Antiviral Research, 4 (1984) 63–70
Elsevier

AVR 00115

Nucleic acid hybridization, a method to determine effects of antiviral compounds on herpes simplex virus type 1 DNA synthesis

H. Gadler[1]*, A. Larsson[2] and E. Sølver[1]

[1]*Department of Virology, National Bacteriological Laboratory, S-105 21 Stockholm, and* [2]*Department of Antiviral Chemotherapy, Research and Developmental Laboratories, Astra Läkemedel AB, S-151 85 Södertälje, Sweden*

(Received 27 May 1983; accepted 3 October 1983)

An application of the nucleic acid hybridization technique to screen effects of antiherpes compounds on herpes simplex virus type 1 (HSV-1) DNA synthesis is described. Whole cells are applied to nitrocellulose filters, their DNA is denatured and fixed to the filter. The resulting DNA spots are hybridized to cloned nick-translated HSV-1 DNA and the amount of hybridization is monitored by autoradiography or scintillation counting. Six antiherpes compounds: bromovinyldeoxyuridine, acyclovir, (*R*)- and (*S*)-enantiomers of 9-(3,4-dihydroxybutyl)guanine, 9-(4-hydroxybutyl)guanine and foscarnet, were evaluated for their effects on HSV-1 DNA synthesis. The most active compounds were bromovinyldeoxyuridine and acyclovir, with mean 50% inhibition values (IC_{50}) for four different HSV-1 strains of 0.3 μM and 0.8 μM, respectively. The (*R*)-enantiomer of the new antiherpes compound 9-(3,4-dihydroxybutyl)guanine was found to be more active than the (*S*)-enantiomer, with mean IC_{50}s of 6.5 and 14 μM, respectively, while mean IC_{50}s of 2.5 and 68 μM were obtained for 9-(4-hydroxybutyl)guanine and foscarnet, respectively.

nucleic acid hybridization; HSV-1 DNA synthesis; antiviral compounds

Introduction

Herpesviruses are known to be responsible for many diseases in man and efforts have been made to find drugs for the treatment of these diseases. Studies of substrate specificities for viral and cellular enzymes have led to new, promising antiherpes compounds such as foscarnet (PFA; [7]), acyclovir (ACV; [4]), bromovinyldeoxyuridine (BVDU; [2]) and 9-(3,4-dihydroxybutyl)guanine (DHBG; [13]). Rapid evalua-

* To whom correspondence should be addressed.

tion of the efficacy and mechanism of antiherpes drugs in cell culture can be difficult if the test procedure is slow, expensive or a large amount of test compound is needed. The most-used assays for this type of investigation have involved measuring cytopathic effects and effects of virus multiplication by plaque counting or by virus yield determinations. To measure the selectivity of inhibition of viral DNA replication, a limited number of compounds have been tested by isopycnic banding of viral and cellular DNA in cesium chloride (CsCl) gradients [3,12].

This study introduces a new application of the nucleic acid hybridization technique, as a rapid and sensitive way of screening effects of antiherpes compounds on HSV-1 DNA synthesis. The results are compared with published results obtained with other techniques.

Materials and Methods

Foscarnet (PFA, phosphonoformic acid), (*R*)- and (*S*)-enantiomers of 9-(3,4-dihydroxybutyl)guanine (DHBG), 9-(4-hydroxybutyl)guanine (HBG) and acyclovir (ACV) were synthesized at Astra Läkemedel, Södertälje, Sweden. (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) was kindly provided by E. de Clercq, The Rega Institute, Leuven, Belgium. Deoxycytidine 5'-[α-^{32}P]triphosphate ([α-^{32}P]dCTP; >7 000 Ci/mmol) was obtained from Amersham International, Amersham, England. All chemicals were of analytical grade. Restriction endonuclease BamHI and T4 DNA ligase were from New England Biolabs, Inc., Beverly, MA, U.S.A. and used in accordance with the manufacturer's specifications.

Cells and viruses

Vero cells (CCL81) were grown in Eagle's minimum essential medium (MEM) containing 0.5% of non-essential amino acids. The medium was supplemented with 10% of fetal calf serum, 20 mM HEPES buffer (pH 7.2), penicillin (120 µg/ml) and streptomycin (100 µg/ml). The cells were grown at 37°C with 5% CO_2; they were screened routinely for mycoplasma contamination by the uridine–uracil incorporation method [16] and found negative. Herpes simplex virus (HSV-1) strains C42, 7935-72, KJ502 and MacIntyre, as well as the plaque assays, have been described earlier [5,7,15,17].

Virus assay

Microtiter culture plates (Titertek, Flow Laboratories Ltd., Irvine, Scotland) with 96 wells and an area/well of 0.28 cm^2, were seeded with Vero cells. After reaching confluence (approximately 4×10^4 cells/well), the plates were exposed to 1–2 plaque forming units (PFU)/cell of different strains of HSV-1 for 1 h at 37°C. The cells were washed once with 0.2 ml phosphate buffered saline (PBS) to remove unadsorbed virus and 0.2 ml MEM, containing 20 mM HEPES, 2% of fetal calf serum and the indicated concentrations of test compounds, was then added. After 16 h of incubation at 37°C in

5% CO_2, the medium was removed and each well was washed once with 0.2 ml PBS. The plates were stored at -70°C or analysed immediately.

Purification of HSV-1 DNA

GMK (green monkey kidney) cells were infected at low multiplicity with HSV-1 strain 9004. In total, 40 glass roller bottles, each with an area of 850 cm^2 and a medium volume of approximately 100 ml, were used. When the cpe (cytopathic effect) affected at least 90% of the monolayer, the medium was harvested and clarified by centrifugation at 2000 × *g* for 20 min. The medium was concentrated in a Millipore Pellicon concentrator (filter pore size 10^6 dalton; Millipore Corporation, Bedford, MA, U.S.A.) to approximately 300 ml and the virions were then pelleted in a Beckman ultracentrifuge, using a SW27 rotor at 131000 × *g* for 60 min. The pellet was resuspended in 12 ml of Tris-buffered saline (TBSE = 0.15 M NaCl–0.005 M EDTA–0.05 M Tris-HCl, pH 7.5) and layered on top of a CsCl step gradient with 15 ml each of CsCl in TBSE, densities 1.35 g/cm^3 and 1.15 g/cm^3, respectively. After a 2-h centrifugation, the virus band was harvested, dialysed and viral DNA extracted. One-half volume of 3% sarkosyl NL-30 (Geigy Industry Chemicals, Ardsley, NY, U.S.A.)–0.025 M EDTA–0.075 M Tris-HCl, pH 9.0 was added and left for 10 min at room temperature. One-quarter volume of predigested pronase (Calbiochem-Behring, San Diego, CA, U.S.A.) was added and incubated for 2 h at 37°C. The material was extracted twice with phenol–chloroform, 1:1, three times with ether and precipitated with one-tenth volume of 3 M sodium acetate and 2 volumes of ice-cold absolute ethanol. The DNA was pelleted at 13000 × *g* for 30 min and dissolved in a small volume of TE (TE = 0.001 M EDTA–0.01 M Tris-HCl, pH 7.5). The viral DNA was separated from contaminating cellular DNA in a CsCl gradient, using a R50Ti rotor at 98700 × *g* for 65 h at room temperature. The viral DNA was localized by measuring the absorbance of fractions at 260 nm. These fractions were pooled, dialysed against TE and DNA was precipitated with sodium acetate and ethanol. The purity of the DNA was checked by measuring the absorbance at 260, 270, 280 and 330 nm; the $(A_{260}-A_{330})/(A_{270}-A_{330})$ quotient was 1.25 and the $(A_{260}-A_{330})/(A_{280}-A_{330})$ quotient, 1.79.

Nucleic acid hybridization probes

HSV-1 DNA purified as described above was cleaved with BamHI to completion. The plasmid pBR322 was likewise cleaved with BamHI and dephosphorylated with calf intestine alkaline phosphatase (Boehringer Mannheim GmbH, Mannheim, West Germany). The viral DNA fragments were ligated with plasmid DNA at a molar ratio of 1.5:1, using T4 DNA ligase. *E. coli* HB101 cells were used for transformation and later spread onto agar plates containing ampicillin. Colonies were picked and checked for tetracyclin resistance. Amp^r- tet^s colonies were screened, using a rapid boiling method [8]. Clones containing the following inserts were picked at random to be used as probes: pHSV 1003 (Q), pHSV 1029 (M and O), and pHSV 1059 (S). Identification of HSV DNA fragments was according to Locker and Frenkel [14]. They were then cultured on a larger scale for purification of plasmid DNA.

Hybridization conditions

The recombinant DNA molecules were nick-translated with 25 μCi[α-^{32}P]dCTP to a specific activity of at least approximately 3×10^7 cpm/μg DNA. A mixture of the 3 clones mentioned above, in equal proportions with regard to radioactivity, was used in all hybridizations. The method for studying the viral DNA synthesis has been described previously [6]. Briefly, the cells were dislodged with trypsin from the microtiter culture plate and filtered through a nitrocellulose filter, using a filtration manifold. The DNA was then denatured in situ by placing the nitrocellulose filter on Whatman 3-MM filter papers soaked with the following solutions: 0.5 M NaOH for 6 min, 0.1 M NaOH–1.5 M NaCl for 10 min, 1 M Tris, pH 7.5 twice for 2 min each and 1.5 M NaCl–0.5 M tris, pH 7.5 for 5 min. After this treatment the nitrocellulose filter was dried at room temperature and then baked in a vacuum oven at 80°C for 2 h. The filter was prehybridized in 6 × SSC–5 × Denhardt's solution (1 × Denhardt = 0.02% polyvinyl-pyrrolidon, PVP, 0.02% bovine serum albumin, BSA, 0.02% Ficoll)–0.5% sodium dodecyl sulphate (SDS)–100 μg/ml of single-stranded salmon sperm DNA. The volume of buffer was 1 ml/10 cm^2 and prehybridization was done for at least 2 h at 65°C. Hybridizations were carried out at the same buffer condition but with denatured recombinant DNA as probe. 0.5×10^6 cpm of radioactivity/10 cm^2 filter was used at 0.5 ml/10 cm^2. The filter was kept in a plastic bag at 65°C overnight and later washed three times in 2 × SSC–0.5% SDS for 45 min at 65°C. Finally, the filter was washed once briefly in 2 × SSC before drying and mounting on thick paper. Autoradiography, using Kodak XAR-5 film and intensifying screens, was performed for times varying between 1 and 5 h at –70°C. The time was chosen to maintain a linear film response to radioactivity. The evaluation could be done visually or by cutting out the spots from the filter and counting them in a scintillation counter.

Results

Nucleic acid hybridization probes

Plasmids pHSV 1003, pHSV 1029 and pHSV 1059 contain HSV BamHI fragments Q, M and O, and S, respectively. These four fragments all originate from different regions in the long unique segment of the HSV genome [14]. No background hybridization to cell DNA was detected using the hybridization conditions described. They were thus considered suitable as probes in hybridization reactions for HSV DNA.

Quantification of spot hybridization

The system used for application of cells and virus to the microtiter plates is shown in Fig. 1. The first 6 wells at a and the last 6 at h are uninfected cells, while the last 6 wells at a and the first 6 at h are HSV-1 infected cells without added compounds. In the following wells each compound was added to the infected cells in duplicate, with increasing concentrations from left to right (as shown in Fig. 1). No hybridization was

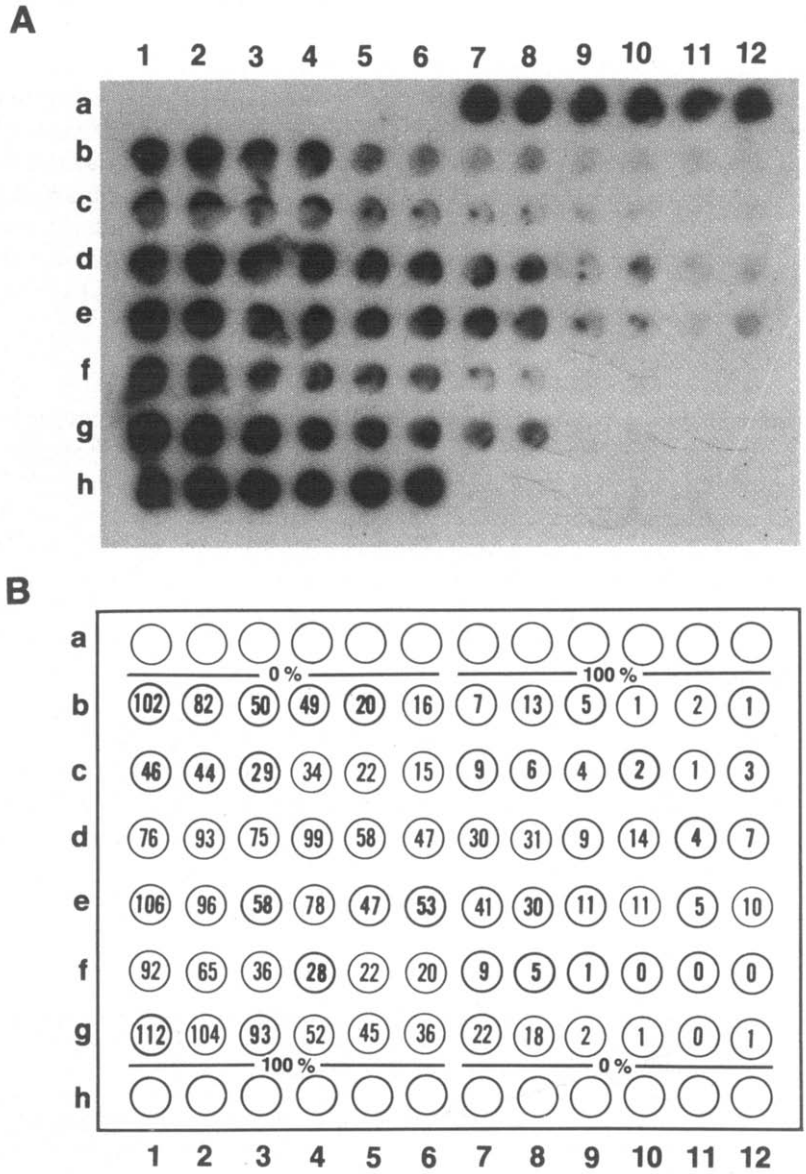

Fig. 1. Effect of antiherpes compounds on HSV-1 DNA synthesis. Vero cells and HSV-1 strain C42 were applied to a 96-well microtiter plate as described below. A. results after autoradiography. B. the same filter after scintillation counting. The percentage of HSV-1 DNA synthesized after treatment with different concentrations of antiherpes compounds, as compared to untreated infected cells, is indicated for each spot. Six concentrations of each compound were tested in duplicate at increasing levels from left to right for each compound. a. Wells no. 1–6 uninfected Vero cells, wells no. 7–12 HSV-1 infected Vero cells. b. BVDU, 0.05, 0.10, 0.25, 0.5, 1.0, 2.5 μM. c. ACV, 0.10, 0.25, 0.50, 1.0, 2.5, 5.0 μM. d. (*R*)-DHBG, 1.0, 2.5, 5.0, 10.0, 25, 50 μM. e. (*S*)-DHBG, 2.5, 5.0, 10, 25, 50, 100 μM. f. HBG, 1.0, 2.5, 5.0, 10.0, 25, 50 μM. g. PFA, 10, 25, 50, 100, 250, 500 μM. h. Wells no. 1–6 HSV-1 infected Vero cells, wells no. 7–12 uninfected Vero cells.

detectable in wells with uninfected cells, indicating that the HSV-1 clones used represent virus-specific DNA sequences.

Quantitation was done both by inspection of the autoradiogram (intensity and size of spots) and by cutting out the spots from the nitrocellulose filter and counting them in a scintillation counter. Autoradiography of the amounts of HSV-1 DNA synthesized after treatment with different concentrations of antiherpes compounds is shown in Fig. 1A. A good estimation of the effects on the viral DNA synthesis was obtained by inspection of the film. Figure 1B shows the results obtained after scintillation counting of the same filter. This gives exact figures for determinations of IC_{50} defined as the concentration of antiviral compound which inhibits viral DNA synthesis by 50%.

Effect of antiherpes compounds

The inhibition of viral DNA synthesis at 1–2 PFU/cell, with 4 different HSV-1 strains, was determined with 6 antiherpes compounds. The IC_{50} values are shown in Table 1. The inhibition by these drugs varied between HSV-1 strains, strain 7935-72 being the most sensitive to all substances. BVDU showed the highest activity, with a mean IC_{50} for the 4 strains of 0.3 μM, while the mean IC_{50} for ACV was 0.8 μM. The (*R*)-enantiomer of DHBG was more active than the (*S*)-enantiomer, with mean IC_{50} values of 6.5 and 14 μM, respectively. The highest IC_{50} was seen for PFA, mean 68 μM.

Comparison of methods

We compared our results with the inhibitory effects on HSV-1 plaque formation and the effects on HSV-1 (strain C42) DNA synthesis as determined by isopycnic banding of viral and cellular DNA. The results summarized in Table 2 indicate a good correlation between the methods.

TABLE 1

Inhibition of HSV-1 DNA synthesis by antiherpes compounds determined by nucleic acid hybridization

Compound	50% inhibition, μM (IC_{50}) of different HSV-1 strains				
	C42	KJ 502	MacIntyre	7935-72	Mean IC_{50}
BVDU	0.2 (0.1–0.4)	0.3	0.6	0.1	0.3
ACV	0.2 (0.1–0.3)	1.3	1.8	0.03	0.8
(*R*)-DHBG	3.6 (1.5–5.6)	11	-	5.0	6.5
(*S*)-DHBG	11 (10–13)	17	21	5.4	14
HBG	2.3 (1.8–2.9)	2.9	6.0	0.4	2.9
PFA	60 (40–80)	42	145	26	68

The determinations were done with the hybridization technique as described in Materials and Methods. The IC_{50} values were calculated from dose–response curves with 6 different concentrations of test compound. With strain C42, the mean values are from three different experiments and the ranges are included.

TABLE 2

Comparison between three methods for measuring 50% inhibition of replication by HSV-1 strain C42

Compound	IC_{50} (μM)		
	Hybridization	Isopycnic DNA gradient[a]	Plaque formation
BVDU	0.2	0.1 [11]	0.3
ACV	0.2	0.03 [11]	0.5
(*R*)-DHBG	3.6	-	4.0
(*S*)-DHBG	11	-	12
HBG	2.3	0.6 [9]	3.0
PFA	60	50 [12]	33

[a] References for the values are given in parentheses.

Discussion

In the search for new antiherpes agents it is important to have a variety of test systems. The nucleic acid hybridization technique has previously been applied for studies on the effects of antiviral compounds on human cytomegalovirus replication [6]. However, since the primary screening of potentially new antiherpes compounds often begins with the evaluation of effects on HSV-1, we decided to compare the results of studies on HSV-1 with those of conventional techniques. When screening the effects of different compounds, this method offers several advantages. Several compounds can be tested at the same time and the amount of test compound needed is small. This is important in the case of compounds which are available in only limited amounts. Furthermore, the determination can be done rapidly by autoradiography and with high sensitivity, as shown in Fig. 1.

Determinations of effects of different antiherpes compounds on viral DNA synthesis have been published earlier. The common method involves measuring the incorporation of [^{3}H]thymidine [3], or ortho[^{32}P]phosphate [10], into both viral and cellular DNA in cell culture in the presence of test compounds. The viral and cellular DNAs are then separated by isopycnic banding in CsCl gradients and quantitated by scintillation counting of fractions. This method gives information on the effect on both viral and cellular DNA in the infected cell. When compared with the effect on the cellular DNA synthesis in the uninfected cell, this can be helpful in an evaluation of the mechanism of action of an antiherpes compound [11].

Using the hybridization technique, 6 different antiherpes compounds were evaluated for the inhibition of HSV-1 DNA synthesis (Table 1) and compared with the results obtained with the two techniques mentioned above (Table 2). A good correlation was observed between the results obtained with these different techniques.

In conclusion, the nucleic acid spot hybridization technique [1] appears to be a suitable method for screening substances for antiherpes effects in cell culture. The method can replace or be combined with existing techniques: analysis of inhibition of viral DNA synthesis by banding viral and cellular DNA in CsCl gradients [3,10,12], or

the inhibition of viral plaque formation. The technique can equally well be applied to other DNA viruses [6] or RNA viruses, once appropriate probes are available.

References

1 Brandsma, J. and Miller, G. (1981) Nucleic acid spot hybridization: rapid screening of lymphoid cell lines for Epstein–Barr viral DNA. Proc. Natl. Acad. Sci. 77, 6851–6855.

2 De Clercq, E., Descamps, J., De Somer, P., Barr, P.J., Jones, A.S. and Walker, R.T. (1979) (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine: a potent and selective antiherpes agent. Proc. Natl. Acad. Sci. 76, 2947–2951.

3 Drach, J.C. and Shipman, Jr., C. (1977) The selective inhibition of viral DNA synthesis by chemotherapeutic agents: an indication of clinical usefulness? Ann. N.Y. Acad. Sci. 284, 396–409.

4 Elion, G.B., Furman, P.A., Fyfe, J.A., De Miranda, P., Beauchamp, L. and Schaeffer, H.J. (1977) Selectivity of action of an antiherpetic agent, 9-(hydroxyethoxymethyl) guanine. Proc. Natl. Acad. Sci. 74, 5716–5720.

5 Eriksson, B. and Öberg, B. (1979) Characteristics of herpesvirus mutants resistant to phosphonoformate and phophonoacetate. Antimicrob. Agents Chemother. 15, 758–762.

6 Gadler, H. (1983) Nucleic acid hybridization for measurement of effects of antiviral compounds on human cytomegalovirus DNA replication. Antimicrob. Agents Chemother. 24, 370–374.

7 Helgstrand, E., Eriksson, B., Johansson, N.-G., Lannerö, B., Larsson, A., Misiorny, A., Norén, J.-O., Sjöberg, B., Stenberg, K., Stening, G., Stridh, S., Öberg, B., Alenius, S. and Philipsson, L. (1978) Trisodium phosphonoformate, a new antiviral compound. Science 201, 819–821.

8 Holmes, D.S. and Quigley, M. (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114, 193–197.

9 Larsson, A., Alenius, S., Johansson, N.-G. and Öberg, B. (1983) Antiherpetic activity and mechanism of action of 9-(4-hydroxybutyl)guanine. Antiviral Res. 3, 77–86.

10 Larsson, A. and Öberg, B. (1981) Selective inhibition of herpesvirus DNA synthesis by foscarnet. Antiviral Res. 1, 55–62.

11 Larsson, A. and Öberg, B. (1981) Selective inhibition of herpesvirus deoxyribonucleic acid synthesis by acycloguanosine, 2′-fluoro-5-iodoaracytosine, and (*E*)-5-(2-bromovinyl)-2′-deoxyuridine. Antimicrob. Agents Chemother. 19, 927–929.

12 Larsson, A. and Öberg, B. (1981) Selectivity of antiherpes compounds on viral and cellular DNA synthesis. Herpesviruses, clinical, pharmacological and basic aspects. In: International Congress Series 571, Eds.: Shiota, H., Cheng, Y.-C. and Prusoff, W.H. (Excerpta Medica, Amsterdam) pp. 211–214.

13 Larsson, A., Öberg, B., Alenius, S., Hagberg, C.-E., Johansson, N.-G., Lindborg, B. and Stening, G. (1983) 9-(3,4-Dihydroxybutyl) guanine, a new inhibitor of herpesvirus multiplication. Antimicrob. Agents Chemother. 23, 664–670.

14 Locker, H. and Frenkel, N. (1979) BamI, KpnI, and SalI restriction enzyme maps of the DNAs of herpes simplex virus strains Justin and F: occurrence of heterogeneities in defined regions of the viral DNA. J. Virol. 32, 429–441.

15 Schnürer, J. and Öberg, B. (1981) Inhibitory effects of foscarnet on herpesvirus multiplication in cell culture. Arch. Virol. 68, 203–209.

16 Stanbridge, E.J. and Scheider, E.L. (1976) A simple biochemical method for the detection of mycoplasms and other microbial contaminants of cell culture. Tissue Cult. Assoc. Manual 2, 371–374.

17 Stening, G., Gotthammar, B., Larsson, A., Alenius, S., Johansson, N.-G. and Öberg, B. (1981) Antiherpes activity of (*E*)-5-(1-propenyl)-2′-deoxyuridine and 5-(1-propenyl)-1-β-D-arabinofuranosyluracil. Antiviral Res. 1, 213–223.