D (K65RM184V) and carry K65R, M184V, and joint K65R and M184V mutations, respectively. Both cord blood mononuclear cells (CBMCs) and MT-2 cells were used for viral replication to high titers and for determinations of drug sensitivity as described elsewhere (9, 21). Previous research has shown that CBMCs may be used in place of peripheral blood mononuclear cells to generate equivalent results in drug sensitivity assays (21). Experiments conducted with CBMCs were carried out according to the protocol of the AIDS Clinical Trials Group and U.S. Department of Defense (12). Drug concentrations that inhibited viral replication by 50% were determined on the basis of p24 antigen levels in culture supernatants by using a kit (Abbott Laboratories, North Chicago, Ill.).

Experiments conducted with MT-2 cells were carried out by a modified assay with 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) (25). Briefly, 96-well plates were set up as previously described, except that assays were done in the absence of Polybrene and that the infection proceeded for 3 h rather than 1 h before seeding. At 5 days postinfection, XTT and *N*-methylphenazonium methosulfate were added to each well for 1 h and then mixed and read at 450 nm. Data were plotted and fitted to a curve with a Deltagraph Professional program. The 50% effective concentrations of the drugs were defined as those concentrations that reduced killing due to virus infection by 50%.

The results in Table 1 show that both the K65R and K65RM184V mutated viruses displayed decreased sensitivity to PMEA, ddC, ddI, and 3TC in both MT-2 cells and CBMCs. Control studies indicated that each of these viruses retained sensitivity to AZT. However, whereas the M184V substitution conferred high-level resistance to 3TC and low-level resistance to ddC and ddI in CBMCs, it did not confer diminished sensitivity to PMEA. It should be noted that the 50% inhibitory concentrations obtained from PMEA varied among the different cell types tested. This may reflect the different efficiencies of diphosphorylation of PMEA to its active form in different cell types.

Parallel studies showed that clinical isolates resistant to AZT did not display reduced sensitivity to PMEA. In contrast, the clinical isolates that were resistant to ddC did display cross-resistance to PMEA.

Cell-free assays. Recombinant mutated forms of RT con-

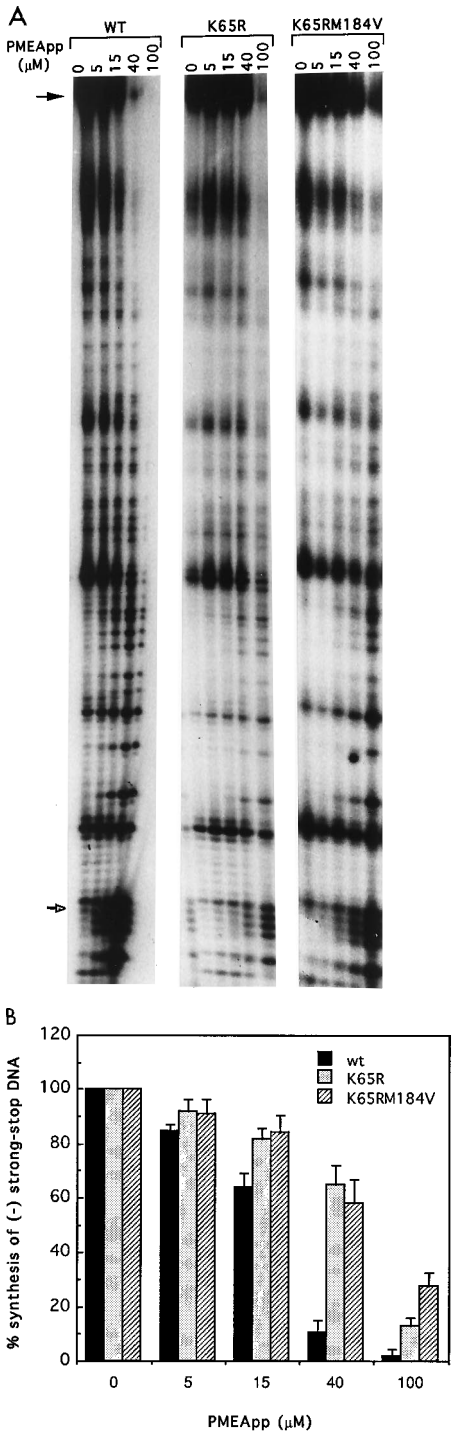

FIG. 1. Effects of PMEApp on reverse transcription. (A) Reverse transcription assays were performed with HIV-PBS RNA template and [γ-^{32}P]ATP-labeled dPR primer in the presence and absence of PMEApp. Full-length (−) strong-stop DNA products are indicated by a solid arrow. The open arrow designates a cluster of chain termination products caused by incorporation of PMEApp. (B) Dose-dependent inhibitory effect of PMEApp on synthesis of (−) strong-stop DNA. The results shown were calculated from the intensities of full-length (−) strong-stop DNA products, as analyzed by phosphorimaging. The relative intensities of the full-length products generated in the presence of PMEApp were divided by those obtained in the absence of drug. Bars show standard deviations.

taining the K65R mutation have previously been shown to cause diminished chain termination efficiency with regard to ddCTP, ddATP, and 3TCTP (8). To determine whether similar results would be obtained in the case of the active metabolite of PMEA, i.e., PMEApp, we employed a cell-free assay to monitor chain termination in the presence of this drug. Both WT and mutated forms of RT, containing either the K65R or M184V mutation or both of these substitutions (i.e., K65RM184V), were assessed in the presence of a viral RNA template, termed HIV-PBS (containing primer-binding site) sequences, 5′ unique and repeat regions of HIV genomic RNA, and oligodeoxynucleotide (dPR) complementary to the PBS as a primer, as previously described (8). The reactions were performed at 37°C in volumes of 20 μl containing 50 mM Tris-HCl (pH 7.8), 75 mM KCl, 10 mM $MgCl_2$, 250 μM (each) the four deoxynucleoside triphosphates, 50 nM HIV-PBS RNA template, 100 nM dPR primer, and different concentrations of PMEApp. After 60 min, the reactions were stopped and the DNA products were electrophoresed on denaturing 5% polyacrylamide gels. Band intensities with regard to (−) strong-stop DNA were analyzed by phosphorimaging. The results shown in Fig. 1A show that PMEApp caused a diminution in the levels of (−) strong-stop DNA product in a concentration-dependent fashion with WT and mutated RT enzymes. The addition of increasing concentrations of PMEApp also led to increased levels of chain termination. However, in each instance we observed that more (−) strong-stop DNA was produced in the presence of PMEApp by mutated K65R and K65RM184V RTs than by WT enzymes. In addition, less chain termination was effected by PMEApp in the case of these mutated enzymes.

Previous findings have shown that the increased synthesis of full-length (−) strong-stop DNA in this assay can be correlated with diminished nucleoside analog-mediated chain termination (8). No apparent differences were shown to exist between the K65R and K65RM184V enzymes with regard to PMEApp in these experiments. In other assays, M184V mutant RT did not display resistance to PMEApp (data not shown). These results suggest that the M184V mutation does not play an important role in the recognition of PMEApp.

We further calculated the inhibitory efficiencies for these various enzymes by dividing the intensities of the full-length (−) strong-stop DNA products formed in the absence of PMEApp by those generated in the presence of various concentrations of this inhibitor. This dose-dependent inhibitory effect is shown in Fig. 1B. These findings are consistent with the tissue culture susceptibility data presented in Table 1.

In summary, our results indicate that the K65R mutation but not the M184V mutation confers resistance against PMEA. It will be of interest to determine whether viruses that are isolated from patients receiving prolonged PMEA therapy will also contain the K65R substitution.

We thank the Medical Research Council of Canada for grant support.

We also thank Mick Hitchcock, Howard Jaffe, John Martin, and Jay Toole for helpful discussions.

REFERENCES

1. **Balzarini, J., A. Karlsson, A.-M. Vandamme, M.-J. Perez-Perez, H. Zhang, L. Vrang, B. Oberg, K. Backbro, T. Unge, A. San Felix, S. Velazquez, M.-J. Camarasa, and E. De Clercq.** 1993. Human immunodeficiency virus type 1 (HIV-1) strains selected for resistance against the HIV-1-specific [2′,5′-bis-*O*-(tert-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)]-β-D-pentofuranosyl (TSAO) nucleoside analogs retain sensitivity to HIV-1-specific nonnucleoside inhibitors. Proc. Natl. Acad. Sci. USA **90:** 6952–6956.
2. **Cherrington, J. M., S. J. W. Allen, N. Bischofberger, and M. S. Chen.** Kinetic interaction of the disphosphates of 9-(2-phosphonylmethoxyethyl) adenine and other anti-HIV active congeners with HIV reverse transcriptase and human DNA polymerases β and γ. Antivir. Chem. Chemother., in press.
3. **De Clercq, E., A. Holy, I. Rosenberg, T. Sakuma, J. Balzarini, and P. C. Maudgal.** 1986. A novel selective broad-spectrum anti-DNA virus agent. Nature (London) **323:**464–467.
4. **De Clercq, E., T. Sakuma, M. Baba, R. Pauwels, J. Balzarini, I. Rosenberg, and A. Holy.** 1987. Antiviral activity of phosphonylmethoxyalkyl derivatives of purines and pyrimidines. Antivir. Res. **8:**261–272.
5. **Fitzgibbon, J. E., R. M. Howell, C. A. Haberzettl, S. J. Sperber, D. J. Gocke, and D. T. Dubin.** 1992. Human immunodeficiency virus type 1 *pol* gene mutations which cause decreased susceptibility to 2′,3′-dideoxycytidine. Antimicrob. Agents Chemother. **36:**153–157.
6. **Gao, Q., Z. Gu, M. A. Parniak, J. Cameron, N. Cammack, C. Boucher, and M. A. Wainberg.** 1993. The same mutation that encodes low-level human immunodeficiency virus type 1 resistance to 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine confers high-level resistance to the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. **37:**1390–1392.
7. **Gao, Q., Z. Gu, M. A. Parniak, X. Li, and M. A. Wainberg.** 1992. In vitro selection of variants of human immunodeficiency virus type 1 resistant to 3′-azido-3′-deoxythymidine and 2′,3′-dideoxyinosine. J. Virol. **66:**12–19.
8. **Gu, Z., E. J. Arts, M. A. Parniak, and M. A. Wainberg.** 1995. Mutated K65R recombinant HIV-1 reverse transcriptase shows diminished chain termination in the presence of 2′,3′-dideoxycytidine-5′-triphosphate and other drugs. Proc. Natl. Acad. Sci. USA **92:**2760–2764.
9. **Gu, Z., Q. Gao, H. Fang, H. Salomon, M. A. Parniak, E. Goldberg, J. Cameron, and M. A. Wainberg.** 1994. Identification of a mutation at codon 65 in the IKKK motif of reverse transcriptase that encodes human immunodeficiency virus resistance to 2′,3′-dideoxycytidine and 2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. **38:**275–281.
10. **Gu, Z., Q. Gao, X. Li, M. A. Parniak, and M. A. Wainberg.** 1992. Novel mutation in the human immunodeficiency virus type 1 reverse transcriptase gene that encodes cross-resistance to 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine. J. Virol. **66:**7128–7135.
11. **Jaffe, H.** 1994. Clinical trials of acyclic nucleotide analogs. Vth International Antiviral Symposium, Nice, France.
12. **Japour, A. J., D. L. Mayers, V. A. Johnson, D. R. Kuritzkes, L. A. Beckett, J.-M. Arduino, J. Lane, R. J. Black, P. S. Reichelderfer, R. T. D'Aquila, C. S. Crumpacker, The RV-43 Study Group, and The AIDS Clinical Trials Group Virology Committee Resistance Working Group.** 1993. Standardized peripheral blood mononuclear cell culture assay for determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. Antimicrob. Agents Chemother. **37:**1095–1101.
13. **Kellam, P., C. A. B. Boucher, and B. A. Larder.** 1992. Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine. Proc. Natl. Acad. Sci. USA **89:**1934–1938.
14. **Larder, B. A., A. G. Darby, and D. D. Richman.** 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science **243:**1731–1734.
15. **Larder, B. A., and S. D. Kemp.** 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science **246:** 1155–1158.
16. **Mitsuya, H., R. F. Jarrett, M. Matsukura, F. D. M. Veronese, A. L. DeVico, M. G. Sarngadharn, D. G. Johns, M. S. Reitz, and S. Broder.** 1987. Long-term inhibition of human T-lymphotropic virus III/lymphadenopathy-associated virus (human immunodeficiency virus) DNA synthesis and RNA expression in T cells protected by 2′,3′-dideoxynucleotides in vitro. Proc. Natl. Acad. Sci. USA **84:**2033–2037.
17. **Nunberg, J. H., W. A. Schleif, E. J. Boots, J. A. O'Brien, J. C. Quintero, J. M. Hoffmann, E. A. Emini, and M. E. Goldman.** 1991. Viral resistance to human immunodeficiency virus type 1-specific pyridinone reverse transcriptase inhibitors. J. Virol. **65:**4887–4892.
18. **Pauwels, R., J. Balzarini, D. Schols, M. Baba, J. Desmyter, I. Rosenberg, A. Holy, and E. De Clercq.** 1988. Phosphonylmethoxyethyl purine derivatives, a new class of anti-human immunodeficiency virus agents. Antimicrob. Agents Chemother. **32:**1025–1030.
19. **Richman, D., C. K. Shih, I. Lowy, J. Rose, P. Prodanovich, S. Goff, and J. Griffin.** 1991. Human immunodeficiency virus type 1 mutant resistant to non-nucleoside inhibitors of reverse transcriptase arise in tissue culture. Proc. Natl. Acad. Sci. USA **88:**11241–11245.
20. **Rooke, R., M. Tremblay, H. Soudeyns, L. DeStephano, X.-J. Yao, M. Fanning, J. S. G. Montaner, M. O'Shaughnessy, K. Gelmon, C. Tsoukas, H. Ruedy, and M. A. Wainberg.** 1989. Isolation of drug-resistant variants of HIV-1 from patients on long-term zidovudine (AZT) therapy. AIDS **3:**411–415.
21. **Salomon, H., A. Belmonte, K. Nguyen, Z. Gu, M. Gelfand, and M. A. Wainberg.** 1994. Comparison of cord blood and peripheral blood mononuclear cells as targets for viral isolation and drug sensitivity studies involving human immunodeficiency virus type 1. J. Clin. Microbiol. **32:**2000–2002.

21a. **Sogocio, K., A. Foli, and R. Yarchoan.** Unpublished observations.
22. **St. Clair, M. H., J. L. Martin, G. Tudor-Williams, M. C. Bach, C. L. Vavro, D. M. King, P. Kellam, S. D. Kemp, and B. A. Larder.** 1991. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science **253:**1557–1559.
23. **Vandamme, A.-M., Z. Debyser, R. Pauwels, K. D. Vreese, P. Goubau, M. Youle, B. Gazzard, P. A. Stoffels, G. F. Cauwenbergh, J. Anne, K. Andries, P. A. Janssen, J. Desmyter, and E. De Clercq.** 1993. Characterization of HIV-1 strains isolated from patients treated with TIBO R82913. AIDS Res. Hum. Retroviruses **10:**39–46.
24. **Vasudevachari, M. B., C. Battista, H. C. Lane, and N. P. Salzman.** 1993. Inhibition of HIV-1 by 9-(2-phosphonylmethoxyethyl) adenine (PMEA) and in vitro selection of variants of HIV-1 resistant to PMEA. Abstract. ASM Conference on Molecular Diagnostics and Therapeutics, Moran, Wyo.
25. **Weislow, O. S., R. Kiser, D. L. Fine, J. Bader, R. H. Shoemaker, and M. R. Boyd.** 1989. New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. J. Natl. Cancer Inst. **81:**577–586.
26. **Zhang, D., A. M. Caliendo, J. J. Eron, K. M. DeVore, J. C. Kaplan, M. S. Hirsch, and R. T. D'Aquila.** 1994. Resistance to 2′,3′-dideoxycytidine conferred by a mutation in codon 65 of the human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. **38:**282–287.