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Structure–activity relationships for the inhibition of DNA polymerase α by aphidicolin derivatives

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ABSTRACT

Aphidicolin and 17 derivatives that have been structurally modified in the A- and D-rings were assessed for their ability to inhibit DNA polymerase α. No derivative surpassed the activity of aphidicolin; derivatives with structural alterations in the A-ring exhibited significantly greater loss of activity relative to derivatives with structural alterations in the D-ring. The conclusions of these studies indicate a critical role for the C-18 function in the interaction of aphidicolin with polymerase α. Molecular modelling studies could not identify structural features of the aphidicolin-dCTP "overlap" that is unique to dCTP, relative to the remaining dNTPs, and that is consistent with the extant structure-activity data.

INTRODUCTION

Aphidicolin 1, a tetracyclic diterpenoid fungal metabolite, has been under intense investigation as a potential therapeutic agent for the treatment of cancer and viral diseases (1-3). Aphidicolin inhibits the replication of eukaryotic and viral nuclear DNA through a specific and high affinity inhibition of the DNA polymerases α and δ of animal cells, the α-like polymerase of plant cells, and the herpes simplex virus- and vaccinia virus-encoded DNA polymerases (1-10). DNA polymerases α and δ are the replicative enzymes responsible for chromosomal DNA duplication and possess undefined functions in the repair of nuclear DNA (4-6,11-13). Aphidicolin does not inhibit the β-polymerase, involved exclusively in DNA repair, or the Γ-polymerase, involved in mitochrondrial DNA synthesis, and has no effect on the growth of prokaryotic organisms (1-3).

The potential of aphidicolin in the treatment of cancer is currently being explored in the clinical trial by the European Organization for Research and Treatment of Cancer of aphidicolin-17-glycinate 2, a water soluble prodrug of aphidicolin (Figure 1). The decision to advance aphidicolin to the clinic was based on only limited success of this agent in the treatment of human tumor xenographs in experimental animals, a finding that is

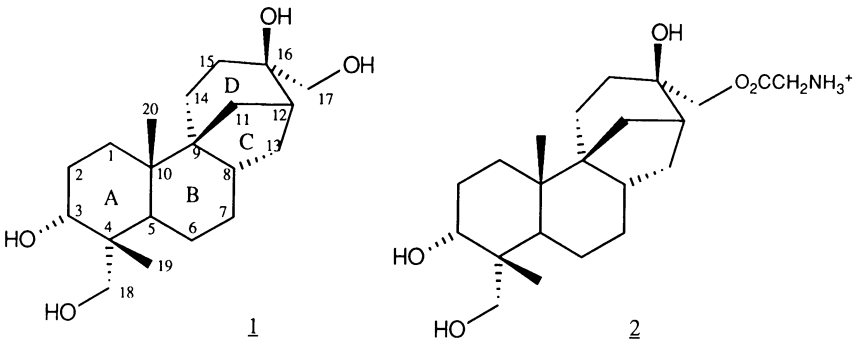


Figure 1. The Structures of aphidicolin (1) and aphidicolin-17-glycinate (2).

inconsistent with the potent growth inhibitory and cytotoxic activities of aphidicolin against human tumor cell lines cultured in vitro. Based on the observation that aphidicolin is inactivated when administered to healthy mice, Pedrali-Noy et al. suggested that the low activity of aphidicolin in many animal tumor models may be a consequence of its rapid metabolic inactivation (1-3). We have recently confirmed this suggestion through our demonstration that aphidicolin undergoes extremely rapid in vitro biotransformation by rat liver systems. We have identified the enzyme responsible for bioinactivation to be a constitutive microsomal cytochrome P-450, postulated to be involved in endogenous steroid regulation (14).

During the course of our studies on aphidicolin metabolism, we identified the initial aphidicolin metabolite, which constitutes >90% of the metabolic profile, and elucidated the chemical sequence leading to the ultimate metabolic disposition of aphidicolin in this in vitro system. These studies indicated the exquisite sensitivity of DNA polymerase α inhibition to aphidicolin structural variation. We have embarked on a program to more precisely define the structure-activity relationships (SAR) of aphidicolin, in order to separate the structural determinants of cytochrome P-450-mediated metabolism and DNA polymerase α inhibition. We wish to report the findings of our studies on the SAR of the aphidicolin skeleton with regard to inhibition of DNA polymerase α.

MATERIALS AND METHODS

Compounds and Methods of Characterization of Aphidicolin Analogs. Aphidicolin, which served as a source for all of the semi-synthetic derivatives described here, was a gift from Dr. Matthew Suffness of the Natural Prod-

ucts Branch of the National Cancer Institute. All of the aphidicolin derivatives were purified to homogeneity on silica gel 60 (230-400 mesh) from E. Merck and analyzed for purity by thin layer chromatographic analysis on silica gel 60 (F-254) TLC plates from E. Merck. All aphidicolin derivatives were fully characterized by the following spectroscropic techniques: ^{1}H NMR spectroscopy recorded on a General Electric QE-300 300 MHz or a Nicolet 360 MHz spectrometer; ^{13}C NMR spectroscopy recorded on a General Electric QE-300 spectrometer at 75 MHz; infrared spectroscopy recorded on a Nicolet FT-IR spectrometer; and mass spectrometry recorded on a Finnigan 4600 spectrometer using chemical ionization with a methane source. Full details of our synthetic procedures and of the spectral characterization of all new compounds will be reported elsewhere (G. Prasad and T. L. Macdonald, in preparation).

Assay for DNA Polymerase α Inhibition. The assay for the inhibition of DNA polymerase α activity was adapted from the method of Pedrali-Noy et al (10). Commercial calf thymus DNA polymerase α (Pharmacia Inc., Piscataway, NJ) was diluted in buffer containing potassium phosphate (60 mM, pH = 7.6), potassium chloride (500 mM), β-mercaptoethanol (50 mM), and glycerol (50% v/v) to provide an activity level of ~25 units/ml and stored in 100µl aliquots at -70°C. Polythymidylic acid (poly [dT]; Sigma Chemical Company, St. Louis, MO) and oligoriboadenylic acid (oligo $[A]_{12-18}$; Pharmacia Inc., Piscataway, NJ) were annealed in a 5 to 1 ratio by weight in water to an A_{260} activity of ~20 and stored at -20°C.

The assay mixture consisted of the following components: Tris buffer (50 mM, pH = 7.8); bovine serum albumin (400 µg/ml); dithiothreitol (1.0 mM), $MgCl_2$ (6.0 mM); KCl (30 mM); the poly [dT]/oligo [A] template-primer (7.2 µg/ml); and [^{3}H]-dATP (ICN Biomedicals, Irvine, CA; 100 µM of a 1.3 mM solution at 246 mCi/mmole). The assay was started by the addition of DNA polymerase α (0.053 units; 9.0 µl) to the assay mixture (40 µl) and an ethanol solution of the aphidicolin derivative (1.0 µl) at 37°C. The total volume of the assay was 50 µl. After a one hour incubation at 37°C, the assay was quenched by the addition of an ice cold solution of 10% trichloroacetic acid/0.1 M sodium pyrophosphate (1.5 ml). The samples were filtered through Millipore microcellulose filters (HAWP 0.45 µm) and washed 3 times with 5.0 ml of the above trichloroacetic acid/pyrophosphate solution and twice with 5.0 ml of cold absolute ethanol. The filters were air dried and transferred to scintillation vials containing 10 ml aqueous counting scintillant. Radioactivity was measured by scintillation spectrometry

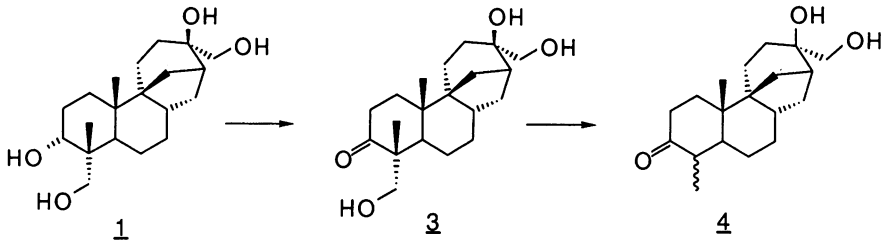


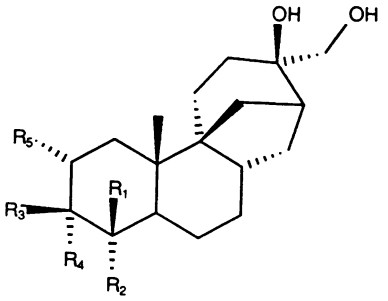
Figure 2. The mechanism of metabolic inactivation of aphidicolin.

on a Beckman LS 3150P liquid scintillation system. The total ethanol content in the polymerase α assay employing the metabolite isolation procedure described above was 2.6%; no loss of DNA polymerase α activity was observed in control studies, in which no aphidicolin was present. The assay was inhibitable to >95% by aphidicolin. Each derivative concentration was determined in triplicate; a minimum of three concentrations was employed to determine relative activity values of the aphidicolin derivatives.

RESULTS AND DISCUSSION

We have recently demonstrated that aphidicolin undergoes rapid metabolic inactivation in in vitro systems from rat liver (14). The sequence of biotransformation is initiated by a cytochrome P-450-mediated oxidation of the C-3 alcohol function and is completed by a retro-aldol reaction of the 18-hydroxymethyl moiety (Figure 2). These metabolic transformations have a profound effect on the activity of the derivative in the inhibition of DNA polymerase α. We therefore initiated a systematic synthetic investigation directed at adjustment of the structural features of the A-ring of aphidicolin in order to expand our understanding of the sensitivity of the aphidicolin binding site on polymerase α to this substructural feature. The diversity of A-ring modified structures that we have developed is presented in Figure 3. We have subsequently explored the sensitivity of polymerase α inhibition to the precise structural features of the D-ring domain; the D-ring aphidicolin analogs prepared for this study are presented in Figure 4.

The data for the inhibition of DNA polymerase α by the aphidicolin derivatives investigated is compiled in the Table. The data is presented for the designated concentration range in terms of two parameters that reflect the activity of the analog relative to that of aphidicolin: the



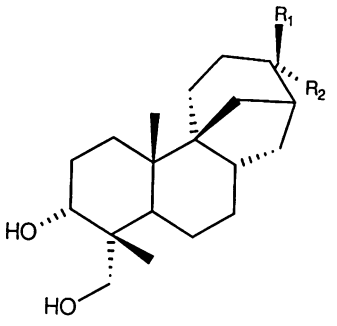
Derivative	R_1=	R_2=	R_3=	R_4=	R_5=
5	CH_3	CH_2OH	H	H	H
6	CH_3	CH_2OH	H	——O——	
7	CH_3	CH_2OH	CH_3	OH	H
8	H	CH_3	H	OH	H
9	CH_3	CH_2OH	C_2H	OH	H
10	CH_3	CH_2OH	OH	H	H
11	CH_3	CH_2OH	H	- ($\Delta^{2,3}$) -	
12	H	CH_3	H	- ($\Delta^{2,3}$) -	
13	CH_3	CH_2OH	H	OH	CH_3
14	CH_3	CH_2OH	OH	CH_3	H

Figure 3. Structures of aphidicolin derivatives (5-14) modified in the A-ring.

relative activity and the IC_{50} value. The relative activity represents the ratio of concentrations (aphidicolin versus derivative) required to effect an identical level of inhibition of DNA polymerase α (expressed as a percentage). The IC_{50} value represents the concentration of the derivative calculated (from a plot of concentration versus polymerase α activity) to effect 50% inhibition of DNA polymerase α activity; the concentration range investigated bracketed the calculated IC_{50} values (with the exception of aphidicolin analog 4 for which an estimated value is presented).

Analysis of Structure-Activity Relationships of A-Ring Modifications of Aphidicolin. From analysis of the data in the Table and of SAR data from other laboratories (15-21), primarily from Hiranuma et al. (20), several prominent, although not absolute, SAR features emerge.

1. The C-18 hydroxyl is the most important functionality in the expression of activity: esterification [e.g. 17,18-diacetoxyaphidicolin (21)], etherification [e.g. 17,18-dibenzylaphidicolin (20)], oxidation [e.g. aphidicolin-17,18-biscarboxaldehyde (20) or removal of this group



Derivative	R_1 =	R_2 =
15	OH	CH_3
16	OH(H)	H(OH)
17	R_2 C=O	R_1
18	OH (n-butyl)	n-butyl (OH)

Figure 4. Structures of aphidcolin derivatives (15-18) modifiedin the D-ring.

[e.g. 18-deoxyaphidicolin (20) and 4, 8, 12 (Table)] significantly diminishes or destroys activity. In addition, the significant difference in activities between 3-deoxyaphidicolin 5 and 2,3-α-epoxyaphidicolin 6 (of $\leq \approx 10X$) or 2,3-dehydroaphidicolin analog 11 (of $\approx 10^5$) must be ascribed to distortions in the in A-ring conformations as a consequence of the C-2/C-3 saturated, epoxy, and unsaturated ring systems. The structural alterations associated with either the 2,3-ene or 2,3 α-epoxy species affect the precise location of the C-18 alcohol functionality in a predictable fashion, and place the 18-hydroxyl group into a pseudo-equatorial orientation (see 20). Thus, the C-18 functional group must be involved in a critical (H-bonding) interaction in the binding site. Additional support comes from Arabshahi et al. (21) who report that 17-acetylaphidicolin showed activity against DNA polymerase α, while 17,18-diacetylaphidicolin was inactive. Rosazza et al. have reported (16,17) 18-acetoxyaphidicolin and aphidicolin-18-carboxylic acid inhibit the uptake of thymidine to P388 leukemic cells, but both compounds were inactive in an in vivo antitumor test system. The in vitro P388 leukemic test system is done in cell culture and the activity of these compounds may be due to metabolic conversion of these compounds back to aphidicolin.

TABLE
INHIBITION OF CALF THYMUS DNA POLYMERASE α
BY SYNTHETIC APHIDICOLIN DERIVATIVES

Aphidicolin Derivative	Concentration Range (mM)	Relative Activity	IC_{50} (μM)
A-ring modified derivatives:			
1	0.370-592(μM)	100	7.4
3	0.01 - 11.4	9 ± 1	75
5	0.15 - 3.50	3.5 ± 0.5	240
6	0.46 - 2.29	1.8 ± 0.7	1,100
7	0.72 - 14.3	1.0 ± 0.7	1,800
8	0.65 - 6.53	0.1 ± 0.05	5,600
4	0.74 - 7.44	0.02 ± 0.02	20 mM*
9	0.19 - 1.91	0.005 ± 0.001	-
10	0.95 - 3.79	$<10^{-4}$	-
11	2.29 - 22.9	$<10^{-4}$	-
12	2.04 - 20.4	$<10^{-4}$	-
13	0.17 - 1.7	$<10^{-4}$	-
14	0.22 - 4.44	$<10^{-4}$	-
D-ring modified derivatives:			
2	0.11 - 5.56	4.2 ± 0.8	160
15	0.12 - 11.4	4 ± 2	560
16	0.55 - 552	0.14 ± 0.07	2,800
17	0.09 - 3.43	$< 10^{-4}$	-
18	0.81 - 8.1	$< 10^{-4}$	-

*estimated

2. The A-ring is highly sensitive to manipulation at the C-2/C-3 positions with the factors contributing to activity being complex. The primary influences of structural manipulation can be ascribed to the influence of steric or polar substituents at the "periphery" of the A-ring

(caused by equatorial substituents at the C-2/C-3 sites) and to conformational effects on the A-ring that influence the positioning of the C-18 hydroxyl moiety (see above). The discovery of 3-deoxyaphidicolin by Ichihara et al. (19) demonstrated that the C-3α alcohol was not essential for expression of polymerase α inhibition; our studies confirm and extend this observation.

The influence of polar equatorial substituents at the C-3 position is represented by the comparison of aphidicolin 1 with 3-epiaphidicolin 10 and 3α-methylepiaphidicolin 14 (both $\approx 10^7$X less active). The influence of sterically demanding substituents is illustrated by the comparison of 1 with 3β-methylaphidicolin 7, 3-ethynylaphidicolin 9, and 2α-methylaphidicolin 13; the loss in activity approximately parallels the anticipated steric demands of the substituents.

3-Ketoaphidicolin 3 retains considerable activity (≈10X less active), which would appear to contradict the postulated influence of steric or polar substituents at C-3. It is possible that the effect of the C-3 carbonyl, while being unfavorable with regard to "peripheral" substitution, favorably adjusts the conformational positioning of the C-18 alcohol function. Hydrophobic interactions of undefined character also appear to play a role in aphidicolin-polymerase α association (1,3,22). Thus, it is possible that the activity series, 3α-hydroxy- 1 > 3-keto- 3 >> 3β-hydroxy-aphidicolin 10 reflects, at least in part, the influence of hydrophobic features of the molecules.

Analysis of Structure-Activity Relationships of D-Ring Modifications of Aphidicolin. The D-ring diol system is significantly less sensitive to structural modification. Although the diol function at the C-16/C-17 sites is not essential for activity, a single alcohol moiety significantly enhances activity (cf. 16 versus 17) and represents the second important functional group component to the pharmacophore. However, the precise location of the C-16/C-17 alcohol functional group appears not to be critical and, in previous studies of aphidicolin SAR, Arabshahi et al. (21) demonstrated that a derivative without a C-16/C-17 hydroxyl retained limited activity. Thus, esterification of the C-17 hydroxyl with glycine (2, ~4%) and removal of the C-17 alcohol (15, ~4%; 16, ~1.5%) provide derivatives with limited activity. Steric constraints in the C-16/C-17 region may play a role in the depressed activity of the n-butyl derivative 18.

Concluding Remarks. The body of SAR data on aphidicolin derivatives reinforces the relative "sensitivity" of structural variation in the A-ring,

particularly at the C-18 alcohol function, and the relative "flexibility" of C,D-ring variation. Little is known regarding structural requirements for the B-ring. The conclusions of these studies indicate a critical role for the C-18 function in the interaction of aphidicolin with polymerase α. Molecular hydrophobicity may also play an important role in the binding interaction.

Only a preliminary understanding of the mechanism through which aphidicolin interacts with polymerase α or δ and inhibits DNA synthesis has emerged. Based on the finding that aphidicolin binds competitively with dCTP at a hydrophobic site on polymerase α, the aphidicolin binding site has been postulated to be at or in the vicinity of the dCTP site or at an allosteric site that exhibits negative cooperativity with the dCTP site (1,2,4,6-8,22). The prevailing assumption--that the aphidicolin binding site is contiguous with the dCTP site--prompted an extensive molecular modelling investigation of the structural relationship between aphidicolin and dCTP. Although structural similarity exists between the aphidicolin A-ring diol functionality and the deoxyribose ring of the dNTPs, our molecular modelling studies could not identify structural features of the aphidicolin-dCTP "overlap" that is unique to dCTP, relative to the remaining dNTPs, and that is consistent with the SAR data of this series. Moreover, an essential structural feature of dCTP is missing in aphidicolin: the triphosphate moiety (PPP). It is possible that inorganic pyrophosphate (PPi) effects aphidicolin inactivation of polymerase α (perhaps as a "co-antagonist" which mimics the PPP ligands at the dCTP binding site). If significant co-factor contributions to aphidicolin binding to polymerase α could be identified, these data may help rationalize the significant difference noted in the inhibition of DNA synthesis in vitro and in vivo and, through an enhanced understanding of the aphidicolin binding site, may help define additional synthetic targets which possess the integrated aphidicolin--co-factor pharmacophore. As yet no evidence for in vivo phosphorylation or pyrophosphate dependence has been presented.

Our lack of knowledge of the aphidicolin pharmacophore and of the structural requirements for binding to the "aphidicolin site" on polymerases α and δ is underscored by the fact that of the approximately fifty semi-synthetic or totally synthetic analogs of aphidicolin currently known, none have approached the parent in activity. Thus, aphidicolin is an exciting lead for the development of antitumor and antiviral agents. However, considerable effort will be required before a full understanding of the

structural determinants responsible for the activity of aphidicolin in the inhibition of the eukaryotic and viral polymerases is elucidated.

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