Antiviral Research 14 (1990) 117–122
Elsevier

AVR 00433

Short Communication

Properties of a 9-(2-phosphonylmethoxyethyl)adenine (PMEA)-resistant herpes simplex virus type 1 virus mutant

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(Received 4 December 1989; accepted 9 April 1990)

Summary

After repeated passages of herpes simplex type 1 (HSV-1) KOS virus in the presence of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) a mutant denoted PMEA^r HSV-1 was isolated which grew well in the presence of 50–100 $\mu g \cdot ml^{-1}$ of the drug. PMEA^r HSV-1 was still sensitive to the related phosphonate analogue (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA). In fact, it was more susceptible to the action of HPMPA than the original virus. PMEA^r HSV-1 also retained sensitivity to 5-bromo-2′-deoxyuridine and other, viral thymidine kinase-dependent substances such as (*E*)-5-(2-bromovinyl)-2′-deoxyuridine. However, PMEA^r HSV-1 was much less sensitive to acyclovir, 1-(β-D-arabinofuranosyl)cytosine and 1-(β-D-arabinofuranosyl)thymine than the parental KOS virus.

Herpes simplex virus; PMEA; 9-(2-Phosphonylmethoxyethyl)adenine; Drug-resistant mutant

Recently, a potent and highly selective antiviral activity has been reported for the *N*-(phosphonylmethoxyalkyl) derivatives of purine and pyrimidine bases (De Clercq, 1989; De Clercq et al., 1986, 1989). These sub-

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stances, e.g. 9-(2-phosphonylmethoxyethyl)adenine (PMEA), (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) and some of their base-modified congeners display a broad-spectrum inhibitory activity against a number of DNA viruses (De Clercq et al., 1987; Baba et al., 1987a,b) and retroviruses (Pauwels et al., 1987; Balzarini et al., 1989). They strongly inhibit viral DNA synthesis at concentrations which are by several orders of magnitude lower than those inhibiting cellular DNA synthesis (Votruba et al., 1987). Although the precise mechanism of action of these substances is as yet unknown, recent studies have demonstrated that the phosphorylated metabolites of these compounds interfere with the virus-encoded HSV-1 DNA polymerase (Merta et al., in press). HSV-1 ribonucleotide reductase (Černý et al., 1990) and AMV (MAV) reverse transcriptase (Votruba et al., in press).

To further elucidate the mode of action of these nucleotide analogues we have started experiments aimed at identifying the viral proteins that interact with the inhibitors or their metabolites. In this paper we describe the selection and some properties of an herpes simplex virus type 1 (HSV-1) mutant which proved to be resistant to the inhibitory action of PMEA.

HSV-1 KOS virus was the same as in previous experiments (Kutinová et al., 1979). For virus cultivation and plaque assays either rabbit embryo fibroblast (REF) cells (Kutinová et al., 1973), VERO cells (kindly supplied by J. Rajčani, Institute of Virology, Bratislava, Czechoslovakia) or CV-1 cells (kindly provided by S. Kit, Baylor College of Medicine, Houston, U.S.A.) were used. They were cultivated as reported previously (Vonka et al., 1976). PMEA and HPMPA were prepared as described previously (Holý and Rosenberg, 1987a,b). They were purified by anion exchange chromatography (Dowex 1) to HPLC homogeneity and used as solid salts. 5-Bromo-2′-deoxyuridine (BDU), 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV), 5-iodo-2′-deoxyuridine (IDU) and 1-(β-D-arabinofuranosyl)thymine (ara-T) were prepared by well established procedures. 1-(β-D-arabinofuranosyl)cytosine (ara-C) and 1-(β-D-arabinofuranosyl)adenine (ara-A) were obtained from Sigma (U.S.A.). (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) was a generous gift from E. De Clercq (Rega Institute, University of Leuven, Belgium). For measuring virus-drug sensitivity, either a plaque reduction assay in REF or VERO cells, using 0.75% methocel medium containing various concentrations of the drug, or a cytopathic effect-inhibition assay was used. In the latter case, VERO cells were grown in 96-well plastic plates (Dynatech) and infected with 100 $TCID_{50}$ of the virus. After 1 h incubation at 37°C, the drugs were added at different concentrations (usually ranging from 0.01 to 50 $\mu g \cdot ml^{-1}$, in two-fold steps). The plates were incubated for 4 days at 37°C in 5% CO_2. The minimum antiviral inhibitory dose (MID) was expressed as the concentration of the drug inhibiting virus-induced cytopathogenicity by 50%.

PMEA-resistant virus was isolated as follows. HSV-1 (KOS) virus was passaged in REF cells at gradually increasing concentrations of the drug. At different passage levels the efficiency of growth of the virus in the presence of the drug was measured by virus titrations. The results suggested a gradual development of resistance up to passage 30. After a total of 33 passages, a virus denoted $PMEA^r$ HSV-1 was isolated and plaque-purified. This virus replicated well in the presence of PMEA

TABLE 1
Influence of PMEA on plaque formation by HSV-1 KOS and its PMEA-resistant (PMEA^r) mutant

HSV-1	Plaque count (log_{10}/ml) at the indicated concentration (μg/ml) of PMEA			
	0	10	25	100
KOS	4.0	2.3	<1.0	<1.0
PMEA^r	3.8	4.1	3.8	3.3

at 50 $\mu g \cdot ml^{-1}$. From the data (Table 1) it appears that plaque formation by the original virus (KOS) was strongly inhibited by PMEA even at a concentration of 10 $\mu g \cdot ml^{-1}$, while the growth of PMEA^r HSV-1 was virtually not affected at concentrations up to 100 $\mu g \cdot ml^{-1}$.

It was of interest to determine the sensitivity of PMEA^r HSV-1 to some of the known HSV-1 inhibitors. These assays were performed in VERO cells, based on the inhibition of viral cytopathogenicity (Table 2). PMEA^r HSV-1 proved susceptible to inhibition by HPMPA, a structurally related analogue of PMEA. Thus, the mutation conferring PMEA resistance was not associated with a loss of sensitivity to HPMPA. On the contrary, PMEA^r HSV-1 was considerably more susceptible to HPMPA than the original virus. This was also demonstrated by a plaque reduction assay and by assays in human diploid fibroblast cells (data not shown). Do these findings suggest that these structurally closely related substances act by different mechanisms? Not necessarily, since it has been shown that some mutants can be resistant to one compound and hypersensitive to a closely related compound with a similar mechanism of action (Coen et al., 1985).

PMEA^r HSV-1 was still sensitive to BDU and BVDU, the action of which is dependent upon phosphorylation by the viral thymidine kinase (TK). However, PMEA^r HSV-1 also showed resistance to ACV and reduced sensitivity to ara-C, ara-A and ara-T. These findings point to the viral DNA polymerase as a possible target for the antiviral action of PMEA. It has been shown that mutations conferring virus-drug resistance because of an altered polymerase exhibit altered sensitivities to other compounds which act as inhibitors of the viral DNA polymerase (Coen and Schaffer, 1980; Coen et al., 1985; Furman et al., 1981).

To obtain more information on the relationship between PMEA and BDU resistance we tried to isolate a recombinant expressing resistance to both substances. Glas virus, a TK$^-$ mutant of the Glasgow 17 strain of HSV-1 (Tenser et al., 1979), was used for this purpose; this virus was kindly provided by R. Tenser (Pennsylvania State University, Hershey, U.S.A.). In agreement with a previous report (De Clercq et al., 1986), the TK$^-$ Glas virus was susceptible to the antiviral

TABLE 2
Comparative sensitivity of HSV-1 (KOS) and PMEA^r HSV-1 to various compounds

Virus	MID (μg/ml)								
	PMEA	HPMPA	BDU	BVDU	IDU	ACV	Ara-C	Ara-A	Ara-T
KOS	8.0	12.5	0.4	0.8	<6.2	0.8	<0.1	25.0	3.1
PMEA^r	>50.0	1.6	0.4	<0.4	<6.2	25.0	0.4	>50.0	25.0

TABLE 3
Comparative sensitivity of HSV-1 (KOS), Glas (TK^-) HSV-1 and P^rB^r HSV-1 to PMEA and BDU

HSV-1	MID (μg/ml)	
	PMEA	BDU
KOS	8.0	0.8
Glas (TK^-)	8.0	>50
P^rB^r	100.0	>50

action of PMEA. To facilitate the isolation of a virus resistant to both substances, the cells were infected with both viruses and then exposed to the compounds. A virus resistant to both PMEA and BDU was finally isolated from CV-1 cells, infected with undiluted UV-irradiated Glas virus (original titer: 3×10^6, survival of less than 10^{-6}) and $PMEA^r$ virus at a multiplicity of infection of 0.0001 PFU per cell, and kept in the presence of BDU (20 $\mu g \cdot ml^{-1}$). At 4 days after infection cytopathic changes developed. The virus from the culture fluid was then passed twice in the presence of a mixture of PMEA (40 $\mu g \cdot ml^{-1}$) and BDU (20 $\mu g \cdot ml^{-1}$). While neither of the parent viruses replicated or formed plaques under these conditions, the virus from the dually infected culture grew well and formed plaques. This virus, denoted P^rB^r, was plaque purified. Its sensitivity to PMEA and BDU is shown in Table 3. Although it is as yet unclear whether the P^rB^r virus is a recombinant or a PMEA-resistant mutant of the reactivated Glas virus, it is clear that both resistance phenomena are controlled by different genetic elements.

To our knowledge $PMEA^r$ and P^rB^r are the first examples of HSV viruses that are able to replicate in the presence of PMEA. The full biochemical characterization of these viruses will be helpful in elucidating the mechanism of action of this drug and related compounds.

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