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**Inhibition of DNA polymerase alpha by aphidicolin derivatives**

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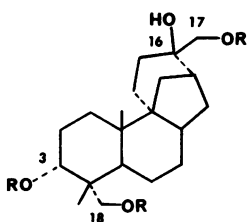
**ABSTRACT**

17-Acetylaphidicolin was 10-fold weaker and two derivatives lacking hydroxyl groups at the 16 and 17 positions were 100-fold weaker than aphidicolin as inhibitors of DNA polymerase  $\alpha$  from HeLa and Chinese hamster ovary cells. 17,18-Diacetyl, 3,17,18-triacetyl and 3-epi derivatives of aphidicolin were inactive. Active compounds were, like aphidicolin, competitive with dCTP and did not inhibit aphidicolin-resistant DNA polymerases.

**INTRODUCTION**

Aphidicolin [1] is a potent inhibitor of eukaryotic DNA polymerase  $\alpha$ . The enzyme from sea urchin embryos (1), rat liver (2) and cultured cells, e.g. HeLa (3,4) and KB (5), is highly sensitive to the drug ( $K_i$  ca. 0.5  $\mu$ M). The recently described DNA polymerase  $\delta$ , derived both from primary mammalian tissues (6-8) and from CV-1 cells (9), has also been reported to be equally sensitive to 1. Although aphidicolin is selective with respect to the other eukaryotic and bacterial DNA polymerases, it does inhibit the DNA polymerases encoded by Herpes simplex type 1 and vaccinia viruses (10) and the  $\alpha$ -like DNA polymerases of yeast (11) and plant cells (12). The mechanism of action of 1 on purified DNA polymerase  $\alpha$  is unknown, but its effect has most often been reported to be competitive with dCTP and noncompetitive with the other dNTP substrates (1,5,10,13), a result also observed with sensitive viral (10) and plant (12) polymerases. We have been intrigued by the spectrum of polymerases sensitive to aphidicolin, by the competitive nature of its action, and by the apparent lack of structural similarity between it and dCTP or any other dNTP. The hydroxyl groups of aphidicolin [1] are situated at opposite ends of a rigid hydrophobic cylindrical structure, and superficially resemble pairs of oxygens in a deoxyribose ring. As a first step in understanding the mechanism of aphidicolin, we sought to measure the effects of changes in its hydroxyl

groups on inhibitory activity. Recently, two naturally occurring derivatives of 1, 17-acetylaphidicolin [2] and 3-deoxyaphidicolin were reported to inhibit sea urchin DNA polymerase  $\alpha$  with potencies similar to that of the parent compound (14). In contrast, a recent paper claimed that 2, among other semisynthetic aphidicolin derivatives, did not inhibit DNA polymerase  $\alpha$  from KB cells (15). We wish to report the effects of blocking or removal of selected hydroxyl groups of aphidicolin on inhibitory activity toward two DNA polymerase  $\alpha$ 's. The results demonstrate that neither intact polar end of 1 is required for expression of inhibitory activity, and that active derivatives demonstrate the same kinetics of inhibition and have selectivity similar to that of aphidicolin.

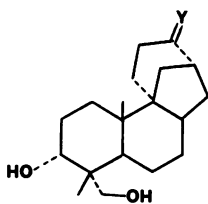


1, R's = H

2, R<sub>17</sub> = COCH<sub>3</sub>, R<sub>3</sub> = R<sub>18</sub> = H

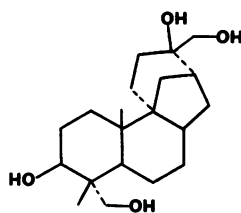
3, R<sub>17</sub> = R<sub>18</sub> = COCH<sub>3</sub>, R<sub>3</sub> = H

4, R's = COCH<sub>3</sub>



5, Y = O

6, Y = H, H



7

Structures

## MATERIALS AND METHODS

Compounds. Aphidicolin [1] was obtained from the Pharmaceuticals Division, Imperial Chemical Industries Ltd. and from the Natural Products Branch, National Cancer Institute. Compound 6 was a gift from Imperial Chemical Industries Ltd., and the other derivatives, 2-5 and 7, were synthesized and purified as described (16).

Thin layer chromatography (TLC). Purity of all compounds was determined by TLC on aluminum-backed silica gel plates (Merck) with 10% methanol in chloroform as eluant. Plates were developed by immersion in a solution of 5% anisaldehyde and 1 M sulfuric acid in ethanol, followed by heating on a hotplate. R<sub>f</sub> values for the compounds in this system were: 0.33 [1], 0.47 [2], 0.52 [3], 0.73 [4], 0.52 [5], 0.61 [6] and 0.27 [7]. As little as 0.01% of aphidicolin could be detected visually in the presence of any other derivative.

DNA polymerases. DNA polymerase  $\alpha$  from HeLa cells (17) and from Chinese hamster ovary (CHO) cells (18) and DNA polymerase III from *B. subtilis* (19) were isolated as described. Reverse transcriptase from avian myeloblastosis virus (AMV RT) was obtained from Life Sciences Inc., St. Petersburg, FL.

Polymerase assays. DNA polymerase assays were done as reported by Khan and Brown (18). Assays typically measured acid-precipitable radioactivity incorporated into DNase-activated calf thymus DNA in the absence of the competitive substrate dCTP ("truncated assay"), and in the presence of 50  $\mu$ M dGTP, dATP and [ $^3$ H]TTP (460 cpm/pmol). Control activities corresponded to the incorporation of 6.57 and 12.0 pmol [ $^3$ H]TMP into activated DNA per 50  $\mu$ l assay tube by the HeLa and CHO enzymes, respectively. Classical  $K_i$  determinations employed variable concentrations of [ $^3$ H]dCTP (650 cpm/pmol) and 50  $\mu$ M of the remaining dNTPs in the presence of several concentrations of test compounds. The results were analyzed as double reciprocal plots, and  $K_i$  values were obtained from Dixon plots as illustrated for compound 5 in the Figure. DNA polymerase III was assayed according to Clements et al. (19) with activated DNA and 50  $\mu$ M dNTPs; control [ $^3$ H]TMP incorporations in the absence and presence of dCTP were 10.2 and 30.3 pmol/assay tube, respectively. AMV RT assays were done as described previously (20) with activated DNA and dNTPs at 50  $\mu$ M; control assays corresponded to incorporation of 5.9 and 17.3 pmol [ $^3$ H]TMP/assay tube in the absence and presence of dCTP, respectively. Inhibitor stock solutions were prepared in dimethylsulfoxide and diluted into enzyme assay mixtures; the solvent (<5%) had no effect on the polymerase control assays.

## RESULTS AND DISCUSSION

### Inhibition of DNA polymerase $\alpha$ by aphidicolin derivatives.

Aphidicolin [1] inhibited both HeLa and CHO cell DNA polymerase  $\alpha$  with  $K_i$  values below 1  $\mu$ M, consistent with results obtained previously (3,4). The results summarized in the Table show that the  $K_i$  values obtained in the truncated, dCTP-deficient assay with both enzymes were identical to those obtained from classical kinetic experiments with the CHO enzyme, as expected if inhibitor is competitive with the deficient substrate (2). Among the synthetic derivatives, 17-acetylaphidicolin [2] inhibited both polymerases, but with about 10-fold lower potency than 1. Two derivatives lacking the hydroxyl groups at the 16 and 17 positions, 3,18-dihydroxy-17-noraphidicolan-16-one [5] and 3,18-dihydroxy-17-noraphidicolane [6], were about 100-fold less active than 1. Inhibition by the derivatives was still competitive with

Table. Inhibitory activity of aphidicolin and derivatives on DNA polymerase  $\alpha$  from HeLa and CHO cells.

$K_i$  values ( $\mu\text{M}$ )<sup>1</sup>

Cmpd.	HeLa (truncated assay)	CHO (truncated assay)	CHO (kinetic)
<u>1</u>	.35	.44	.65
<u>2</u>	3.6	8.5	5.0
<u>3</u>	inactive <sup>2</sup>	inactive	
<u>4</u>	inactive	inactive	
<u>5</u>	40	33	37
<u>6</u>	68	36	nd <sup>3</sup>
<u>7</u>	nd	inactive	

<sup>1</sup> $K_i$  values (truncated) were determined in assays lacking dCTP and represent drug concentrations causing half-maximal inhibition of <sup>3</sup>enzyme activity.  $K_i$  values (kinetic) were determined in assays using [<sup>3</sup>H]dCTP as variable substrate and from Dixon plots of the results. See MATERIALS AND METHODS for details. <sup>2</sup>Inactive compounds gave <10% inhibition at 100  $\mu\text{M}$  concentration. <sup>3</sup>not done

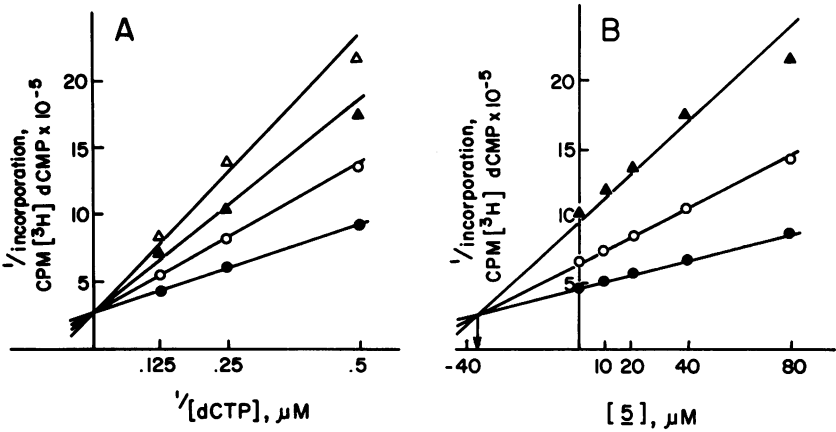


Figure. Kinetic analysis of inhibition of CHO<sub>3</sub> DNA polymerase  $\alpha$  by 5. The enzyme was assayed with activated DNA and [<sup>3</sup>H]dCTP as variable substrate with 50  $\mu\text{M}$  of the other dNTPs (see MATERIALS AND METHODS). Control assays with labelled substrate at 2, 4 and 8  $\mu\text{M}$  incorporated 15.1, 23.1 and 33 pmol of [<sup>3</sup>H]dCMP per assay tube, respectively. Panel A: Lineweaver Burk plot of enzyme activity in the presence of no drug (●) and 20 (○), 40 (▲) and 80 (△)  $\mu\text{M}$  5. Panel B: Dixon plot of enzyme activity in the presence of 5 assayed with [<sup>3</sup>H]dCTP at 2 (▲), 4 (○) and 8 (●)  $\mu\text{M}$ .

dCTP, as illustrated by the results shown in the Figure for 5. Blocking of both ends of 1 to produce 17,18-diacetyl [3] and 3,17,18-triacetylaphidicolin [4] did, however, completely abolish inhibitory activity (see Table). Our observation of potent inhibition of DNA polymerase  $\alpha$  by 2 is consistent with the report of Haraguchi et al. (14) that this compound inhibited the sea urchin enzyme with  $K_i$  2.6  $\mu$ M, but contradicts that of Hiranuma et al. (15) in which 2, 5 and other 17-substituted aphidicolin derivatives were inactive against KB cell DNA polymerase  $\alpha$ . We are confident that the activities reported in the Table for 2, 5 and 6 are indeed due to the derivatives and not, for example, to residual aphidicolin. TLC analyses (see MATERIALS AND METHODS) confirmed that the derivatives contained less than 0.01% of aphidicolin or another derivative. A derivative in which the configuration about C-3 is altered, 3-epiaphidicolin [7], was inactive against CHO DNA polymerase  $\alpha$  at concentrations up to 100  $\mu$ M (Table). Recently, 7 was also reported to be inactive against the KB cell enzyme (15). Although this result indicates a high degree of stereospecificity of interaction of this part of aphidicolin with DNA polymerase  $\alpha$ , the 3 $\beta$ -OH group does not appear to be essential for activity: for example, 3-deoxyaphidicolin, lacking the relevant hydroxyl group, was a potent inhibitor ( $K_i$  = 1.3  $\mu$ M) of the sea urchin enzyme (14). In addition, both 3-deoxyaphidicolin and the 3-oxo derivative were reported to have "one-third" of the activity of 1 against the KB cell DNA polymerase  $\alpha$  (15).

Our results for compounds 2, 5 and 6 and the results from the literature cited above for the 3-deoxy and 3-oxo derivatives of aphidicolin clearly show that neither intact functional end of 1 is absolutely required for expression of DNA polymerase  $\alpha$  inhibition. Simultaneous blocking of both ends of 1 (compounds 3 and 4) did, however, completely abolish inhibitory activity.

Active derivatives are competitive with dCTP.

We questioned if alterations of 1 might have produced active compounds that inhibited DNA polymerase  $\alpha$  with different kinetics. In assays of HeLa DNA polymerase  $\alpha$  under truncated, dCTP-deficient conditions, addition of 0.5 mM of both dGTP and dATP caused no decrease in inhibition by 1, 2, 5 or 6 at concentrations that inhibited the enzyme by 80-90%. However, addition of 0.5 mM dCTP in these assays completely reversed inhibition (results not shown). Substantially the same results were obtained with the CHO enzyme for 1, 2 and 5 by classical kinetic analysis. As summarized in the Table and illustrated in the Figure for compound 5, inhibition was competitive with dCTP and noncompetitive with the other dNTPs (latter results not shown).

### Aphidicolin derivatives do not inhibit aphidicolin-resistant DNA polymerases.

Aphidicolin at 120  $\mu\text{M}$  did not inhibit the *E. coli* DNA polymerases, nor at 240  $\mu\text{M}$  did it inhibit AMV reverse transcriptase (10). We repeated similar assays of aphidicolin and several of its derivatives with *B. subtilis* DNA polymerase III and AMV RT to determine if structural modification might reveal sensitivity of enzymes normally resistant to 1. Neither aphidicolin nor compounds 2, 3 or 5 at 200  $\mu\text{M}$  inhibited the bacterial enzyme or the reverse transcriptase when assayed with activated DNA under truncated (-dCTP) or complete mix conditions. Aphidicolin at 200  $\mu\text{M}$  also did not inhibit AMV RT in reactions employing oligo dT-poly A and [ $^3\text{H}$ ]TTP as template and substrate, in agreement with the results of reference 10 (results not shown).

### CONCLUSIONS

The results reported in this paper and those of related papers (14,15) suggest that 1 binds via both functional (hydroxylated) ends to two sites on DNA polymerase  $\alpha$ , at least one of which is a site that binds dCTP, probably as a substrate. Either polar end of the molecule can be modified, but not both, and the resulting compounds can retain strong to moderate ability to bind and inhibit DNA polymerase  $\alpha$ . Modifications of 1 reported in this paper did not alter the kinetics of inhibition by active compounds, nor did any of the derivatives inhibit enzymes that are normally resistant to 1. Acetylation of the 17-OH group at the "upper end" of 1 (see structure) decreased activity 10-fold, whereas removal of the 17-carbon, leaving 16-oxo or 16-(H<sub>2</sub>) groups, decreased activity an additional 10-fold, suggesting the loss of hydrogen bonding interactions that may stabilize the aphidicolin:enzyme complex. Specific interactions of this part of aphidicolin with enzyme are, therefore, probably not crucial to its binding, and inactivity of compounds bearing 17-substituents more bulky than acetyl (15) may result from steric repulsion. The "lower end" of 1, in contrast, cannot tolerate either a bulky substituent (compounds 3 and 4) or a change in configuration at C-3 (compound 7). Paradoxically, activity is little affected by oxidation of the 3-OH group, according to published results (15), or to its complete removal: the latter compound, 3-deoxyaphidicolin, was reported to be both a potent inhibitor of sea urchin and HeLa DNA polymerase  $\alpha$  and competitive only with dCTP (14). The observations that the inhibitory activity of compounds modified at the "upper end" was also competitive with dCTP and that a configurational change at C-3 abolished

activity would argue that the "lower end" of the molecule is that region that occupies or overlaps with a dCTP binding site on DNA polymerase  $\alpha$ .

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