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In vitro selection and molecular characterization of human immunodeficiency virus type 1 with reduced sensitivity to 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA)

Andrea Foli[a], Kristina M. Sogocio[a], Barry Anderson[a], Mark Kavlick[a], M. Wayne Saville[a], Mark A. Wainberg[b], Zhengxian Gu[b], Julie M. Cherrington[c], Hiroaki Mitsuya[a], Robert Yarchoan[a,*]

[a] *Medicine Branch, National Cancer Institute, Bethesda, MD 20892-1906, USA*
[b] *McGill University AIDS Centre, Jewish General Hospital, Montreal, Quebec, Canada H3T 1E2*
[c] *Gilead Sciences Inc., Foster City, CA 94404, USA*

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Abstract

9-[2-(Phosphonomethoxy)ethyl]adenine (PMEA) is an acyclic nucleotide with potent in vitro activity against human immunodeficiency virus type 1 (HIV-1). The present study was undertaken to determine whether HIV-1 resistance to PMEA could be generated by in vitro selection and if so, to determine which mutations in reverse transcriptase (RT) were responsible. HIV-1_{LAI} was serially passaged for 10 months in the presence of increasing concentrations of PMEA up to a maximum of 40 μM. After 40 passages, the 50% inhibitory concentration (IC_{50}) of PMEA had increased almost 7-fold from 4.45 to 30.5 μM. Some cross-resistance to 2′,3′-dideoxycytidine (ddC, zalcitabine), 2′,3′-dideoxyinosine (ddI, didanosine), and 3′-thiacytidine (3TC, lamivudine) was also observed, but no cross-reactive resistance to 3′-azido-3′-thymidine (AZT, zidovudine). Sequencing of the RT encoding region of each of eight *pol* clones from resistant isolates revealed a Lys-65 → Arg (K65R) substitution. HIV with the K65R mutation inserted by site-directed mutagenesis also had decreased sensitivity to PMEA in H9 cells and a similar cross-resistance profile. Thus, HIV can develop decreased sensitivity to PMEA after long-term in vitro exposure and this change is associated with a K65R substitution. Additional studies will be needed to determine whether a similar mutation in HIV RT develops in patients receiving PMEA or its orally bioavailable prodrug adefovir dipivoxil (bis-POM PMEA).

Keywords: HIV-1; Drug-resistant virus; Reverse transcriptase mutations

* Corresponding author. Bldg. 10, Rm. 12N226, National Institutes of Health, Bethesda, MD 20892-1906. Tel.: +301 4960328; fax: +301 4023645.

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1. Introduction

9-[2-(Phosphonomethoxy)ethyl]adenine (PMEA), a member of the family of phosphonomethylalkylpurines, has potent in vitro activity against a broad range of retroviruses and herpesviruses (De Clercq et al., 1986; Pauwels et al., 1988). This acyclic nucleotide has antiretroviral activity in several animal models and, like the related acyclic drug (*R*)-9-(2-phosphonylmethoxypropyl)adenine (PMPA), can prevent de novo infection of macaques by simian immunodeficiency virus (Balzarini et al., 1989, 1990, 1991a; Tsai et al., 1994, 1995). PMEA has also been found to have clinical activity against human immunodeficiency type-1 (HIV-1) in early trials, and further studies are ongoing to evaluate an orally bioavailable pro-drug of PMEA, 9-[2-(bispivaloyloxymethyl)phosphonylmethoxyethyl]adenine (bis-POM PMEA, adefovir dipivoxil), in patients with HIV infection (Collier et al., 1993; Starrett et al., 1992; Walker et al., 1993). Upon entering cells, PMEA is phosphorylated to its active moiety, PMEA diphosphate (PMEApp) which acts as a competitive inhibitor of HIV reverse transcriptase (RT) with regard to deoxyadenosine-5′-triphosphate (dATP)(Balzarini et al., 1991b).

HIV RT exhibits considerable infidelity during viral replication. The clinical use of RT inhibitors is associated with the emergence of resistant HIV strains with RT mutations (Boucher et al., 1990; Larder and Kemp, 1989; Richman, 1993; Shirasaka et al., 1993). There is variation among drugs in the degree and rapidity of resistance, and consideration of this phenomenon is proving to be important in the design of effective antiretroviral strategies. Resistant strains of HIV can commonly be generated in vitro by propagation in the presence of increasing drug concentrations; moreover, the mutation patterns in such strains can predict those developing in patients receiving the drug (Gao et al., 1993a; Richman et al., 1991; Schinazi et al., 1993). With this background, we undertook to generate HIV resistance to PMEA by in vitro passage and to characterize the cross-resistance patterns and RT mutations in the generated resistant virus.

2. Materials and methods

2.1. Cells and virus

H9 cells (gift of Robert C. Gallo) were used to propagate virus in the presence of PMEA, and MT-2 cells (Harada et al., 1985) were used to assay for the presence of infectious virus by its cytopathic effect (CPE). The cells were maintained in culture in RPMI-1640 medium (GIBCO Laboratories, Gaithersburg, MD) supplemented with 15% heat-inactivated fetal calf serum (FCS) (Hyclone Laboratories, Logan UT), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco), referred to as complete medium (CM). The strains of HIV-1 used were HIV-1_{LAI} (ABI, Columbia, MD), cloned wild-type HXB2D (gift of Robert C. Gallo), and the constructed virus HXB2D(K65R) in which the K65R mutation has been inserted into HXB2D by site-directed mutagenesis (Gu et al., 1994). The HIV-1_{LAI} used for the initial infection of the H9 cells was concentrated 1000-fold by pelleting. The 50% tissue culture infectious dose ($TCID_{50}$) of the virus preparations was determined in the relevant target cells using standard techniques (Johnson and Byington, 1990).

2.2. Anti-HIV drugs

PMEA was provided by Gilead Sciences, Foster City, CA. 3′-Azido-2′,3′-dideoxythimidine (AZT), 2′,3′-dideoxycytidine (ddC), and 2′,3′-dideoxyinosine (ddI) were purchased from Calbiochem, San Diego, CA. The negative enantiomer of 2′,3′-dideoxy-3′-thiacytidine (3TC) was the gift of Dr Raymond F. Schinazi, Veterans Affairs Medical Center, Decatur, GA, and nevirapine was provided by Dr Vincent J. Merluzzi, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT. All drugs were diluted in PBS and stored at −20°C prior to use; dimethyl sulfoxide (DMSO) was used to help solubilize the nevirapine.

2.3. Selection and testing of PMEA-resistant isolates

1×10^6 H9 cells were exposed for 1 h to 100

$TCID_{50}$/cell of HIV_{LAI} and then cultured in the presence or absence of 1 μM PMEA in 50 ml of CM in 75-cm^2 flasks (Corning Inc., Corning, NY) at 37°C in humidified air supplemented with 5% CO_2. After 2 days, the cells were washed to remove residual virus and resuspended in fresh media with the addition of PMEA as appropriate. After 5 additional days, the culture supernatant was harvested and filtered through a 0.45-μm filter (Nalgene, Rochester, NY). Subsequent passages were performed in the same manner except that 25 ml of the culture supernatant from the previous passage were used to infect the fresh H9 cells. The time for each passage was 1 week. Aliquots of supernatant were also periodically frozen at −80°C. The concentration of PMEA in the cultures was increased over 40 passages from 1 to 40 μM. The initial starting concentration was based on reported values of the 50% inhibitory concentration (IC_{50}) of PMEA in H9 cells in the range 0.4–8 μM (Balzarini et al., 1991a; Smith et al., 1989). The supernatants were monitored for the presence of infectious HIV by assessing the CPE in MT-2 cells. Also, p24 production was monitored in the supernatants by radioimmune assay (DuPont, Wilmington, DE). As a control for spontaneous changes in the HIV that may have occurred by serial passage, HIV was passaged in parallel in the absence of PMEA (medium-passaged virus).

Testing of viral sensitivities to drugs in peripheral blood mononuclear cells (PBMC) was performed according to the protocol of the AIDS Clinical Trials Group and the US Department of Defense (Japour et al., 1993). The degree of viral replication was assessed on the basis of p24 antigen production. Testing of drug sensitivities in H9 cells was performed using a modification of the method of Shirasaka et al. (1993). The H9 cells were pre-exposed to virus for 1 h, washed, and cultured in the presence of various concentrations of drugs in quadruplicate for 7 days. Virus production was assessed by p24 antigen production, and the IC_{50} calculated by linear regression.

2.4. Viral RT nucleotide sequencing

Cellular DNA was extracted from H9 cells infected with HIV, and a 690-bp fragment of the HIV-1 *pol* gene was amplified by 35 cycles of polymerase chain reaction (PCR) using the primer pairs AS-71 (5′-GTACCAGTAGAATTCA-AGCCAGGA-3′) and OS-11 (5′-TTCATAACC-CATGAGGAATGG-3′). This segment includes nearly all the HIV mutations that have been previously identified as being associated with resistance to nucleoside and non-nucleoside RT inhibitors (Schinazi et al., 1994). The PCR product was extracted, digested with *Xba*I and *Eco*RI, and ligated into the pTZ19R-based vector pTZT. Competent *Escherichia coli* cells (strain DH5α; GIBCO/BRL) were then transformed with the *pol*-inserted pTZT and cloned on ampicillin-selective agar plates. Plasmid DNA was isolated after colony expansion by the use of Qiagen Plasmid Kit (Chatsworth, CA) and sequenced by the dideoxy-chain termination method using the Sequenase DNA Sequencing Kit, Version 2.0 (United States Biochem, Cleveland, OH).

3. Results

3.1. In vitro selection of a PMEA-resistant strain

To determine whether a PMEA-resistant strain could be established in vitro, $HIV\text{-}1_{LAI}$ was cultured in H9 cells in the presence of successively greater concentrations of drug, starting at 1 μM. Parallel cultures of virus were maintained in the absence of PMEA. After two passages, the concentration of PMEA was increased to 4 μM; then, after passage 17, the concentration was increased 1.5–2.5-fold every 6–8 passages until a concentration of 40 μM was attained at passage 40. Each time the concentration of PMEA was increased in the cultures, the level of HIV-1 p24 Ag production initially declined but then increased again after several further passages (results not shown). By the 40th passage, HIV was able to replicate well in the presence of 40 μM PMEA, a concentration that was completely inhibitory for the pre-passaged virus. As higher concentrations of PMEA inhibited H9 cell proliferation, this passage-40 virus was utilized for studies of drug sensitivity.

Table 1
Susceptibility of HIV-1 variants to anti-HIV drugs[a]

Virus	IC_{50} (μM):					
	PMEA	AZT	ddI	ddC	3TC	Nevirapine
$HIV\text{-}1_{LAI}$ pre-cultured	4.45 (0.65)	0.032 (0.027)	0.465 (0.3)	0.038 (0.036)	0.022 (0.012)	0.028 (0.004)
HIV-1 media-passaged[b]	7.93 (5.57)	0.044 (0.027)	1.65 (0.640)	0.068 (0.022)	0.086 (0.024)	0.111 (0.049)
HIV-1 PMEA-passaged[c]	30.5 (5.30)	0.010 (0.008)	4.64 (0.135)	0.295 (0.115)	0.805 (0.100)	0.024 (0.017)

[a] Susceptibilities were tested in H9 cells as described in Section 2. IC_{50} represents the inhibitory concentration 50% calculated on the basis of the HIV-1 p24 Ag levels in culture supernatants; values shown are the average of two independent experiments, number in parentheses represents $\pm$ S.E.M..
[b] $HIV\text{-}1_{LAI}$ after 40 passages in medium without PMEA.
[c] $HIV\text{-}1_{LAI}$ after 40 passages in the presence of increasing concentrations of PMEA as described in Section 2.

3.2. Assessment of drug activity against passaged virus

After passage 10, the IC_{50} of PMEA was no different than for the pre-selected virus (results not shown). However, as shown in Table 1, the IC_{50} of PMEA for passage-40 virus was 30.5 μM, a 6.9-fold increase compared to pre-selected virus (4.45 μM). Increases over pre-cultured virus in the IC_{50} for ddI (9.9-fold), ddC (7.8-fold), and 3TC (36-fold) were also observed. By contrast, PMEA-passaged HIV-1 showed no cross-resistance to either AZT or nevirapine (Table 1).

3.3. Sequence analysis of the RT region of PMEA-passaged virus

To investigate the molecular basis for resistance to PMEA, we sequenced codons 21–240 of RT of each of eight clones of PCR *pol* products from PMEA-passaged HIV-1 obtained after the 39th passage. As controls, four clones of PCR *pol* products from the media-passaged HIV (40th passage) and four clones of PCR *pol* products from the pre-passaged HIV were sequenced. As shown in Fig. 1, each of the eight PMEA-passaged clones (P) had a substitution of arginine for lysine at codon 65 (K65R). This substitution was not observed with any of the clones from pre-passaged control HIV (C) or medium-passaged (M) virus. Five of the eight clones of PMEA-passaged HIV also had a substitution of phenylalanine for leucine at codon 214 (L214F); however, this substitution was also observed in one of the four clones of pre-passaged HIV.

3.4. Studies with constructed HIV variants

To clarify the contribution of the K65R mutation to the change in sensitivity to PMEA, we studied the recombinant virus HXB2D(K65R) in which the K65R mutation had been inserted by site-directed mutagenesis into the RT of HXB2D (Gu et al., 1992, 1994). Similar to results previously found in MT-2 cells (Gu et al., 1995), K65R conferred an 8.9-fold increase in the IC_{50} to PMEA when tested in H9 cells (Table 2). A similar increase in the IC_{50} with K65R was observed in PBMC. In addition, like the virus selected by passage with PMEA, HXB2D(K65R) had an increase in IC_{50} to ddI, ddC, and 3TC, but not to AZT, as compared with HXB2D (Table 2). Thus, the K65R mutation was sufficient to reproduce a pattern of resistance and cross-resistance observed in the PMEA-passaged HIV.

4. Discussion

In this paper, we demonstrate that HIV with reduced sensitivity to PMEA can be generated in vitro by exposure to increasing concentrations of drug and that this altered sensitivity profile is associated with a K65R mutation in HIV RT. We also show that HIV with K65R manifests its resistance in PBMC. HIV with K65R had previ-

SEQUENCES OF HIV PASSAGED WITH PMEA AND CONTROL STRAINS

VIRUS STRAIN	30	40	50	60	70	80	90	100	110	120	130
WT	VKQWPLTEEK	IKALVEICTE	MEKEGKISKI	GPENPYNTPV	FAIKKKDSTK	WRKLVDFREL	NKRTQDFWEV	QLGIPHPAGL	KKKKSVTVLD	VGDAYFSVPL	DEDFRKYTAF
P Clone 1	----------	----------	----------	----------	----R-----	----------	----------	----------	----------	----------	----------
P Clone 3	----------	----------	----------	----------	----R-----	----------	----------	----------	----------	----------	----------
P Clone 4	----------	----------	----------	----------	----R-----	----------	----------	----------	----------	----------	----------
P Clone 5	----------	----------	----------	----------	----R-----	----------	----------	----------	----------	----------	----------
P Clone 8	----------	----------	----------	----------	----R-----	----------	----------	----------	----------	----------	----------
P Clone 10	----------	----------	----------	----------	----R-----	----------	----------	----------	----------	----------	----------
P Clone 11	----------	----------	----------	----------	----R-----	----------	----------	----------	----------	----------	----------
P Clone 12	----------	----------	----------	----------	----R-----	----------	----------	----------	----------	----------	----------
C Clone 2	----------	----------	----------	----------	--V-------	----------	----------	----------	----------	----------	----------
C Clone 4	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
C Clone 8	----------	----------	----------	----------	----------	----------	----------	----------	-R--------	----------	----------
C Clone 9	----------	----------	----------	----------	--V-------	----------	----------	----------	----------	----------	----------
M Clone 2	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
M Clone 7	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
M Clone 8	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
M Clone 9	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------

	140	150	160	170	180	190	200	210	220	230
	TIPSINNETP	GIRYQYNVLP	QGWKGSPAIF	QSSMTKILEP	FRKQNPDIVI	YQYMDDLYVG	SDLEIGQHRT	KIEELRQHLL	RWGLTTPDKK	HQKEPPFLWM
P Clone 1	----------	----------	----------	----------	----------	----------	----------	----------	---F------	----------
P Clone 3	----------	----------	----------	----------	----------	----------	----------	----------	---F------	----------
P CLone 4	----------	----------	----------	----------	----------	----------	----------	----------	---F------	----------
P Clone 5	----------	----------	----------	----------	----------	----------	----------	----------	---F------	----------
P Clone 8	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
P Clone 10	----------	----------	----------	----------	----------	----------	----------	----------	---F------	----------
P Clone 11	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
P Clone 12	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
C Clone 2	----------	----------	----------	----------	--E-------	--------I-	----------	----------	----------	----------
C Clone 4	----------	----------	----------	----------	-K--------	----------	----------	----------	----------	----------
C Clone 8	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
C Clone 9	----------	----------	----------	----------	----------	----------	----------	---K------	---F------	----------
M Clone 2	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
M Clone 7	----------	----------	----------	----------	-K--------	----------	----------	----------	----------	----------
M Clone 8	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
M Clone 9	----------	----------	----------	----------	----------	----------	-----R----	----------	----------	----------

Fig. 1. Sequence of codons 21–230 of the RT of HIV-1_{LAI} passaged in the presence of PMEA (P), of control HIV-1_{LAI} that was not passaged (C), and of HIV-1_{LAI} that was passaged in the absence of drug (M). A 690-bp fragment of the RT genome of HIV-1 was amplified by PCR and then expanded and sequenced. Each sequence is of a different clone.

Table 2
Susceptibility of the K65R recombinant virus to anti-HIV drugs[a]

Virus	Cells	IC_{50} (μM)				
		PMEA	AZT	ddI	ddC	3TC
HXB2D	H9	1.2 (0.30)	0.01 (0.002)	ND[b]	0.6 (0.10)	0.4 (0.05)
HXB2D(K65R)	H9	10.7 (1.50)	0.005 (0.001)	ND	5.2 (1.20)	14.5 (1.30)
HXB2D	PBMC	0.8 (0.15)	0.006 (0.001)	7.3 (0.90)	ND	0.5 (0.08)
HXB2D(K65R)	PBMC	12.5 (2.60)	0.01 (0.002)	26.8 (3.10)	ND	11.3 (1.9)

[a] Susceptibility of HXB2D, a wild-type cloned infectious HIV and HXB2D(K65R), a construct of this virus into which K65R was inserted by site-directed mutagenesis, to various anti-HIV drugs. Susceptibilities were determined in H9 cells and in PBMC. Results were calculated on the basis of the HIV-1 p24 Ag levels in culture supernatants; values shown are the average of two separate experiments, number in parentheses represents ± S.E.M..
[b] ND, not determined in these experiments.

ously been found to arise upon exposure to increasing concentrations of ddC (Gu et al., 1992, 1994; Zhang et al., 1994), and it had been shown to have cross-resistance to ddI, 3TC, and PMEA (Gu et al., 1994, 1995; Zhang et al., 1994). Such cross-resistance, however, does not necessarily predict that a mutation will arise under selective pressure; for example, in vitro or in vivo exposure to 3TC generally induces selection of M184V or M184I rather than K65R (Kavlick et al., 1995; Schinazi et al., 1993; Schuurman et al., 1995; Tisdale et al., 1993; Wainberg et al., 1995). In the case of PMEA, we now show that K65R is the principal mutation developing in response to the in vitro exposure described here. It is conceivable that the L214F mutation seen in some clones in the present study also played a role in the resistance pattern. However, as this variant is seen in numerous wild-type strains of HIV (Myers et al., 1992), as it was seen prior to PMEA exposure, and as K65R can by itself cause the resistance pattern observed, it seemed unlikely that L214F contributes substantially to the phenotype of the PMEA-exposed virus, although this possibility could not be completely ruled out. Cherrington et al. (1995) have recently found that exposure of HIV-1_{IIIB} to PMEA in MT2 cells selected for a resistant strain with a different mutation, K70E, but not with K65R. Thus, it appears that either of two separate RT substitutions can lead to PMEA resistance. It will be of interest to see if there is any interaction between these two RT substitutions.

It is worth noting that the in vitro selection in this study was performed in H9 cells, which are not known to be infected with other retroviruses, and it is thus unlikely that this mutation pattern arose by recombination. Also, we were not able to utilize concentrations of PMEA higher than 40 μM in selecting resistant virus because of cytotoxicity to the H9 cells in long-term culture; under these conditions, however, only a 7-fold decreased sensitivity to PMEA was attained even after 39 passages. Because of the acyclic structure of PMEA (which is different from the sugar ring of the other dideoxynucleosides), one might have perhaps expected the in vitro drug exposure to have selected for resistant HIV with a novel *pol* mutation. However, in this study as well as in preliminary results reported by Vasudevachari et al. (1993), it appears that a mutation previously reported to arise following exposure to ddC is a principal resistance-conferring mutation arising in PMEA-exposed HIV.

Additional studies will be needed to determine whether K65R also arises in patients receiving PMEA (or its pro-drug bis-POM PMEA). However, in the case of ddC, this same mutation was found to arise both in tissue culture and in patients (Gu et al., 1994; Zhang et al., 1994), and one may hypothesize that the clinical use of PMEA will induce at most low-level resistance and that this will be associated with K65R. It is noteworthy that while K65R has cross-resistance with ddI, ddC and 3TC, it has no cross-resistance with AZT, and this suggests that the combination

of PMEA (or bis-POM PMEA) and AZT may be worth exploring (Lush et al., 1996). In this regard, it will be of interest to determine whether the development of K65R affects AZT sensitivity in HIV strains with established AZT resistance. Additional potential advantages of this combination are that the drugs are synergistic and that they appear to act preferentially in different cells (Gao et al., 1993b; Perno et al., 1992; Smith et al., 1989). AZT is especially active in replicating cells while PMEA may be equally active in resting and replicating cells. Clinical studies of the combination of AZT and bis-POM PMEA are now underway.

In conclusion, the present studies indicate that K65R is a mechanism by which low-level HIV resistance to PMEA arises upon in vitro exposure to the drug. Further studies are needed to determine whether K65R (or alternatively K70E) will be selected in patients receiving PMEA.

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