Antiviral Research, 5 (1985) 93–101
Elsevier

AVR 00149

In vivo distribution and activity of aphidicolin on dividing and quiescent cells*

S. Spadari[1], F. Focher[1], C. Kuenzle[2], E.J. Corey[3], A.G. Myers[3], N. Hardt[4], A. Rebuzzini[1], G. Ciarrocchi[1] and G. Pedrali-Noy[1]

[1]*Istituto di Genetica Biochimica ed Evoluzionistica, Consiglio Nazionale delle Ricerche, Pavia, Italy,* [2]*Department of Pharmacology and Biochemistry, University of Zurich, Zurich, Switzerland,* [3]*Department of Chemistry, Harvard University, Cambridge, MA, U.S.A., and* [4]*Department of Molecular Biology, Université Libre de Bruxelles, Rhode-St-Genèse, Belgium*

(Received 23 November 1983; accepted 29 May 1984)

Summary

In view of a possible use of aphidicolin, an inhibitor of DNA polymerases (including viral DNA polymerases), to control excessive cell proliferation we have investigated: (i) the effect of the drug on the growth of several human neoplastic cells; (ii) the activity of synthetic analogs aimed at relating the structural feature of aphidicolin to cytotoxicity; (iii) the in vivo fate and distribution of aphidicolin in different fluids, organs and tissues of mice following parenteral and/or peroral administration.

aphidicolin; cell proliferation; DNA polymerase α; tissue distribution

Introduction

The use of pure natural drugs to control excessive cell proliferation has a history of about 35 years. Despite the fact that some of them have been found clinically active and several are in various phases of preclinical development, the need for novel and more selective anticancer drugs continues. A rational design of novel and effective anticancer drugs is still limited by our scant knowledge of cancer biology and biochemistry, and a major goal in cancer chemotherapy is not only to learn how to affect the fraction of proliferating cells and the length of the cell cycle (tumor cells usually proliferate faster than normal cells originating from the same type of tissue), but also drug levels and intervals between doses.

*Presented at the International Symposium on Antiviral Compounds, Universitäts-Augenklinik Eppendorf, Hamburg, F.R.G., June 13–16, 1983.

Correspondence to: Dr. Silvio Spadari, Istituto di Genetica Biochimica ed Evoluzionistica, Via Abbiategrasso, 207, 27100 Pavia, Italy (Tel. 382-422411).

In recent years it has been reported that aphidicolin [2,3], a mycotoxin that can be extracted from the culture filtrates of fungi such as *Cephalosporium aphidicola* and *Nigrospora oryzae*, specifically inhibits DNA replication in eukaryotes, due to its selective inhibition of the replicative eukaryotic DNA polymerase α (see reviews in [7] and [17]).

Thus aphidicolin is specifically cytotoxic during the 'S' phase of the cell cycle, i.e. during the synthesis of nuclear DNA. Unlike most other inhibitors of DNA synthesis the drug does not bind directly to DNA, nor does it interfere with other metabolic activities of the cells such as RNA, proteins and ribo- or deoxyribonucleotide synthesis. The synthesis of mitochondrial DNA (or chloroplast DNA in plants) is also fully resistant to the drug [5,16].

Because of its properties we have proposed the use of aphidicolin to control excessive cell proliferation, for example in patients with cancer, psoriasis or other varieties of dermatitis [17]. Also, some viral DNA polymerases (such as those of herpes and vaccinia viruses) are sensitive to aphidicolin, although the drug exhibits no antiviral selectivity. However, variant DNA polymerases, resulting from active site alterations, and isolated from HSV mutants resistant to antiviral drugs (such as PFA) were found to be more sensitive to aphidicolin than the parental DNA polymerase (Cheng, Y.C., personal communication), suggesting the possible use of this drug in viral chemotherapy, alone or in combination with other antiviral drugs.

In this article we report some studies undertaken to relate the structural features of aphidicolin to cytotoxicity, to evaluate its activity on proliferating or resting cells, and to determine the in vivo fate and distribution of aphidicolin in different tissues and organs of mice that will allow selection of optimal dose levels and intervals between doses.

Materials and Methods

Cell lines

The effect of aphidicolin was tested on the growth of the following cultured cell lines: Molt-4 (human T-lymphoblastoid cells); Ltk, RAJI and TO-117 (human B-lymphoblastoid cells); COLO-38 and MAI (human melanoma cells); P3/X63-Ag8 (murine myeloma cells) and HeLa cells.

Culture media

Cells were grown in suspension with Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal calf serum, L-glutamine (300 mg/l), and gentamicin (200 μg/ml). Exponentially growing cells were used in all experiments.

Measurement of the rate of DNA synthesis

The incorporation of [^{3}H]thymidine into acid-insoluble material during 30-min pulse periods was used as a measure of DNA synthesis. [^{3}H]Thymidine (20 μCi; 24 Ci/mmol) was added to 6×10^5 cells in 1.5 ml of medium. The cells were incubated at 37°C, and two 100-μl aliquots were removed after 0, 5, 10, 20 and 30 min, spotted onto

Whatman GF/C filters which were washed with trichloroacetic acid. Remaining radioactivity was measured in a Beckman LS-7000 scintillation counter using an Omnifluor-based scintillation fluid.

Preparation of rat liver microsomes

Rat liver oxidative microsomal enzymes were prepared as previously described [1].

Administration of aphidicolin

5 mg of aphidicolin, dissolved in DMSO at 100 mg/ml, were injected intraperitoneally into 2-month-old mice of 40–45 g body weight. Equal amounts, dissolved in olive oil, were administered to other mice orally, i.e. through a cannula inserted via the oesophagus into the stomach.

Quantitative determination of aphidicolin in body fluids and animal tissues

Aphidicolin was extracted from tissues and body fluids, removed at different times after administration of the drug, as described in Fig. 3. Varying amounts of extract were tested for inhibition of DNA polymerase α in the presence of 5 μM dCTP. The results were compared with similar amounts of control extracts prepared from body fluids or tissues of a mouse which did not receive aphidicolin. This was done to rule out the presence of soluble dissociable inhibitors or activators of DNA polymerase α. The inhibition of DNA polymerase α by increasing concentration of aphidicolin in the presence of 5 μM dCTP served as a standard curve for the determination of aphidicolin. This method allows quantitative determination of aphidicolin even in the presence of inactive metabolite(s) with similar chemical structure(s) such as those generated by liver microsomal oxidases [1].

Results

Aphidicolin inhibits DNA replication and growth of neoplastic cells without interfering with immunoglobulin secretion and expression of HLA antigens

In view of a possible clinical use of aphidicolin we have investigated the effect of aphidicolin on the growth of several human and murine neoplastic cells measured by incorporation of [^{3}H]thymidine into DNA [15]. A concentration of aphidicolin between 0.042 and 0.09 μg/ml was sufficient to inhibit DNA synthesis and cell growth by 50% in all neoplastic cells tested (Table 1). A similar concentration of aphidicolin inhibited to the same extent the in vitro activity of DNA polymerase α purified from neoplastic cells.

Aphidicolin concentrations 100 times higher than that causing 50% inhibition of cell growth had no effect on RNA synthesis, protein synthesis (including the immunoglobulins G secreted into the culture medium), and the expression of HLA antigens that are involved in relevant phenomena of the immune response [15].

Aphidicolin: structure–activity relationship

A vexing problem associated with in vivo studies and with the possible clinical use

TABLE 1

Concentration of aphidicolin causing 50% inhibition of DNA synthesis in different neoplastic cell lines

Cell line	Aphidicolin (μg/ml)
Molt-4	0.066
Lik	0.060
RAJI	0.058
TO-117	0.083
COLO-38	0.090
MAI	0.075
P3/X63-Ag8	0.042
HeLa	0.070

Molt-4 (human T-lymphoblastoid cells); Ltk, RAJI and TO-117 (human B-lymphoblastoid cells); COLO-38 and MAI (human melanoma cells); P3/X63-Ag8 (murine myeloma cells).

of aphidicolin is its deactivation by liver microsomal oxidase (Fig. 1) in a reaction which is temperature-dependent and requires NADP [11]. Microsomal oxidases present in 25 mg of Aroclor-induced rat liver are capable of inactivating 25 μg of aphidicolin within 10 min at 30°C.

The unique structure and activity of aphidicolin and its unfavorable metabolic inactivation by liver microsomal oxidases, make it a useful model for the development of synthetic analogs.

The metabolic inactivation by liver oxidases and the fact that 3-deoxy-aphidicolin and aphidicolin-16–17 ester derivatives are less active than aphidicolin towards α-polymerase in vitro [6] suggest that the OH groups of the molecule are important for

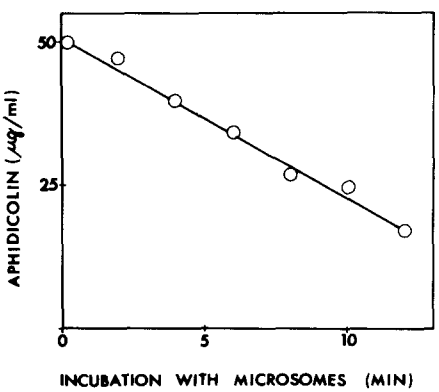

Fig. 1. Inactivation of aphidicolin by rat liver microsomes. Aphidicolin (50 μg/ml) was incubated at 30°C with rat liver homogenates (9000 × *g* supernatant or S-9) after induction by Aroclor 1254 [1] in a reaction mixture containing 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose 6-phosphate, 4 mM $NADP^+$, 100 mM sodium phosphate, pH 7.4. 100 μl of S-9 was added to 1 ml of this reaction mixture. 1 ml of S-9 fraction (15 mg proteins/ml) contained microsomes from 250 mg of wet liver. The concentration of residual active aphidicolin, following incubation with liver microsomes, was determined by measuring the inhibition of purified HeLa cell DNA polymerase α [14].

the drug's activity. Since total synthesis of aphidicolin [10,18,4,9] is difficult and cannot yet be a substitute for its isolation from culture filtrates, we have assayed some simpler compounds both in vivo and in vitro.

One such compound is shown in Fig. 2 and was chosen because it has all four hydroxyl groups in the same steric configuration as aphidicolin. Unfortunately, this analog was found inactive at up to 10^3 times the effective concentration of aphidicolin, both in vitro against cellular DNA polymerase α or HSV-1 or HSV-2 DNA polymerases and in vivo against [^{3}H]thymidine incorporation into cellular DNA.

The critical question of the relationship between structure and in vivo activity is certainly hampered by the limited number of compounds (synthetic or natural) that have been tested in vivo and by a lack of analogs within the structural class of aphidicolin. We hope, however, that our studies will stimulate some interest in the development of aphidicolin analogs so that the therapeutic potential of aphidicolin may be increased by chemical modifications reducing its sensitivity to liver microsomal oxidases or increasing its solubility in aqueous solvents.

Aphidicolin as a potential therapeutic agent

Aphidicolin, through its selective inhibition of DNA polymerase α responsible for the replication of cellular nuclear DNA, is specifically cytotoxic during the 'S' phase of the cell cycle but apparently has no adverse effect upon non-multiplying cells, because it allows other important cellular functions (like RNA, protein and nucleotide biosynthesis) to proceed [12].

Aphidicolin has therefore aroused considerable interest for its possible use in the control of tumor growth which is essentially tied both to the length of the cell cycle and to the higher fraction of proliferating cells. The clinical use of this drug seems limited by its rapid inactivation by the liver. However, because of the ability of aphidicolin to block cells in the 'S' phase and to synchronize cells in culture [12], its possible use in combination with other cell-cycle-specific drugs is suggested. Alternatively, aphidicolin could first be used for a short period to synchronize target cells that might

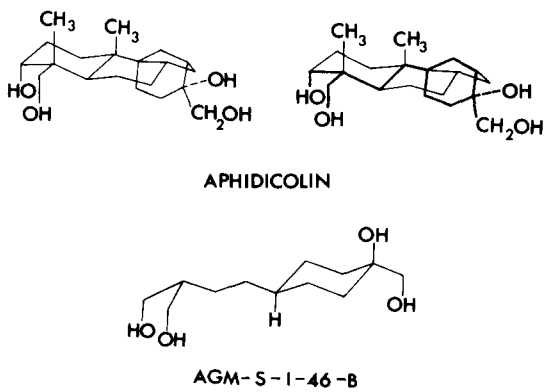

Fig. 2. Structure of aphidicolin (top) and of the analog AGM-S-I-46-B (bottom) whose structure corresponds to the dark lines in the aphidicolin formula at the top right.

subsequently be attacked, for instance, by compounds known to block the mitotic process.

Selective accumulation of aphidicolin in target cells might also be achieved if regional infusions of drugs via an arterial route are possible. This would have the advantage that aphidicolin entering the systemic circulation would be inactivated by liver microsomes.

It is possible that the therapeutic potential of aphidicolin may be increased by chemical modification reducing its sensitivity to liver microsomal oxidases. However, the metabolic inactivation of aphidicolin may be a positive advantage for certain applications. In particular, an aphidicolin ointment could be useful in the control of excessive cell division in the skin which is observed in psoriasis, other forms of dermatitis, and skin tumors.

Aphidicolin absorbed through the skin (i.e., beyond the therapeutic target) would be inactivated quickly, thereby minimizing possible adverse side-effects of the treatment. We have obtained evidence that aphidicolin can be found in the skin when administered intraperitoneally to mice and that it is absorbed through the skin when applied topically as an ointment. The drug could control the proliferation of keratinocytes in culture where cell division appears to be confined, as in skin, to keratinocytes of the basal layer.

Determination of aphidicolin in various tissues, organs and body fluids of mice

In view of a possible use of aphidicolin in cancer or viral chemotherapy (aphidicolin also inhibits HSV-1 and HSV-2 DNA polymerases [13]), it is important to learn how to select dose levels and intervals between doses. Chemotherapeutic concentrations of the drug must be reached and maintained in different tissues, organs or body fluids without overdosing the host organism. An 'S'-phase-specific drug like aphidicolin, for instance, would have to reach effective serum levels at intervals of just less than 'S' phase duration in order to affect all proliferating tumor cells. Basic kinetic information obtained from animal models is useful to optimize therapeutic designs for controlling local or disseminated cancers.

Since the reported deactivation of aphidicolin by microsomal oxidases precludes the use of radioactive aphidicolin to follow the distribution and fate of the drug in living organisms (active and inactive compounds would both retain the label), we have developed an enzymatic method for microdetermination of aphidicolin in body fluids and tissue biopsies [14]. The method is essentially based on: (i) complete acetone extraction of aphidicolin from body fluids, tissues and organ biopsies; (ii) the ability of aphidicolin to bind to and to inhibit DNA polymerase α.

Aphidicolin was administered to mice either intraperitoneally or orally and its concentration in various organs, tissues (Fig. 3) and body fluids was determined at several hours after administration. The results can be summarized as follows: the highest levels of aphidicolin were found in tissues and organs (like spleen, intestine, stomach, skin, testis, etc.) with the highest number of proliferating cells (Fig. 3A–D) and, therefore, the highest levels of the replicative α-polymerase. High levels of aphidicolin were also found in liver, although hepatocytes are long-lived in normal circumstances and can be regarded as slow- or non-dividing cells containing low levels

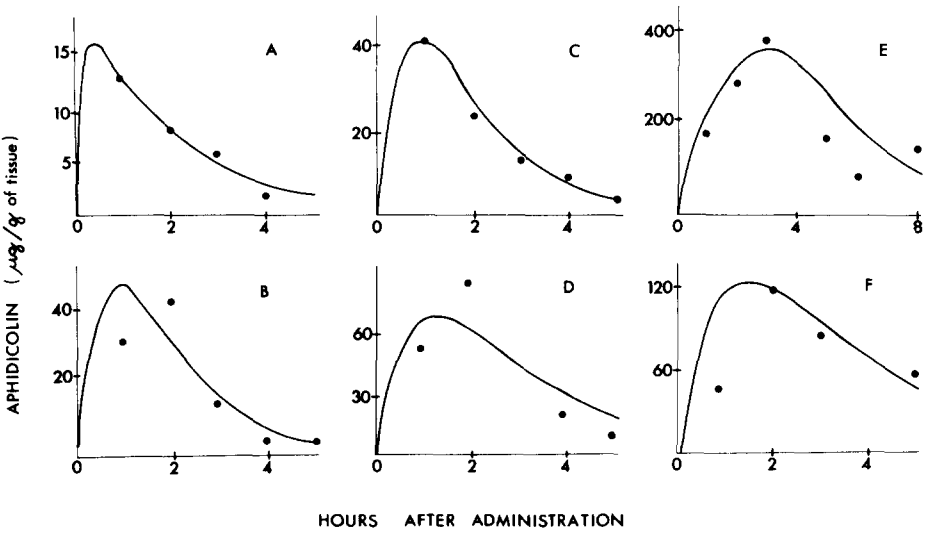

Fig. 3. Distribution of aphidicolin in liver, spleen, stomach and large intestine at several hours following administration either intraperitoneally (A–D) or perorally (E,F). 5 mg of aphidicolin dissolved in 100 μl DMSO were injected intraperitoneally into 2-month-old mice of 40–45 g body weight. Equal amounts of aphidicolin, dissolved in olive oil, were administered to other mice orally, through a cannula inserted via the oesophagus into the stomach. Body fluids and organs were removed at different times after administration of aphidicolin and were used within a few days after storage at -70°C. Stomach and large intestine (cecum) of mice that received aphidicolin perorally, were cut open and the mucosa washed extensively with repeated jets of 0.9% NaCl from a wash bottle until completely free from adhering content. Tissues and organs were then homogenized in 5 mM potassium phosphate, pH 7.2, 1 mM EDTA and 0.02% sodium azide (which completely inhibits microsomal oxidase, thus avoiding inactivation of aphidicolin during extraction, but has no effect on purified DNA polymerase α). Cell debris was removed by centrifugation, the acetone evaporated, the volume restored with the above buffer, and varying amounts of solution were tested for inhibition of DNA polymerase α. The values represent averages of three experiments and curves were calculated according to the least-squares method applied to first-order absorption and first-order elimination kinetics of drug levels after a single dose. A = liver, B = spleen, C and E = stomach, D and F = large intestine.

of α-polymerase. This most probably reflects the fact that liver receives most of the cardiac output and, in principle, aphidicolin should not cause hepatocellular necrosis because it does not block essential hepatocellular functions such as RNA, protein and mt (mitochondrial)-DNA synthesis.

No aphidicolin or else very low levels of the drug were found in resting cells like neurons and ventricular cardiac muscle cells that do not divide after birth and no longer contain the α-polymerase [8].

Aphidicolin also reached high levels in blood and urine after parenteral administration despite its poor solubility in water. Like many other drugs aphidicolin seems to be eliminated from the circulation by metabolism (by liver hepatocytes), excretion (as revealed by high urinary concentrations) and accumulation in tissues. It is still difficult to determine on the basis of these preliminary experiments the rate of each of those processes that contribute to the termination of the drug's action. The rate of

onset and the duration of drug action also depend on the route of administration. We have found that when aphidicolin is given perorally, its local concentration in stomach and intestinal mucosa (Fig. 3E and F) and feces (results not shown) is much higher than when similar amounts are given intraperitoneally, although peak levels are delayed by 1–2 h. The results are probably due to the high lipid solubility of aphidicolin. In contrast, aphidicolin levels in body fluids and several tissues other than the gastrointestinal tract, are lower when the drug is given perorally as compared to intraperitoneally.

Conclusion

Aphidicolin is a new drug that may be valuable therapeutically for controlling cell proliferation because it is specifically cytotoxic during the 'S' phase of the cell cycle, due to a selective inhibition of the replicative DNA polymerase α. Other important cellular functions (mt-DNA, RNA, protein and nucleic acids precursor synthesis) are not affected suggesting that aphidicolin has no adverse effects upon non-multiplying cells. Its unique structure and activity make aphidicolin a useful model for the development of synthetic analogs that could overcome the poor solubility in water and the unfavorable metabolic inactivation of aphidicolin by liver microsomal oxidases. We have started some studies that should stimulate interest in the development of aphidicolin analogs.

The inactivation of aphidicolin by microsomal oxidases precludes the use of radioactive aphidicolin to follow the drug's distribution and fate in vivo. However, an enzymatic method developed in our laboratory allows quantitative determination of pmol amounts of aphidicolin and is suitable for the determination of the drug in fluids and tissue biopsies from living humans. Preliminary experiments in mice have shown that this drug is found preferentially in highly proliferating cells, probably reflecting its ability to bind to the DNA polymerase α responsible for nuclear DNA replication. The high lipid solubility of aphidicolin probably explains the much higher concentrations found in the stomach and intestinal mucosa when the drug is administered via the gastrointestinal tract.

Acknowledgements

This work was supported by grants of the Progetto finalizzato 'Controllo delle Malattie da Infezione' and 'Oncologia' del Consiglio Nazionale delle Ricerche, Italy.

References

1 Ames, B.N., McCann, J. and Yamasaki, E. (1975) Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. Mutat. Res. 31, 347–364.

2 Brundret, K.M., Dalziel, W., Hesp, P., Jarvis, J.A. and Neidle, S. (1972) X-ray crystallographic

determination of the structure of the antibiotic aphidicolin: a tetracyclic diterpenoid containing a new ring system. J. Chem. Soc. Chem. Commun., 1027–1028.

3 Bucknall, R.A., Moores, H., Simms, R. and Hesp, P. (1973) Antiviral effects of aphidicolin, a new antibiotic produced by *Cephalosporium aphidicola*. Antimicrob. Agents Chemother. 4, 294–298.

4 Corey, E.T., Tius, M.A. and Das, J. (1980) Total synthesis of (±)-aphidicolin. J. Am. Chem. Soc. 102, 1742–1744.

5 Geuskens, M., Hardt, N., Pedrali-Noy, G. and Spadari, S. (1981) An autoradiographic demonstration of nuclear DNA replication by DNA polymerase α and of mitochondrial DNA synthesis by DNA polymerase γ. Nucleic Acids Res. 9, 1599–1613.

6 Haraguchi, T., Oguro, M., Nagano, H., Ichihara, A. and Sakamura, S. (1983) Specific inhibitors of eukaryotic DNA synthesis and DNA polymerase alpha, 3-deoxy-aphidicolin and aphidicolin 17-monoacetate. Nucleic Acids Res. 11, 1197–1209.

7 Hubermann, J.A. (1981) New views of the biochemistry of eukaryotic DNA replication revealed by aphidicolin, an unusual inhibitor of DNA polymerase alpha. Cell 23, 647–648.

8 Hubscher, U., Kuenzle, C.C. and Spadari, S. (1977) Variation of DNA polymerases alpha, beta and gamma during perinatal tissue growth and differentiation. Nucleic Acids Res. 4, 2917–2929.

9 Ireland, R.E., Godfrey, J.D. and Thaisrivongs, S. (1981) An efficient stereoselective total synthesis of (±)-aphidicolin. J. Am. Chem. Soc. 103, 2446–2448.

10 McMurry, J.E., Andrus, A., Ksander, G.M., Musser, J.H. and Johnson, M.A. (1979) Stereospecific total synthesis of aphidicolin. J. Am. Chem. Soc. 101, 1330–1332.

11 Pedrali-Noy, G., Mazza, G., Focher, F. and Spadari, S. (1980) Lack of mutagenicity and metabolic inactivation of aphidicolin by rat liver microsomes. Biochem. Biophys. Res. Commun. 93, 1094–1103.

12 Pedrali-Noy, G., Spadari, S., Miller-Faurés, A., Miller, A.O.A., Kruppa, J. and Koch, G. (1980) Synchronization of HeLa cell cultures by inhibition of DNA polymerase alpha with aphidicolin. Nucleic Acids Res. 8, 377–387.

13 Pedrali-Noy, G. and Spadari, S. (1980) Mechanism of inhibition of Herpes simplex virus and Vaccinia virus DNA polymerases by aphidicolin, a highly specific inhibitor of DNA replication in eukaryotes. Virology 36, 457–464.

14 Pedrali-Noy, G., Kuenzle, C.C., Focher, F., Belvedere, M. and Spadari, S. (1981) An enzymatic method for microdetermination of aphidicolin: a promising anticancer drug. J. Biochem. Biophys. Methods 4, 113–121.

15 Pedrali-Noy, G., Belvedere, M., Crepaldi, T., Focher, F. and Spadari, S. (1982) Inhibition of DNA replication and growth of several human and murine neoplastic cells by aphidicolin without detectable effect upon synthesis of immunoglobulins and HLA antigens. Cancer Res. 42, 3810–3813.

16 Sala, F., Galli, M.G., Levi, S., Burroni, D., Parisi, B., Pedrali-Noy, G. and Spadari, S. (1981) Functional roles of the plant alpha-like and gamma-like DNA polymerases. FEBS Lett. 124, 112–118.

17 Spadari, S., Sala, F. and Pedrali-Noy, G. (1982) Aphidicolin: a specific inhibitor of nuclear DNA replication in eukaryotes. Trends Biochem. Sci. 7, 29–32.

18 Trost, B.M., Nishimura, Y., Yamamoto, K. and McElvain, S.S. (1977) A total synthesis of aphidicolin. J. Am. Chem. Soc. 101, 1328–1330.