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Mutually Exclusive Inhibition of Herpesvirus DNA Polymerase by Aphidicolin, Phosphonoformate, and Acyclic Nucleoside Triphosphates

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Dual inhibitor studies were performed to examine the interaction of aphidicolin, phosphonoformate, 9-(2-hydroxyethoxymethyl)guanine triphosphate, and 9-(1,3-dihydroxy-2-propoxymethyl)guanine triphosphate with herpes simplex virus DNA polymerase. Kinetic data indicated that inhibition by one agent prevents simultaneous inhibition by a second agent, producing a mutually exclusive inhibition pattern. This suggested that binding sites on the DNA polymerase molecule for these compounds are kinetically overlapping. These findings should be taken into consideration for the design of future antiviral compounds and combination chemotherapy protocols.

Herpes simplex virus (HSV) DNA polymerase is a logical target for the development of antiviral drugs since it is necessary for viral replication and is biochemically distinct from host DNA polymerases. Several classes of antiherpetic agents have been developed which interact with virus DNA polymerase. They can be grouped according to mechanism of interaction as follows. (i) The first group is nucleoside analogs; these compounds are phosphorylated in virus-infected cells and interact with virus DNA polymerase as nucleoside triphosphates (NTPs) by competing with naturally occurring deoxynucleoside triphosphates (dNTPs) as substrate for incorporation into replicating DNA. The nucleosides 9-(2-hydroxyethoxymethyl)guanine (ACG; acyclovir) and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) are examples which, as NTPs (ACGTP and DHPGTP), compete with dGTP for incorporation into DNA (10, 12, 15, 17). (ii) Aphidicolin and its derivatives make up the second group; aphidicolin has been found to competitively inhibit incorporation of dATP, dCTP, and dTTP by virus DNA polymerase into activated DNA template (13), but is noncompetitive with dGTP and uncompetitive with low concentrations of DNA (13, 21). (iii) Pyrophosphate analogs constitute the third group; compounds such as phosphonoacetate and its cogener phosphonoformate (PFA) inhibit pyrophosphorolysis competitively with pyrophosphate but are noncompetitive with dNTPs and uncompetitive with DNA (9, 11, 20, 22).

It appears that these three classes of compounds interact with virus DNA polymerase by different mechanisms. Several laboratories have investigated the inhibition of virus replication in culture by combinations of these compounds. In the case of phosphonoacetate and ACG, antiviral synergism has been observed (7, 26). Other studies indicate that resistance to PFA can result in both collateral hypersensitivity to aphidicolin (2, 4, 18) and cross-resistance to ACG (5, 6, 8, 14, 19, 24). It is not clear why resistance to one class of compound affects resistance to a different class of compound. One possibility is that binding sites on the DNA polymerase molecule for these compounds are closely associated. To investigate this possibility, we examined the kinetic interaction of HSV DNA polymerase with combinations of ACGTP, DHPGTP, aphidicolin, and PFA. The results are presented in this communication.

MATERIALS AND METHODS

Chemicals. All chemicals used were reagent grade or better. dNTPs, calf thymus DNA, PFA, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo. ^{3}H-labeled dNTPs were purchased from ICN Pharmaceuticals Inc., Irvine, Calif. Aphidicolin was obtained from Imperial Chemical Industries Limited, Cheshire, U.K., dissolved in dimethyl sulfoxide as a 30 mM stock, and stored at −20°C. ACG was obtained from Wellcome Research Laboratories, Research Triangle Park, N.C. DHPG was obtained from Syntex Laboratories, Inc., Palo Alto, Calif.

Substrate preparation. ACG was converted to the triphosphate by chemical methods (23), and DHPG was converted to the triphosphate by using HSV type 1 thymidine kinase and human erythrocyte lysate as described previously (3). NTPs were purified by anion-exchange high-pressure liquid chromatography. Activated DNA was prepared as previously described (1) and end labeled by incubation with [^{3}H]dGTP (6×10^3 cpm/pmol) and HSV DNA polymerase (10). Reactions were terminated by heating to 65°C for 15 min, followed by slow cooling to 23°C. Unincorporated dNTP was separated from polynucleotide by Sephadex G-25 filtration. Substrate prepared in this manner had 7×10^3 cpm/μg of nucleotide.

Enzyme purification and assay. DNA polymerase induced by HSV type 1 (KOS) was purified by DEAE, phosphocellulose, and double-stranded DNA-cellulose chromatography as previously described (8). The specific activity of enzyme used in these studies was at least 1.2×10^4 U/mg. Standard HSV DNA polymerase reaction mixtures contained the following: 50 mM Tris-hydrochloride, pH 8.0; 4 mM $MgCl_2$; 0.5 mM dithiothreitol; 0.2 mg of bovine serum albumin per ml; 0.2 M KCl; 10 μg of activated calf thymus DNA; 100 μM each dATP, dCTP, and dGTP; and 5 μM [^{3}H]dTTP in a volume of 100 μl. One unit of DNA polymerase activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of dTMP per h at 37°C in the standard assay.

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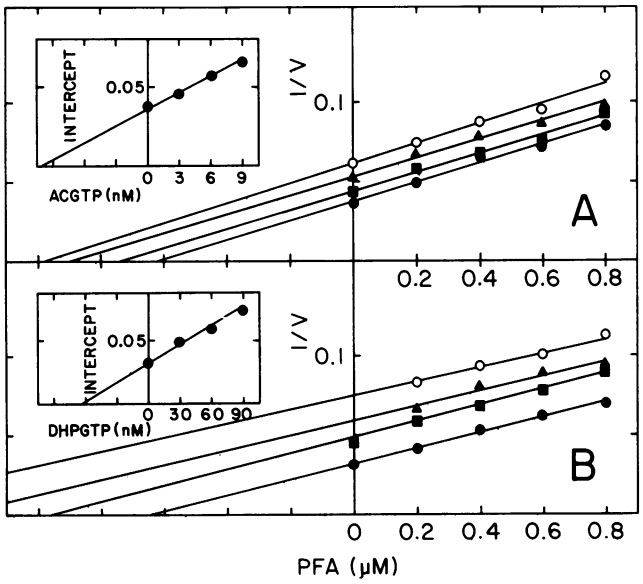

FIG. 1. Inhibition of virus DNA polymerase by combinations of PFA and acyclic nucleotide. DNA polymerase (0.03 U) was assayed under conditions described in the text. PFA was present at the concentrations indicated. (A) ACGTP was added to produce the following concentrations: 0 (●), 3 (■), 6 (▲), and 9 (○) nM. (B) DHPGTP was added to produce the following concentrations: 0 (●), 30 (■), 60 (▲), and 90 (○) nM. Reaction velocity is expressed as picomoles of dGMP incorporated per hour. Lines were fitted to the data by the method of least squares.

For kinetic studies, dATP, dCTP, and dTTP were present at 100 μM each and [^{3}H]dGTP (1,000 cpm/pmol) was present at 0.5 μM. Aphidicolin, PFA, ACGTP, and DHPGTP were added at various concentrations, and reactions were allowed to proceed for either 10 or 20 min at 37°C. Samples of 50 μl were spotted onto GF/A filter disks and processed to determine trichloroacetic acid-insoluble radioactivity.

Nuclease assays were performed under conditions identical to those used for DNA polymerase assays except that dNTPs were omitted and 2 μg of [^{3}H]dGMP end-labeled DNA was used in place of activated DNA. After incubation at 37°C for 30 min, trichloroacetic acid-soluble radioactivity was determined.

RESULTS AND DISCUSSION

To examine the interaction of aphidicolin, PFA, ACGTP, and DHPGTP with HSV DNA polymerase, DNA polymerase and polymerase-associated nuclease activity was assayed in the presence of various concentrations of one inhibitor and different fixed concentrations of a second inhibitor. The reciprocals of reaction velocities were then plotted. This method of analysis has been described by Segel (25) and is valid for all combinations of inhibitors, whether competitive or noncompetitive with substrate. The effect of the second inhibitor upon slopes of the lines observed indicates whether enzyme is simultaneously inhibited by both inhibitors or whether inhibition by one agent prevents inhibition by a second agent. In the former case, addition of a second inhibitor results in a series of intersecting lines with increasing downward slopes, due to the formation of a dual inhibitor-enzyme complex in addition to the complexes formed between enzyme and single inhibitors. If binding of one inhibitor prevents binding of a second inhibitor, however, then inhibition is mutually exclusive and the addition of a second inhibitor has no effect on the slopes of the lines observed.

Previous studies have shown that incorporation of ACGTP or DHPGTP into template may result in product inhibition (10, 12) and possibly enzyme inactivation (16) when high concentrations of ACGTP are used. In the present study reaction velocities in the presence of ACGTP and DHPGTP decreased relative to controls upon prolonged incubation. However, reaction velocities in the presence of 3, 6, and 9 nM ACGTP remained constant for at least 10 min and reaction velocities were constant for at least 20 min in the presence of 30 nM DHPGTP. Reaction velocities in the presence of 60 and 90 nM DHPGTP were constant only if aphidicolin or PFA was present (data not shown). Under these conditions steady state was achieved, so DNA polymerase reactions involving ACGTP were incubated for only 10 min, whereas those involving DHPGTP in combination with aphidicolin or PFA were incubated for 20 min.

The interaction of HSV DNA polymerase with PFA and ACGTP or DHPGTP during DNA synthesis is shown in Fig. 1. The K_i value for PFA was 0.6 μM. This was determined by the intercept ($-K_i$) of the line obtained in the absence of a second inhibitor, since PFA is noncompetitive with substrate, and is in reasonable agreement with previously published values (9, 11, 20, 22). Since ACGTP and DHPGTP are competitive with dGTP, which was used at a concentration approximately 2.5-fold above the K_m, apparent K_i values of 10 nM for ACGTP and 60 nM for DHPGTP are higher than the true K_i values of 3 and 30 nM published previously (10, 12). A set of parallel lines were observed, indicating that inhibition of DNA polymerase by acyclic nucleotide and PFA was mutually exclusive (25). This suggests that the PFA binding site on the DNA polymerase molecule may be overlapping with the acyclic nucleotide binding site.

A similar set of experiments, designed to investigate the interaction of HSV DNA polymerase with aphidicolin and acyclic NTP, is shown in Fig. 2. Reciprocal plots of reaction velocities indicated that inhibition of DNA synthesis was mutually exclusive with apparent K_i values of 2 to 5 μM, 10nM and 60 nM for aphidicolin, ACGTP, and DHPGTP. Since aphidicolin is noncompetitive with dGTP (13), apparent K_i values were estimated by the abscissa intercept method.

Dual inhibitor studies were also performed to examine the interaction of PFA and aphidicolin with HSV DNA polymerase. The results presented in Fig. 3A indicate mutually exclusive inhibition of DNA polymerase activity with a K_i value of 0.6 μM for PFA and an apparent K_i of 5 μM for aphidicolin. This experiment was also performed with [^{3}H]dTTP as labeled precursor and mutually exclusive kinetics were observed (data not shown). In addition, the effect of PFA and aphidicolin upon hydrolysis of DNA by HSV DNA polymerase-associated nuclease is shown in Fig. 3B. The interaction was mutually exclusive with K_i values of 8 and 0.22 μM for PFA and aphidicolin. Since neither PFA nor aphidicolin is competitive with DNA substrate (9, 11, 13, 20–22) K_i values were determined by the abscissa intercept and by replot of ordinate intercepts.

These results indicated that PFA, aphidicolin, ACGTP, and DHPGTP acted upon HSV DNA polymerase in such ways that inhibition of nucleotide incorporation by one compound interfered with inhibition by a second compound. This was also true for inhibition of DNA polymerase-associated nuclease activity by PFA and aphidicolin. This does not prove that binding sites for the three classes of compound are identical or that they are located physically close

together, since mutually exclusive kinetics could result from allosteric effects. However, if the binding sites are overlapping it would be possible for a single change at the active site of DNA polymerase to result in altered affinities for PFA, aphidicolin, and acyclic NTPs; for example, if a basic amino acid at the active site is involved in the binding of PFA and acyclic NTP, it is conceivable that a change to a more neutral amino acid would lessen the binding affinities of acidic compounds such as phosphonoacetate and acyclic NTPs, whereas affinities to hydrophobic compounds such as aphidicolin would increase. In this event, the reported cross-resistance of PFA-resistant virus strains and DNA polymerases isolated from these strains to inhibition by acyclic nucleotide (5, 6, 8, 14, 19, 24) and collateral hypersensitivity to aphidicolin (2, 4, 18) would be more than coincidental.

Based on the mutually exclusive interaction of PFA, aphidicolin, and acyclic NTP with virus DNA polymerase, we would predict that inhibition of virus by combinations of these compounds should not be more than additive. However, several reports have indicated synergistic interaction of PFA and ACG (7, 26). One possible explanation for these observations is that these compounds may act by other mechanisms in addition to inhibition of HSV DNA polymerase. There is no evidence at present to support this. It is also possible that the synergistic antiviral activity, which was observed using plaque reduction assays, reflects the action of combinations of these compounds against different populations of virus variants in the virus preparations used. It will be of interest to see if similar results are obtained with yield reduction assays.

The kinetic data presented in these studies suggest that the binding sites for these compounds could be physically overlapping. High-resolution mapping of drug resistance loci of the HSV DNA polymerase gene or sequencing of enzyme purified to homogeneity will be necessary to further investigate this possibility. For the present time, these data shed light on the complexity of virus DNA polymerase and should be considered during the design of combination chemotherapy protocols.

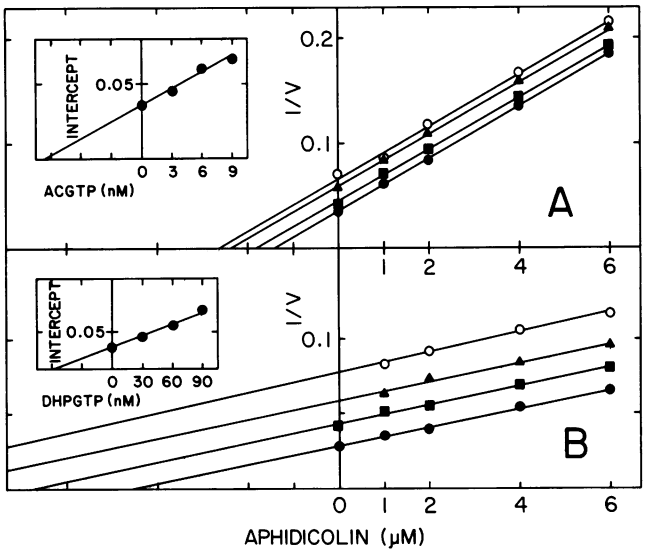

FIG. 2. Inhibition of virus DNA polymerase by combinations of aphidicolin and acyclic nucleotide. DNA polymerase (0.03 U) was assayed under conditions described in the text. Aphidicolin was present at the concentrations indicated. (A) ACGTP was added to produce the following concentrations: 0 (●), 3 (■), 6 (▲), and 9 (○) nM. (B) DHPGTP was added to produce the following concentrations: 0 (●), 30 (■), 60 (▲), and 90 (○) nM. Reaction velocity is expressed as picomoles of dGMP incorporated per hour. Lines were fitted to the data by the method of least squares.

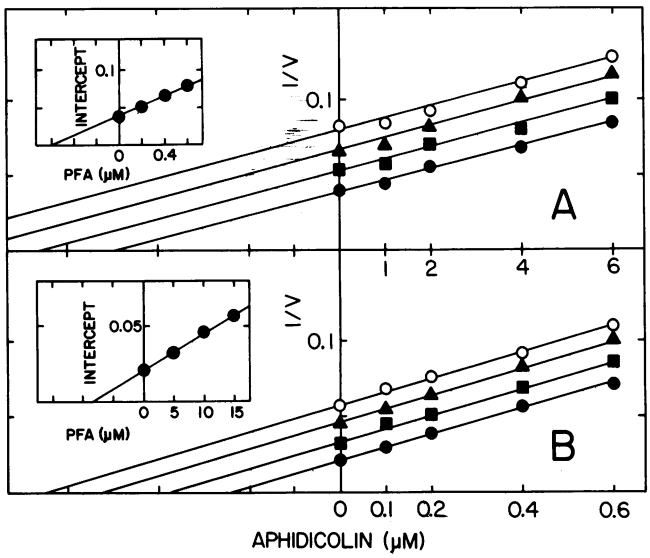

FIG. 3. Inhibition of virus DNA polymerase and polymerase-associated nuclease activity by combinations of aphidicolin and PFA. (A) DNA polymerase (0.03 U) was assayed under conditions described in the text. Aphidicolin was present at the concentrations indicated. PFA was added to produce the following concentrations: 0 (●), 0.2 (■), 0.4 (▲), and 0.6 (○) μM. Reaction velocity is expressed as picomoles of dGMP incorporated per hour. (B) DNA polymerase-associated nuclease was assayed under conditions described in the text. Reaction mixtures contained 0.05 U of DNA polymerase (assayed under standard DNA polymerase conditions) and aphidicolin was present at the concentrations indicated. PFA was added to produce the following concentrations: 0 (●), 5 (■), 10 (▲), and 15 (○) μM. Reaction velocity is expressed as picomoles of dGMP released from acid-precipitable polynucleotide per hour. Lines were fitted to the data by the method of least squares.

ACKNOWLEDGMENT

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