JOURNAL OF VIROLOGY, Nov. 1980, p. 457-464
0022-538X/80/11-0457/08$02.00/0
Vol. 36, No. 2

Mechanism of Inhibition of Herpes Simplex Virus and Vaccinia Virus DNA Polymerases by Aphidicolin, a Highly Specific Inhibitor of DNA Replication in Eucaryotes

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The inhibition in vitro of herpes simplex virus 1 and vaccinia virus DNA polymerases by aphidicolin is primarily noncompetitive with dGTP, dATP, dTTP, DNA, and Mg^{2+} and competitive with dCTP in analogy with the mode of inhibition of cellular α-polymerase. The degree of inhibition of viral or cellular growth in vivo can be quantitatively predicted by the degree of inhibition of the isolated replicative DNA polymerases at the same concentration of aphidicolin in suitable conditions (limiting dCTP concentration). Thus, the only in vivo target for aphidicolin is probably the replicative DNA polymerase, and aphidicolin is a highly specific inhibitor of replicative nuclear DNA synthesis in eucaryotes. This, coupled with the lack of mutagenic effect, represents a valuable property for an anticancer drug. The specificity of inhibition (contrary to the aspecific effect on almost all DNA polymerases by a true competitive inhibitor, such as 1-β-D-arabinofuranosylcytidine 5'-triphosphate) and the structure of the drug, which does not resemble that of the triphosphates, suggest that aphidicolin must recognize a site common only to the replicative DNA polymerases of eucaryotes and different from the binding site for deoxyribonucleic triphosphates and DNA, which should be similar in reparative and procaryote-type DNA polymerases; the aphidicolin binding site is probably very near to, or even overlapping with, the binding site for dCTP so that the drug mimics a competitive effect with this nucleotide.

Aphidicolin, a tetracyclic diterpenoid obtained from *Cephalosporium aphidicola* (4), inhibits growth of cultured human cells (5, 17) as well as growth of herpes simplex and vaccinia viruses (5). In vitro, it inhibits specifically the α-polymerase of animal cells (12, 15, 17), which, by all evidence (2, 9, 10, 11, 25, 26), is the replicative enzyme for chromosomal DNA. It does not inhibit the β-polymerase (12, 15, 17), most likely involved in DNA repair (10, 11), nor the γ-polymerase (12, 15, 17), involved in mitochondrial (and hence procaryote-like) DNA synthesis (10, 11). We have recently shown that herpes simplex virus- and vaccinia virus-coded DNA polymerases are also inhibited by aphidicolin (17) and that this inhibition is probably the basis of its antiviral activity. The inhibition by aphidicolin of DNA synthesis in vivo (17) or of DNA polymerase α in vitro (15, 17) is reversible, but the mechanism of inhibition is still uncertain. The report that the inhibition of DNA polymerase α in vitro is noncompetitive with dATP, dGTP, dTTP and competitive with dCTP (13) contrasts with the inhibition of DNA synthesis in isolated nuclei by aphidicolin, which is surprisingly noncompetitive with respect to each deoxyribonucleoside triphosphate (dNTP) (14). Furthermore, the K_i values of aphidicolin on the isolated herpes simplex virus DNA polymerase and on the α-polymerase are higher by a factor of 10 than the concentrations giving half-maximal inhibition in vivo of viral or cellular DNA replication (17). We therefore investigated the mechanism of inhibition of the DNA polymerases of herpes simplex and vaccinia viruses comparatively with that of the α-polymerase of the host human cells; we also studied the comparative effects of aphidicolin and 1-β-D-arabinofuranosylcytidine 5'-triphosphate (ara-CTP) on these, on the other animal DNA polymerases (β and γ), on reverse transcriptase, on deoxynucleotidyl terminal transferase, and on bacterial DNA polymerases. As the data presented here will show, we have come to the conclusion that aphidicolin is a specific inhibitor of the replicative eucaryotic DNA polymerases and that its in vivo activity can be predicted solely and quantitatively on the basis of the effect on the isolated DNA polymerases. The mechanism of inhibition is apparently competitive with dCTP in all sensitive DNA polymerases, so the drug must recognize a specific por-

tion of the active site of the enzyme unique to the replicative eucaryotic DNA polymerases.

MATERIALS AND METHODS

Chemicals. Unlabeled dNTP's were purchased from C. F. Boehringer & Soehne GmbH, Mannheim, Germany. Dithiothreitol (DDT) was obtained from Miles Laboratories, Inc., Kankakee, Ill. Bovine serum albumin, grade A, was obtained from Calbiochem, Lucerne, Switzerland. [^{3}H]dNTP's and [*methyl*-^{3}H]-thymidine (24 Ci/nmol) were from Radiochemical Centre. Polyadenylate and oligodeoxythymidylate (chain length, 12 to 18) were obtained from P-L Biochemicals, Milwaukee, Wis. Herpes simplex virus 1 (HSV-1) DNA polymerase and vaccinia virus DNA polymerase were purified by the methods of Yamada et al. (27) and Citarella et al. (7), respectively, and were a gift of A. Weissbach of the Roche Institute of Molecular Biology, Nutley, N.J. HeLa cell DNA polymerase α was a DNA-cellulose fraction with 22,000 U/mg. Deoxynucleotidyl terminal transferase was purified from calf thymus and was a gift of Dr. Ramel of the Research Division of Hoffmann-La Roche Inc., Nutley, N.J. Avian myeloblastosis virus reverse transcriptase was a gift of G. E. Houts of the National Institutes of Health, Bethesda, Md.

Preparation of templates. Activated calf thymus DNA was prepared as described by Pedrali-Noy and Weissbach (20).

Assay of DNA polymerases. Each reaction was carried out at 37°C. The α-polymerase was assayed in a mixture of 20 mM potassium phosphate (pH 7.2), 0.5 mM DTT, 7 mM $MgCl_2$, 150 μg of bovine serum albumin per ml, 200 μg of activated calf thymus DNA per ml, 100 μM dGTP, dCTP, and dATP, and 50 μM [^{3}H]dTTP (500 cpm/pmol) or with the modifications indicated in the figure legends.

The vaccinia virus DNA polymerase was assayed by the method of Citarella et al. (7) in a reaction mixture containing 50 mM potassium phosphate (pH 7.5), 0.5 mM DTT, 7 mM $MgCl_2$, 150 μg of bovine serum albumin per ml, 200 μg of activated DNA per ml, [^{3}H]dTTP (1,000 cpm/pmol) at 50 μM, and 100 μM nonradioactive dNTP's or with the modifications indicated in the figure legends.

For the HSV-1 DNA polymerase assay, 0.25 M KCl was included in the α-polymerase assay mixture, and potassium phosphate buffer was replaced by 50 mM Tris-hydrochloride (pH 8.3) according to Yamada et al. (27). Samples of the reaction mixture were placed on GF/C filters and batch washed with trichloroacetic acid as described previously by Bollum (1). The background level was less than 100 cpm when the labeled dNTP was present at the highest specific activity. A unit of DNA polymerase incorporates 1 nmol of total dNTP into acid-insoluble form in 60 min at 37°C.

RESULTS

Mode of inhibition of HSV-1 and vaccinia virus DNA polymerases by aphidicolin. To obtain information on the mechanism of inhibition of HSV-1 and vaccinia virus DNA polymerases, we studied the effect of increasing concentrations of aphidicolin on the polymerization rates at various concentrations of activated DNA, dNTP's, or Mg^{2+}. The results were compared throughout with parallel tests on the DNA polymerase α, purified from HeLa cells.

With various concentrations of activated DNA, the behavior of aphidicolin inhibition of HSV-1 and vaccinia virus DNA polymerases was relatively complex (Fig. 1). At high concentra-

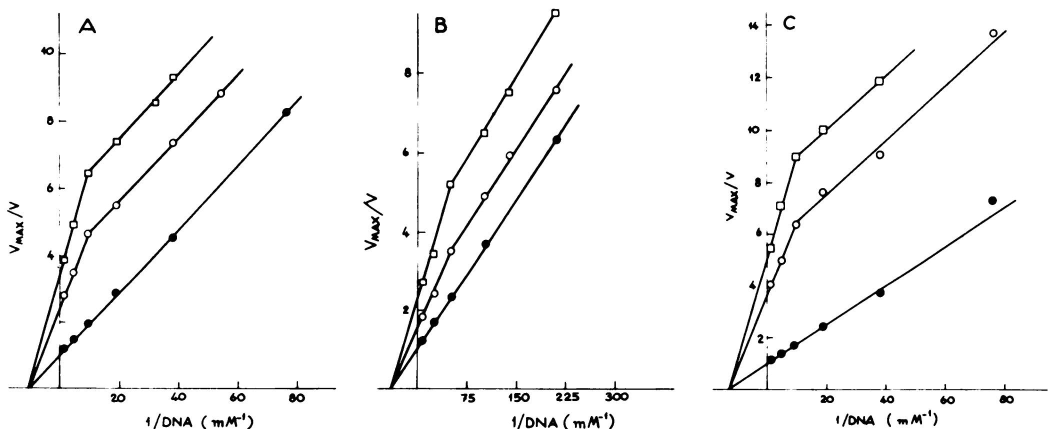

FIG. 1. *Double reciprocal plots of the effects of aphidicolin on the polymerization rates of HSV-1 (A) and vaccinia virus (B) DNA polymerases or HeLa cell DNA polymerase α (C) in the presence of various concentrations of activated DNA. V_{max} is 19,000 (A), 11,500 (B) or 75,000 (C) cpm of [^{3}H]dTMP incorporated per hour. In this as well as in subsequent figures each point is the average of two determinations; all lines represent least-squares fits of the experimental values, and time points were taken at 30 and 60 min, when the kinetics were linear. Symbols: no aphidicolin (●); (A) 2.96 (○) and 7.4 (□) μM aphidicolin; (B) 11.8 (○) and 34 (□) μM aphidicolin; and (C) 4.44 (○) and 7.4 (□) μM aphidicolin.*

tions of DNA substrate (the apparent K_m for the DNA is 90 ± 6 and 25 ± 2 μM in terms of nucleotide for the HSV-1 and vaccinia virus polymerases, respectively), the inhibition seemed purely noncompetitive, but at low concentrations of DNA, aphidicolin behaved as an uncompetitive inhibitor. A similar inhibition at various concentrations of activated DNA was also observed for HeLa cell α-polymerase: with this enzyme the apparent K_m for activated DNA was 76 ± 5 μM in terms of nucleotide, and the inhibition was similar to that exerted on HSV-1 and vaccinia virus DNA polymerases; the inhibition was thus of the purely noncompetitive type at a high ratio of DNA template to enzyme, but at low concentrations of DNA it became uncompetitive (Fig. 1C). The results obtained at low concentrations were similar to those described by Oguro et al. (13) for the DNA polymerase α from sea urchin blastulae, but these authors did not perform experiments at higher DNA concentrations. A possible explanation for this behavior would be that the polymerase, when bound to DNA, might have the right configuration to bind aphidicolin and could retain this configuration for a short time after it is released from DNA. At high DNA concentrations (as is the case inside the nucleus) the polymerase would remain unbound to DNA for very short times and therefore would bind aphidicolin, and the inhibition would appear noncompetitive; at low DNA concentrations, the unmodified form of the enzyme would dominate, and aphidicolin would behave as an uncompetitive and less efficient inhibitor. It would therefore be of interest in this respect to perform affinity measurements with labeled aphidicolin, but the radioactive compound is not yet available.

With different concentrations of dGTP (the data with dATP were similar and are not shown) or dTTP (Fig. 2), the inhibitions of HSV-1 and vaccinia virus DNA polymerases by aphidicolin were of the mixed type because the straight lines intersect at a point to the left of the origin slightly above the 1/dGTP or 1/dTTP axis. This indicated a slight deviation from purely noncompetitive behavior and a somewhat higher affinity of the inhibitor for the free enzyme than for the enzyme-dNTP complex (24). The comparative experiments with DNA polymerase α at different concentrations of dGTP (and dATP, data not shown) showed that aphidicolin affected the slope of the line of the double reciprocal plot, but not the intersect on the abscissa (Figure 2C). This indicated that the effect was purely noncompetitive in this case and suggested a complete lack of effect of aphidicolin on the affinity of DNA polymerase α for dGTP (and dATP). With dTTP the inhibition was of the mixed type because the straight lines intersected at a point to the left of the origin slightly above the 1/dTTP axis, thus indicating a somewhat higher affinity of the inhibitor for the free enzyme than for the enzyme-dTTP complex, as was the case for the viral polymerases. When various concentrations of [^{3}H]dCTP were used with saturating concentrations of dGTP, dATP, and dTTP, the inhibition was apparently competitive for all three tested polymerases (Fig. 3), as has been recently reported for the DNA polymerase α of sea urchin blastulae and mouse myeloma (13).

With respect to Mg^{2+} concentrations, aphidicolin behaves as a noncompetitive inhibitor in DNA synthesis catalized by both viral DNA polymerases and by DNA polymerase α (data not shown).

Replicative HSV-1, vaccinia virus, and human DNA polymerases are probably the only targets for aphidicolin in vivo. We previously reported (17) that 50% inhibition of HSV-1 growth or of DNA synthesis in HeLa cells was obtained in vivo at 0.59 or 0.22 μM aphidicolin, respectively, i.e., at concentrations nearly 10 times lower than those observed for the in vitro inhibition of purified HSV-1 DNA polymerase and human α-polymerase, respectively. Vaccinia virus DNA polymerase was inhibited 50% at 22 μM aphidicolin, a concentration only two times higher than that required for 50% inhibition of vaccinia growth. We interpreted this as being due to compartmentation of the drug in the nucleus, to an additional target of aphidicolin in the replication fork, or to metabolic conversion of aphidicolin to an even more active inhibitor in vivo. The last hypothesis is ruled out by a recent report (16) that rat liver microsomal oxidases can convert aphidicolin to inactive metabolite(s), whereas HeLa cells neither inactivate nor convert aphidicolin to more active derivative(s).

Now, in the light of the observed competitive inhibition with dCTP of HSV-1, vaccinia virus, and HeLa cell replicative DNA polymerases by aphidicolin, we studied the inhibition of these viral and cellular DNA polymerases in vitro at 3 μM dCTP (which is the in vivo concentration of this dNTP in HeLa cells [3]) and found that concentrations of 0.5, 10, and 0.22 μM aphidicolin, respectively, were required for 50% inhibition (Fig. 4). These concentrations of aphidicolin are identical to those causing 50% reductions in the growth rates of HSV-1, vaccinia virus, and HeLa cells, respectively. This suggests that both viral and cellular replicative DNA polymerases are probably the only target for

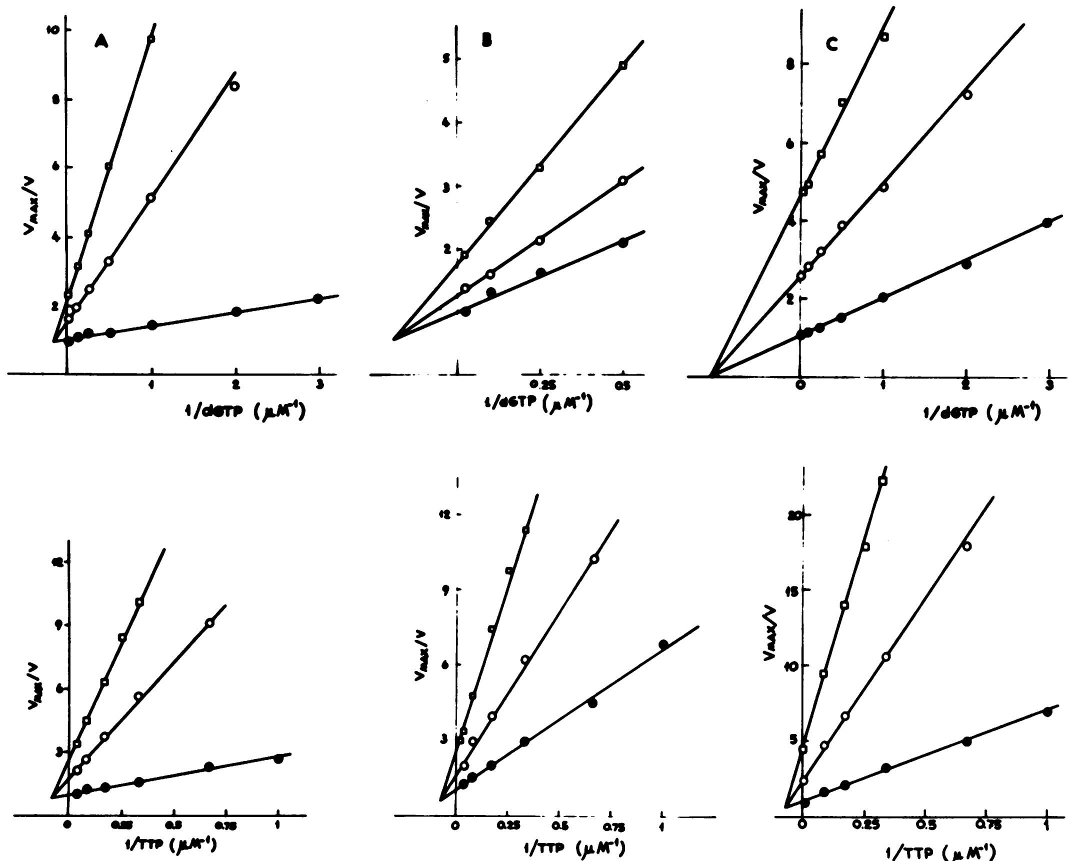

FIG. 2. *Double reciprocal plots of the effects of aphidicolin on the polymerization rates of HSV-1 (A) and vaccinia virus (B) DNA polymerases or HeLa cell DNA polymerase α (C) in the presence of various concentrations of [³H]dGTP or [³H]dTTP. The other three nonradioactive dNTP's were 100 μM. V_{max} values are 20,000 (A), 12,500 (B), and 45,000 (C) cpm of [³H]dGMP incorporated per h and 30,000 (A), 9,500 (B), and 52,500 (C) cpm of [³H]dTMP incorporated per h. Symbols: no aphidicolin (●); (A) 2.96 (○) and 7.4 (□) μM aphidicolin; (B) 11.8 (○) and 34 (□) μM aphidicolin; and (C) 2.96 (○) and 7.4 (□) μM aphidicolin.*

aphidicolin also in vivo and explains our previously reported (17) lower susceptibility of these DNA polymerases to aphidicolin. Unaware of the competitive inhibition with dCTP, in our previous work we measured the in vitro inhibition of these polymerases with 100 μM dCTP in the reaction mixture.

Aphidicolin as a specific inhibitor of DNA replication in eucaryotes (or eucaryote-type chromosomes) due to interference with only replicative eucaryotic DNA polymerases. Aphidicolin is a specific inhibitor of replicative DNA synthesis in eucaryotes; this conclusion issues from the following observation: this drug inhibits in vivo the DNA synthesis of animal (5, 12, 17) and plant (22a) cells as well as the growth of viruses, such as herpes simplex and vaccinia viruses (5, 17), whereas it does not affect bacterial growth (see below). In vitro, it inhibits specifically the replicative α-polymerase of animal (12, 15, 17) and plant (22a) cells and the DNA polymerases coded by HSV-1 and vaccinia virus (17), which are essential for the replication of their DNA. The replicative α-like DNA polymerase A_1 (6) of a lower eucaryote, *Saccharomyces cerevisiae*, is also inhibited by the drug (P. Plevani, personal communication). DNA polymerase β most likely involved in DNA repair (10, 11), DNA polymerase γ (involved in mitochondria DNA synthesis, and hence procaryote-like because of the absence of nucleosomal structure in mitochondrial DNA [10, 11]), the template-independent deoxynucleotidyl terminal transferase, and the reverse transcriptase from avian myeloblastosis virus are all resistant to aphidicolin (Table 1).

Conversely, the bacterial DNA polymerases, either nonreplicative or replicative (*Escherichia*

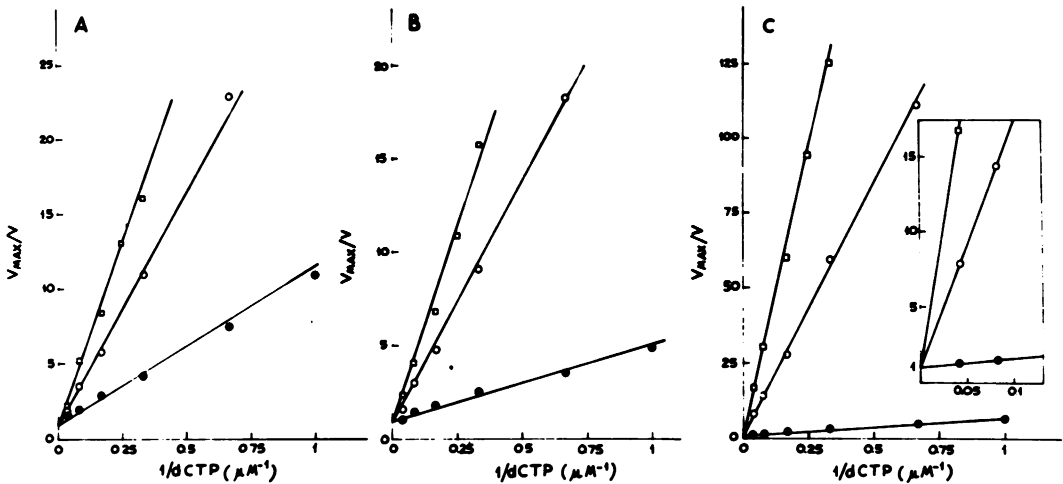

FIG. 3. *Double reciprocal plots of the effects of aphidicolin on the polymerization rates of HSV-1 (A) and vaccinia virus (B) DNA polymerases or HeLa cell DNA polymerase α (C) in the presence of various concentrations of [³H]dCTP (1,000 cpm/pmol). The other three nonradioactive dNTP's were 100 μM. V_{max} is 16,400 (A), 10,000 (B), or 50,000 (C) cpm of [³H]dCMP incorporated per h. Symbols: no aphidicolin (●); (A) 1* (○) *and 2.96* (□) *μM aphidicolin; (B) 12* (○) *and 30* (□) *μM aphidicolin; and (C) 2.96* (○) *and 7.4* (□) *μM aphidicolin.*

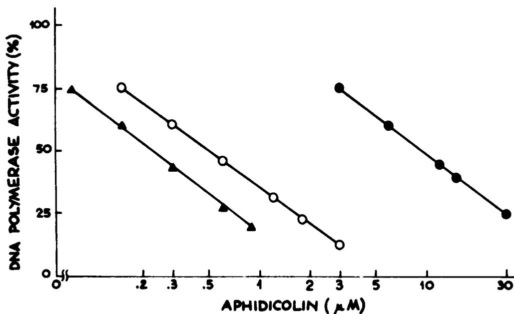

FIG. 4. *Inhibition of HSV-1* (○) *and vaccinia virus* (●) *DNA polymerases or HeLa cell DNA polymerase α* (▲) *by aphidicolin. Each DNA polymerase was tested under the assay conditions described in the text except that dCTP was present at 3 μM.*

coli DNA polymerases I, II, and III and DNA polymerase III holoenzyme), and the T4 phage DNA polymerase are all insensitive to this drug (Table 1). Furthermore, we have shown that bacterial growth is resistant even to 250 μM aphidicolin (remembering that the K_i values for HeLa cell, HSV-1 and vaccinia virus growth are 0.22, 0.59, and 11 μM, respectively). The possibility that the insensitivity of bacteria to aphidicolin may be due to inactivation of the drug is ruled out by a recent report that, in contrast to rat liver microsomes, bacteria do not inactivate aphidicolin after incubation for several hours (16). Another reason for the insensitivity of bacteria could be a lack of permeation by the drug. However, this is unlikely since a study of DNA synthesis in *E. coli* permeabilized by toluene shows that *E. coli polA1* cells (derived from W3110), treated by a procedure known to give a physiologically active growing point sensitive to other specific replication inhibitors, like nalidixic acid (21), incorporate 52 pmol of dTMP per 5 $\times 10^8$ cells per min and 54 and 58 pmol with 30 and 300 μM aphidicolin, respectively.

What is the basis for the specificity of recognition by aphidicolin for the replicative polymerases of eucaryotes? The question is puzzling, since the kinetic measurements reported above demonstrate that the in vitro inhibition of HSV-1, vaccinia virus, and human replicative DNA polymerases by aphidicolin is primarily noncompetitive with respect to dGTP, dATP, dTTP, DNA, and Mg^{2+}, but is competitive with dCTP. This seems a surprising observation in light of the specificity of inhibition of the replicative DNA polymerases of eucaryotes by aphidicolin. We have therefore compared the effects of aphidicolin and ara-CTP (a well known true competitor of dCTP) on several bacterial, viral, and animal replicative and nonreplicative DNA polymerases (Table 1). From the data it is evident that ara-CTP is conspicuously nonselective with respect to the inhibition of DNA polymerases, being active indifferently on bacterial, viral, and animal enzymes and on reparative or replicative enzymes, whether DNA directed, RNA directed, or not requiring template (with the notable exception of *E. coli* DNA polymerase I). Con-

TABLE 1. *Effects of aphidicolin and ara-CTP on several DNA polymerase activities*

Enzyme	Aphidicolin		ara-CTP	
	Sensitivity (+) or resistance (−)	Reference	Sensitivity (+) or resistance (−)	Reference
E. coli DNA polymerase I	−	This paper[a, b]	−	22
E. coli DNA polymerase II	−	This paper[a, b]	+	22
E. coli DNA polymerase III	−	This paper[a, b]	+	22
E. coli DNA polymerase III holoenzyme	−	This paper[a, b]		
T4 DNA polymerase	−	This paper[a]	+	22
Deoxynucleotidyl terminal transferase	−	This paper[a]	+	8
Reverse transcriptase	−	This paper[a]	+	23
DNA polymerase β	−	12, 15, 17	+	23, 28
DNA polymerase γ	−	12, 15, 17	+	23
DNA polymerase α	+	12, 15, 17	+	28
Herpes simplex 1 DNA polymerase	+	17	+	This paper
Vaccinia DNA polymerase	+	17	+	This paper
Plant cell α-like DNA polymerase	+	Sala et al.[c]	+	This paper
Plant cell γ-like DNA polymerase	−	Sala et al.[c]		

[a] These negative results refer to a series of experiments in which up to 120 μM aphidicolin was used in the assays of bacterial and phage DNA polymerases and up to 240 μM aphidicolin was used in the assays of terminal transferase, reverse transcriptase, and DNA polymerases β and γ. Reverse transcriptase was insensitive to aphidicolin when copying both ribo- and deoxyribosynthetic templates or activated DNA.

[b] In collaboration with U. Hübscher.

[c] Sala et al. (22a).

versely, aphidicolin recognizes with high specificity the eucaryotic enzymes and, among these, the nuclear and DNA-dependent enzymes and those involved in replicative synthesis. Thus, a simple competition with the dCTP site cannot explain the results with aphidicolin, considering that its structure is highly hydrophobic, that it does not resemble the triphosphate structure, and that the binding site for dNTP's is probably similar also in the reparative and procaryote-type DNA polymerases. It thus seems reasonable to suggest that aphidicolin must recognize a hydrophobic site which is common only to the replicative DNA polymerases of eucaryotes; this binding site is probably so near or even overlapping with the binding site for dCTP that this agent mimics competitive behavior.

DISCUSSION

We have shown that the in vitro inhibition of HSV-1 and vaccinia virus DNA polymerases by aphidicolin is primarily noncompetitive with respect to dGTP, dATP, dTTP, DNA, and Mg^{2+} and competitive with respect to dCTP. The mechanism of inhibition is therefore similar for animal DNA polymerase α (13), plant cell α-like DNA polymerase (Sala et al., in press), and viral replicative DNA polymerases. In addition, our results indicate that aphidicolin is a specific inhibitor of nuclear DNA replication in eucaryotes (or eucaryote-type chromosomes) because it interferes only with replicative eucaryotic DNA polymerases.

We have also compared the effects of aphidicolin and ara-CTP (a known competitor of dCTP) on several bacterial, viral, and cellular replicative and nonreplicative DNA polymerases and concluded that aphidicolin must recognize a hydrophobic region common only to the replicative DNA polymerases of eucaryotes (and hence different from the binding sites for dNTP's and DNA, which probably have to be similar in the reparative and procaryote-type DNA polymerases) in the reverse transcriptase and deoxynucleotidyl terminal transferase as well as in replicative eucaryotic DNA polymerases. Under this hypothesis, the slightly different sensitivities to aphidicolin of HSV-1, vaccinia virus, and HeLa cell replicative DNA polymerases should reflect slight modifications of the common binding site for aphidicolin rather than a different capacity to recognize the dCTP substrate.

In the light of these findings we could predict the in vivo inhibition of DNA synthesis or of viral and HeLa cell growth from the in vitro inhibition of viral and cellular replicative DNA polymerases performed at 3 μM dCTP, which is the in vivo concentration of this dNTP in host HeLa cells. We have thus found that the concentrations of the drug causing 50% reductions in growth of HSV (0.59 μM), vaccinia virus (11 μM), and HeLa cells (0.2 μM) are identical to the

concentrations required to inhibit 50% of the purified HSV-1, vaccinia virus and HeLa cell DNA polymerase α activities in vitro when performed at 3 μM dCTP. These results, coupled with recent observations that the in vivo inhibition by aphidicolin is reversible (17), that the inhibition of the replicative DNA polymerase α allows the synchronization of cell cultures (19), and that when DNA replication is inhibited by aphidicolin, the cells retain the capacity to perform repair synthesis due to nuclear DNA polymerase β (18) and to synthesize mitochondrial DNA due to DNA polymerase γ (M. Geuskens, N. Hardt, G. Pedrali-Noy, and S. Spadari, unpublished data), strongly suggest that, also in vivo, the replicative DNA polymerases of eucaryotes (viral or cellular) are the only target of aphidicolin and that there is no need of additional hypothetical factors modulating the sensitivity of DNA replication to the drug.

The same inhibition for the viral DNA polymerase and α-polymerase by aphidicolin also suggests the existence of similar recognition sites for aphidicolin on these enzymes, probably quite specific for eucaryotic replicative polymerases (Table 1). The fact that other sites of these three replicative polymerase molecules are nevertheless different (absence of immunological cross-reaction) leaves the hope that some chemical modifications of the drug might affect these replicative enzymes differently and, perhaps, supply an inhibitor more active on the viral polymerases than on the host enzymes.

Finally, the selective inhibition of human and viral replicative DNA polymerases by aphidicolin described in this work—coupled with our recent observation that neither aphidicolin nor its metabolic derivative(s) induces DNA repair synthesis in HeLa cells (16, 18), the absence of mutagenicity in the Ames *Salmonella*-microsome test (16), the lack of effect on DNA repair synthesis by human cells (18) and on mitochondrial DNA replication (see above), and the lack of interference with the production of antibodies and the activity against several neoplastic human cell lines (including leukemia and melanoma) (G. Pedrali-Noy, M. Belvedere, N. Hardt, F. Focher, and S. Spadari, manuscript in preparation)—makes aphidicolin a potentially attractive anticancer drug.

ACKNOWLEDGMENTS

We thank A. Todd and B. Hesp of the Imperial Chemical Industries, England, for the gift of aphidicolin and A. Falaschi for discussion and critical reading of the manuscript.

This work was supported in part by the Programma Finalizzato "Controllo della crescita neoplastica" del Consiglio Nazionale delle Ricerche and by EURATOM contract 152-76-1 BIO I.

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