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In vitro and in vivo antiviral activity of scopadulcic acid B from *Scoparia dulcis*, Scrophulariaceae, against herpes simplex virus type 1

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Summary

The antiviral activity of five diterpenoids isolated from *Scoparia dulcis* L., Scrophulariaceae, was examined in vitro against herpes simplex virus type 1. Among these compounds, only scopadulcic acid B was found to inhibit the viral replication with the in vitro therapeutic index of 16.7. The action of scopadulcic acid B was not due to a direct virucidal effect or inhibition of virus attachment to host cells. Single-cycle replication experiments indicated that the compound interfered with considerably early events of virus growth.

The influence of scopadulcic acid B on the course of the primary corneal herpes simplex virus infection was investigated by means of a hamster test model. When the treatment was initiated immediately after virus inoculation, scopadulcic acid B, when applied orally or intraperitoneally, effectively prolonged both the appearance of herpetic lesions and the survival time at the dose of 100 and 200 mg/kg per day.

Scopadulcic acid B; Herpes simplex virus; In vitro assay; Hamster model; Corneal infection

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Introduction

Many selective antiviral agents, especially nucleoside analogues such as vidarabine, acyclovir and bromovinyl deoxyuridine (Coen et al., 1986), have been developed to enable the chemotherapy of human herpes simplex virus infection. The fact that herpes simplex virus type 1 (HSV-1) mutants, which acquire resistance to acyclovir, have arisen during the drug treatment (Darby et al., 1981; Field, 1982) is one of the reasons of a continuing search for new antiviral compounds.

Five new diterpenoids were recently isolated as constituents of a Paraguayan medicinal plant, *Scoparia dulcis* L. (Hayashi et al., 1987a,b; Kawasaki et al., 1987). We have examined the inhibitory effects of these compounds on replication of HSV-1 in HeLa cell cultures. Furthermore, the in vivo antiviral activity of scopadulcic acid B, the most effective compound of the five diterpenoids, against experimental HSV-1 infection in hamsters was determined.

Materials and Methods

Compounds

The diterpenoids tested were isolated from a Paraguayan crude drug 'Typychá kuratũ' (whole plant of *Scoparia dulcis* L., Scrophulariaceae) (Hayashi et al., 1987a,b; Kawasaki et al., 1987). Chemical structures of the compounds are shown in Fig. 1. Stock solutions (10 mg/ml) for testing in vitro were prepared in dimethylsulfoxide and stored at 4°C.

Cell culture

HeLa 229 cell monolayers were cultured in Eagle's minimal essential medium (MEM) containing 5% fetal calf serum (FCS) for use in growth of virus or plaque assay.

Virus

Stock suspensions of the HF strain of HSV-1 were prepared from infected HeLa cells.

Assay for antiviral activity in cell culture

Monolayers of HeLa 229 cells in 24-well culture plates were washed and infected using HSV-1 with an m.o.i. of 0.5, adsorbed for 1.5 h at room temperature, and refed with maintenance medium (MEM plus 2% FCS) containing various amounts of the compound. Cultures were incubated at 34°C for 24 h in 5% CO_2, harvested and disrupted by three cycles of freezing and thawing.

Virus yields were determined by plaque assay. Each compound was assayed at least twice. The ED_{50} was determined as the least drug concentration which reduced plaque numbers by 50% in the treated cultures compared to untreated infected cultures. In one series of experiments the cells were pretreated with varying concentrations of scopadulcic acid B for 3 or 24 h at 37°C prior to virus infection.

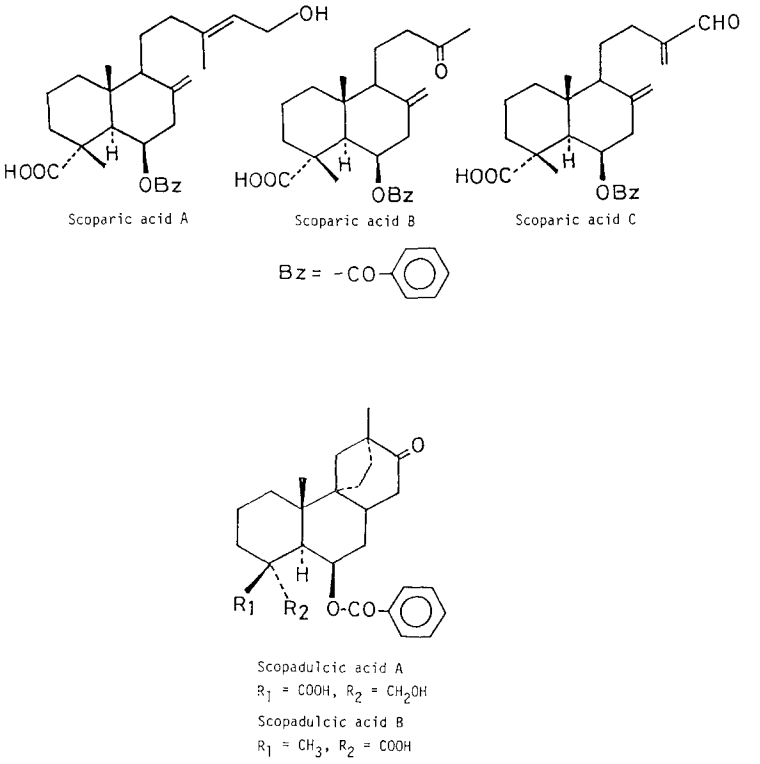

Fig. 1. Chemical structures of diterpenoids.

In other experiments, the virus was treated with varying concentrations of the compound for 3 h at 4°C prior to the virus being added to the cell monolayers.

Cytotoxicity assay

For growth inhibition studies, 5×10^4 cells in 0.5 ml MEM supplemented with 5% FCS were seeded into each well of 24-well plates, cultured for 24 h at 37°C, and allowed to grow for additional 24 h in the presence of increasing amounts of the compound. After the medium was removed, cell monolayers were detached with 0.05% trypsin and the viable cells were calculated by the trypan blue-exclusion method. The inhibition data were plotted as dose–effect curves (not shown) from which the 50% inhibitory doses (ID_{50}) were obtained. The ID_{50} are the average of three assays with four concentrations within inhibitory range of the compounds (duplicate cultures).

DNA-DNA hybridization

E. coli HB101 cells were transformed with HSV-1 (strain F) DNA *Bam*HI Q fragment cloned in pBR 322. They were then cultured on a larger scale for purification of plasmid DNA (Maniatis et al., 1982). The recombinant DNA molecules were labeled with biotin-dUTP (BRL, Maryland, U.S.A.) by nick translation

(Rigby et al., 1977) using commercially available reagents (Nick Translation Kit, No. 8160SP, BRL, Maryland, U.S.A.).

The procedures for hybridization and detection of viral DNA used in this study were described previously (Nago et al., 1988). Briefly, HeLa cells infected with HSV-1 were suspended in Tris buffer (pH 7.5) containing 0.6% Nonidet P-40. After centrifugation, the supernatant was incubated with proteinase K (0.1 mg/ml) and denatured by boiling in 0.3 M NaOH. The specimens were applied to nitrocellulose filter fixed to HYBRI-SLOT™ manifold (BRL, Maryland, U.S.A.). The filter was prehybridized for at least 2 h at 42°C in prehybridization solution. The solution was then replaced with hybridization solution containing biotin-labeled probe. Hybridization was carried out at 42°C for 16–20 h. A series of slots containing known amounts of the plasmid DNA was prepared on each filter as a control. The detection of viral DNA by means of a streptavidin/alkali phosphate kit (Blu GENE, No. 8279SA, BRL, Maryland, U.S.A.) was performed under instructions provided by the manufacturer. The evaluation could be done by determining the peak height with a densitometer (Clini Scan™, Helena Laboratories, Texas, U.S.A.).

Animals

Four-week-old female golden hamsters (55–60 g) obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan) were used.

Preparation of drug for animal treatment

For intraperitoneal applications, scopadulcic acid B was suspended in sterile saline. For oral dosage, the drug was suspended in sterile distilled water. Placebo treatment was saline or water.

Inoculation of animals and evaluation of HSV-1 infection

For infection, 10 μl of virus suspension in MEM containing 3×10^4 to 5×10^4 plaque-forming units (PFU) was placed on the right eyes of hamsters. For each animal 45 linear corneal scarifications and a further 45, perpendicular to the first, were made with twenty 23-gauge needles. The development and severity of the facial lesions and survival times were recorded every day. The scores of the lesions are as follows: 0, no lesion; 1, slight and local erosion; 2, mild erosion; 3, moderate erosion; and 4, severe erosion. The average lesion score was calculated as the mean severity for all animals in a treatment group.

Results

Anti-HeLa cell growth and anti-HSV-1 activity of diterpenoids

Table 1 summarizes the 50% inhibitory doses for cell growth (ID_{50}) and the 50% effective doses for virus replication (ED_{50}) determined for five diterpenoids, scoparic acid A, B and C, and, scopadulcic acid A and B. The chemical structures of these compounds are shown in Fig. 1. All of them showed relatively higher cytotoxicity. Especially, scopadulcic acid B was a potent inhibitor with ID_{50} of 0.2 μg/ml.

TABLE 1

ID_{50} and ED_{50} of compounds for cell growth and virus replication[a]

Compound	Cytotoxicity (ID_{50}± SD)[b]	Antiviral activity (ED_{50}± SD)[c]	In vitro therapeutic index (ID_{50}/ED_{50})
	μg/ml		
Scoparic acid A	1.80±0.17	1.26±0.10	1.4
Scoparic acid B	0.94±0.11	1.12±0.14	0.8
Scoparic acid C	1.10±0.12	3.20±0.24	0.3
Scopadulcic acid A	0.78±0.13	0.95±0.11	0.8
Scopadulcic acid B	0.20±0.03	0.012±0.002	16.7

[a] HeLa 229 cells were employed for both cytotoxicity and plaque assays.
[b] Dose necessary to inhibit 50% of the growth of HeLa 229 cells.
[c] Dose necessary to inhibit 50% of the plaque-forming units.

In vitro therapeutic indexes (ID_{50}/ED_{50}) were calculated for the compounds against HSV-1. The values for scoparic acid A, B and C, and scopadulcic acid A are ranged from 1.4 to 0.3 when examined under the conditions mentioned in Materials and Methods. Only scopadulcic acid B, which had an in vitro therapeutic index of 16.7, showed higher selective toxicity.

Inhibition of HSV-1 replication by scopadulcic acid B

The changes in amounts of intracellular viruses, viruses released into culture medium and intracellular viral DNA were determined when HeLa cells were infected with HSV-1 and incubated in the presence of various concentrations of scopadulcic acid B. The results indicate that those three yields are reduced with the increment of the compound added. The ED_{50} for viral DNA synthesis, and virus

TABLE 2

Effect of pretreatment of cells with scopadulcic acid B on viral replication

Concentration in medium (μg/ml)	Time of pretreatment (h)	Changes in virus yield[a]
0.01	0	42
0.01	3	47
0.01	24	32
0.05	0	15
0.05	3	21
0.05	24	28
0.1	0	8
0.1	3	12
0.1	24	12

Cells used were HeLa 229 cells.
[a] Percentage PFU change in plaque number when compared to cultures without drug. Cultures were inoculated in triplicate with an m.o.i. of 0.5. Untreated controls gave an average yield of 75×10^6 PFU/well.

yield in cells or culture medium were 0.041, 0.024 and 0.009 μg/ml, respectively. Thus, virus release appeared to be most affected by scopadulcic acid B.

Table 2 shows the effect of the pretreatment of host cells on the replication of HSV-1. The cells were pretreated for 3 h or 24 h at 37°C with varying concentrations of scopadulcic acid B, then infected with virus followed by incubation with medium supplemented with the drug. When the infected cells were continued to be cultured with the same concentrations as those of pretreatment, we observed no appreciable effect on virus yield at each concentration compared with the cells without pretreatment. Following adsorption to cells, unadsorbed viruses were assayed. There was no difference in unadsorbed virus number between pretreated cells and untreated ones (data not shown).

In order to determine the direct virucidal activity of scopadulcic acid B, the stock solution was diluted in MEM to provide final concentrations ranging from 0.5 to 50 μg/ml. Virus was added to the solution and incubated for 3 h at 4° C, then assayed in HeLa cells. No significant virucidal action of the solution against HSV-1 was observed (data not shown).

The effect of time of addition of scopadulcic acid B on the reduction of virus yield was examined. The drug was effective when added immediately after virus adsorption or 0.5 h later. The drug was less effective when it was added after 1 h or 2 h, but when added after 3 h its effect was not seen. These results suggest that scopadulcic acid B must interfere with an earlier event of HSV-1 replication.

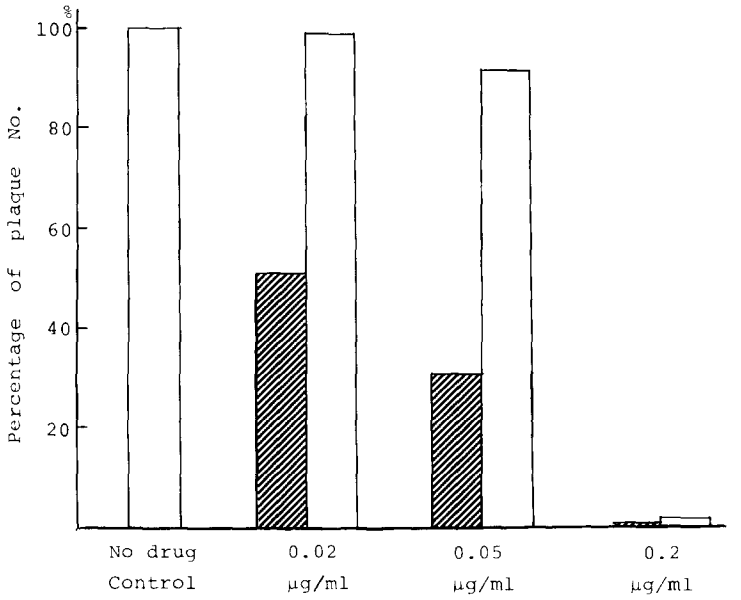

Fig. 2. Reversibility of effect of scopadulcic acid B. HeLa cells were treated with various concentrations of drug for 24 h (hatched bar), then released into drug-free medium and incubated for an additional 6 h (open bar). Virus yield was determined by plaque assay. The plaque number for control cultures was 82×10^6 PFU/well.

To assess whether the inhibitory effect of scopadulcic acid B was reversible, HeLa cells were treated with the compound at varying concentrations for 24 h and then placed in drug-free medium to be incubated for an additional 24 h. Fig. 2 shows that the inhibitory effect of scopadulcic acid B at 0.02 and 0.05 μg/ml was readily reversed upon removal of the compound. At the concentration of 0.2 μg/ml, however, the effect appeared not to be reversed. The growth of host cells was significantly inhibited at this concentration (approximately 20% of untreated control). Therefore, the effect at 0.2 μg of scopadulcic acid B/ml should be considered to reflect the cytotoxicity against HeLa cells.

Therapeutic efficacy of scopadulcic acid B against experimental HSV-1 corneal infection of hamsters

Initially, the efficacy of scopadulcic acid B at a dose of 20 mg or 200 mg/kg per day against experimental infection with HSV-1 was evaluated. As shown in Fig. 3, two daily i.p. doses of 10 mg or 100 mg/kg of the drug continuing for 7 days were effective in delaying the appearance of the primary facial erosions compared with saline-treated control group where the lesions were seen in two of five animals at day 3 and 4 days after infection all animals showed moderate or severe lesions. Drug treatment with 20 mg/kg per day (group I) decreased significantly the average lesion score at 4th day ($P<0.01$, Student's t-test). Also, at the dose of 200 mg/kg per day (group II), significant reduction in the average lesion score was recorded on days 4, 5 and 6 at the level of 0.01, 0.01 and 0.05, respectively. In this experiment, while all hamsters in control group died by day 8, all animals in group I died by day 10 and three of five animals in group II by day 12. The remaining two animals in group II survived.

Table 3 summarizes the therapeutic efficacy of scopadulcic acid B when administered orally two times per day beginning immediately after viral infection for 7

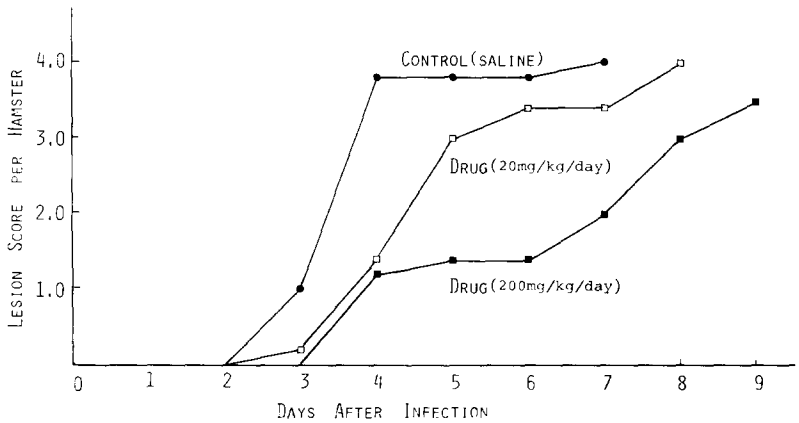

Fig. 3. Effect of scopadulcic acid B on the development of facial lesions after corneal inoculation of HSV-1 into golden hamsters. Treatment was started just after inoculation and consisted of 2 daily applications (i.p.) of 0.5 ml of drug.

TABLE 3

Effect of scopadulcic acid B on experimental HSV-1 corneal infection

Treatment group	Dose (mg/kg/day)	Duration of treatment (days)	Survivors /total	Mean lag phase of hamster that developed facial lesion (days ± S.D.)	Mean survival time of hamsters that died (days ± S.D.)	Time interval of death (days)
H_2O	0	7	0/5	4.0 ± 0.71	7.6 ± 0.55	7 - 8
Scopadulcic acid B	20	7	0/5	5.4 ± 1.34	8.2 ± 0.84	7 - 9
Scopadulcic acid B	100	7	2/5	5.2 ± 3.29	9.3 ± 1.53	8 -11
H_2O	0	5	0/9	3.4 ± 0.88	6.7 ± 0.71	6 - 8
Scopadulcic acid B	100	5	2/9	4.6 ± 0.50[a]	8.4 ± 1.51[b]	7 -10

Hamsters were infected with 3×10^4 PFU of HSV-1 HF strain and treated perorally two times per day.
[a] Statistically different from the H_2O-treated animals ($P<0.01$; Student's t-test).
[b] Statistically different from the H_2O-treated animals ($P<0.05$).

or 5 days. The efficacy was assessed in terms of the time required for appearing herpetic lesions and survival times.

When hamsters were treated for 7 days using oral doses of 20 mg or 100 mg/kg per day, the appearance of the primary lesions in all animals took longer time in both scopadulcic acid B-treated groups compared with placebo (water)-treated control group where all animals showed the lesions by 5th day of infection. The mean survival times of the hamsters were found to be prolonged by the drug.

A similar experiment was performed using oral doses of 100 mg/kg per day for 5 days. In this experiment, significant delay on both the appearance of primary lesions ($P<0.01$) and death ($P<0.05$) was achieved (Table 3).

Golden hamsters treated for 5 or 7 days using intraperitoneal or oral doses of 100–200 mg scopadulcic acid B/kg per day did not show evidence of toxicity (change in activity or appetite) over the survival period. The primary lesions of the survived animals began to be cured since two weeks after viral inoculation.

Discussion

In the antiviral research, several assay systems have been developed (Grunert, 1979). These systems make use of animals, culture cells and purified virus-specific enzymes (Chandra et al., 1979; Goswami et al., 1982; McKenna et al., 1987). In this paper, we employed plaque assay and nucleic acid hybridization (Nago et al., 1988) for in vitro assay, and experimental corneal infection in hamsters for in vivo assay.

Five diterpenoids were tested for antiherpes activity. While scoparic acids A, B and C are different in the structures of the side chain, all of them have similar bio-

logical effects in terms of cytotoxicity against HeLa cells and anti-HSV-1 activity. Thus, the differences are not likely to affect at least both bioassay systems. On the other hand, 50% inhibitory concentrations for cellular growth and virus replication were approximately 4- and 80-fold lower for scopadulcic acid B than those for scopadulcic acid A. It might be interesting to elucidate the structure-activity relationship of these compounds.

In order to understand the mechanism of inhibition, the effect of scopadulcic acid B was studied under various in vitro experimental conditions. The antiviral effect of the compound is suggested not to be attributed to a direct inactivation of the viral particles or inhibition of attachment. The compound was found to inhibit the growth of HSV-1 when added within 1 h post-infection. This means that the compound might act at much earlier events such as fusion of the viral envelope with the plasma membrane (Morgan et al., 1968), transport of the capsids through the nuclear pores and release of the DNA into the nucleus (Batterson et al., 1983).

The accurate step(s) of viral replication in vitro which are blocked by scopadulcic acid B are yet unknown. However, since this compound has chemical structure similar to aphidicolin, a tetracyclic diterpenoid, which is known as a specific inhibitor of DNA polymerase (Ikegami et al., 1978; Ohashi et al., 1978; Pedrali-Noy and Spadari, 1979), it is interesting to study the effect of the compound on DNA polymerases derived from viruses and cells.

The influence of scopadulcic acid B on the course of the corneal HSV-1 infection was investigated by means of a golden hamster test model. Since Alenius (1980) reported that maximum tissue virus titers are reached 48 h after inoculation, the effectiveness of medication exceeding 48 h is not expected. Thus, we administered the drug immediately after viral inoculation.

Scopadulcic acid B was able to delay the appearance of primary disease significantly when applied orally or intraperitoneally, and prolonged the survival time of animals with the survival rates of ca. 20–40%.

We have demonstrated using in vitro and in vivo assay systems that scopadulcic acid B can be counted one of the promising antiviral chemicals derived from plants. It is important to understand the mechanism of the antiviral activity and evaluate the utility of the drug.

References

Alenius, S. (1980) Inhibition of herpesvirus multiplication in guinea pig skin by antiviral compounds. Arch. Virol. 65, 149–156.

Batterson, W., Furlong, D. and Roizman, B. (1983) Molecular genetics of herpes simplex virus. VIII. Further characterization of a temperature sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. J. Virol. 45, 397–407.

Chandra, P., Ebener, U. and Gericke, D. (1979) Molecular mechanisms for control of RNA tumor viruses. In: P. Chandra (Ed.), Antiviral Mechanisms in the Control of Neoplasia, pp. 523–537. Plenum Press, New York.

Coen, D.M., Chiou, H.C. and Gibbs, J.S. (1986) Molecular genetics of antiviral chemotherapy of herpesviruses. In: C. Lopez and B. Roizman (Eds.), Human herpesvirus infections: Pathogenesis, diagnosis, and treatment, pp. 117–128. Raven Press, New York.

Darby, G., Field, H.J. and Salisbury, S.A. (1981) Altered substrate specificity of herpes simplex virus thymidine kinase confers acyclovir-resistance. Nature (London) 289, 81–83.

Field, H.J. (1982) Development of clinical resistance to acyclovir in herpes simplex virus-infected mice receiving oral therapy. Antimicrob. Agents Chemother. 21, 744–752.

Goswami, B.B., Crea, R., Van Boom, J.H. and Sharma, O.K. (1982) 2′,5′-linked oligo (adenylic acid) and its analogs. A new class of inhibitors of mRNA methylation. J. Biol. Chem. 257, 6867–6870.

Grunert, R.R. (1979) Search for antiviral agents. Ann. Rev. Microbiol. 33, 335–353.

Hayashi, T., Kawasaki, M., Kishi, M., Arisawa, M., Shimizu, M., Morita, N., Tezuka, Y., Kikuchi, T. and Berganza, L.H. (1987a) Structure of new diterpenoids from Paraguayan crude drug 'Typychá Kuratũ' (*Scoparia dulcis* L.). Symposium papers, 29th Symposium on the Chemistry of Natural Products, Sapparo, Japan, pp. 544–551.

Hayashi, T., Kishi, M., Kawasaki, M., Arisawa, M., Shimizu, M., Suzuki, S., Yoshizaki, M., Morita, N., Tezuka, Y., Kikuchi, T., Berganza, L.H., Ferro, E. and Basualdo, I. (1987b) Scopadulcic acid-A and -B, new diterpenoids with a novel skeleton, from a Paraguayan crude drug 'Typychá Kuratũ' (*Scoparia dulcis* L.). Tetrahedron Lett. 28, 3693–3696.

Ikegami, S., Taguchi, T. and Ohashi, M. (1978) Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase-α. Nature (London) 275, 458–459.

Kawasaki, M., Hayashi, T., Arisawa, M., Shimizu, M., Horie, S., Ueno, H., Syogawa, H., Suzuki, S., Yoshizaki, M., Morita, N., Tezuka, Y., Kikuchi, T., Berganza, L.H., Ferro, E. and Basualdo, I. (1987) Structure of scoparic acid A, a new labdane-type diterpenoid from a Paraguayan crude drug 'Typychá Kuratũ' (*Scoparia dulcis* L.). Chem. Pharm. Bull. 35, 3963–3966.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York.

McKenna, C.E., Khawli, L.A., Bapat, A., Harutunian, V. and Cheng, Y.-C. (1987) Inhibition of herpesvirus and human DNA polymerases by α-halogenated phosphonoacetates. Biochem. Pharmacol. 36, 3103–3106.

Morgan, C., Rose, H.M. and Mednis, B. (1968) Electron microscopy of herpes simplex virus. I. Entry. J. Virol. 2, 507–516.

Nago, R., Hayashi, K., Ochiai, H., Kubota, Y. and Niwayama, S. (1988) Detection of herpes simplex virus type 1 in herpetic ocular diseases by DNA-DNA hybridization using a biotinylated DNA probe. J. Med. Virol. 25, 259–270.

Ohashi, M., Taguchi, T. and Ikegami, S. (1978) Aphidicolin: a specific inhibitor of DNA polymerases in the cytosol of rat liver. Biochem. Biophys. Res. Commun. 82, 1084–1090.

Pedrali-Noy, G. and Spadari, S. (1979) Effect of aphidicolin on viral and human DNA polymerases. Biochem. Biophys. Res. Commun. 88, 1194–1202.

Rigby, P.W., Dieckmann, M., Rhodes, N. and Berg, P. (1977) Labeling deoxyribonucleic acid on high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113, 237–251.

Whitley, R.J., Soong, S.-J., Dolin, R., Galasso, G.J., Chien, L.J. and Alford, C.A. (1977) Adenosine arabinoside therapy of biopsy-proved herpes simplex encephalitis. N. Engl. J. Med. 297, 289–294.