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Anti-herpesvirus activity of citrusinine-I, a new acridone alkaloid, and related compounds

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Summary

Citrusinine-I, a new acridone alkaloid isolated from the root bark of the citrus plant (Rutaceae), exhibited potent activity against herpes simplex virus (HSV) type 1 and type 2 at low concentrations relative to their cytotoxicity; 50% effective concentrations (ED_{50}) of citrusinine-I were 0.56 μg/ml and 0.74 μg/ml against HSV-1 and HSV-2, respectively. Inhibitory action was also demonstrated against cytomegalovirus (CMV) and thymidine kinase-deficient or DNA polymerase mutants of HSV-2. The compound markedly suppressed HSV-2 and CMV DNA synthesis at concentrations which did not inhibit the synthesis of virus-induced early polypeptides. However, citrusinine-I had no inhibitory activity against HSV and CMV DNA polymerases in cell-free extracts. Although the target of this inhibitor remains to be elucidated, the most plausible candidate is a virus-coded ribonucleotide reductase. Citrusinine-1, when combined with acyclovir or ganciclovir, synergistically potentiated the antiherpetic activity of these agents. Based on a comparative study of the antiherpetic activity of citrusinine-1 and 28 related compounds, a structure-activity relationship could be established.

Citrusinine-I; Acridone alkaloid; Herpes simplex virus; Human cytomegalovirus

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Introduction

A number of selective agents have been developed against herpes simplex virus (HSV) and varicella-zoster virus (VZV) (De Clercq, 1982, 1986, 1988; Trousdale et al., 1983; Wildiers and De Clercq, 1984). Most of these compounds are nucleoside analogues, and their antiviral activity depends on two virus-induced enzymes, thymidine kinase and DNA polymerase. The most successful agent, acyclovir (ACV), has proved clinically beneficial in the treatment of a variety of herpesvirus infections (Reichman et al., 1983; Fiddian et al., 1984). However, the widespread use of the drug might give rise to the emergence of drug-resistant variants. In fact, even at the initial stage of ACV therapy, drug-resistant virus strains have been isolated from immunocompromised patients and normal subjects (Burns et al., 1982; Wade et al., 1983; McLaren et al., 1985; Svennerholm et al., 1985; Ellis et al., 1987).

Certain acridone alkaloids are known to possess a variety of biological activities, including antitumor, antiviral and anti-inflammatory activities (Dunn et al., 1973; Kramer et al., 1976; Gerfzon et al., 1983). When evaluating the antiviral activity of newly isolated acridone alkaloids, we found that citrusinine-I isolated from the root bark of *Citrus sinensis* Osbeck var. brasiliensis Tanaka (Rutaceae) had potent antiherpetic activity. In this report, we describe the activity of citrusinine-I against HSV and cytomegalovirus (CMV). It is a unique inhibitor with a different action mechanism from that of the nucleoside analogs ACV and bromovinyldeoxyuridine (BVDU).

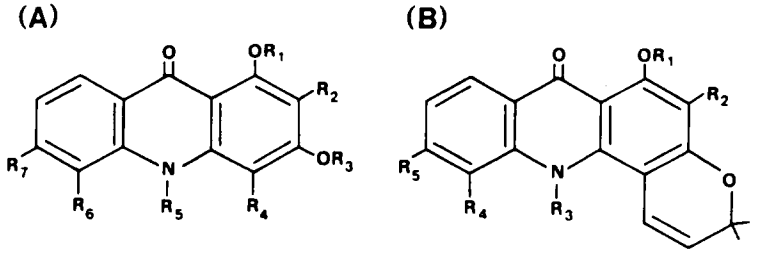
Materials and Methods

Test compounds and chemicals

Citrusinine-I and other acridone alkaloids were isolated from the root and/or stem barks of some genus *Citrus*, according to published procedures (Wu et al., 1982, 1983a–d). The chemical structure of citrusinine-I is shown in Fig. 1. All acridone alkaloids were initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/ml and stored at −20°C until use. L-[^{35}S]methionine (600 Ci/mmol), [methyl-^{3}H]dTTP (85 Ci/mmol) and [α-^{32}P]dCTP (410 Ci/mmol) were purchased from Amersham Laboratories, U.K.

Cell and virus

Human embryonic fibroblasts (HEF) were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), and used in passage of 5 to 20. VERO cells, a line of African green monkey kidney cells, were grown in MEM supplemented with 7% calf serum. Mouse leukemia L1210 cells were grown in RPMI 1640 supplemented with 10% FCS. A plaque-purified clone



Compounds	R_1	R_2	R_3	R_4	R_5	R_6	R_7
(A)							
1	H	H	H	H	H	H	H
2	H	H	H	H	CH_3	OCH_3	OH
3	H	H	H	C_5H_9	CH_3	H	H
4	H	H	CH_3	H	CH_3	OH	OCH_3
5	H	H	CH_3	H	CH_3	OCH_3	OH
6	H	H	CH_3	H	CH_3	OCH_3	OCH_3
7	H	H	CH_3	C_5H_9	CH_3	H	H
8	H	H	CH_3	C_5H_9	CH_3	OH	H
9	H	H	CH_3	C_5H_9	CH_3	OCH_3	OH
10	H	H	CH_3	OCH_3	CH_3	OH	H
11	H	C_5H_9	H	C_5H_9	CH_3	OH	H
12	H	OCH_3	CH_3	OCH_3	CH_3	OCH_3	OH
13	CH_3	H	CH_3	H	CH_3	H	H
14	CH_3	H	CH_3	H	CH_3	OCH_3	OCH_3
15	$COCH_3$	H	$COCH_3$	H	H	H	H
16	$COCH_3$	H	$COCH_3$	H	CH_3	H	H
(B)							
1	H	H			H	H	H
2	H	H			H	OH	H
3	H	H			CH_3	H	H
4	H	H			CH_3	OH	H
5	H	H			CH_3	OCH_3	OH
6	H	H			CH_3	OCH_3	OCH_3
7	H	C_5H_9			H	H	H
8	H	C_5H_9			H	OH	H
9	H	C_5H_9			CH_3	H	H
10	H	C_5H_9			CH_3	OH	H
11	CH_3	H			CH_3	H	H
12	CH_3	H			CH_3	OCH_3	H
13	CH_3	H			CH_3	OCH_3	OCH_3

Fig. 1. Structures of acridone alkaloids.

of HSV type 2 strain 186 was used as a wild-type throughout this study. HSV-2 mutants, phosphonoacetic acid (PAA)-resistant, aphidicolin (Aph)-resistant and thymidine kinase-deficient mutants, were isolated in our laboratory (Nishiyama et al., 1984, 1985). HSV type 1 strain HF, CMV strain AD169, vesicular stomatitis virus (VSV), Sindbis virus and parainfluenza virus type 2 were also used in this study. The virus stocks were prepared by infecting the cells at low multiplicities (0.001 to 0.1 PFU per cell), as described previously (Nishiyama and Rapp, 1981).

Plaque reduction assays

To determine the dose-response of antiviral activity of inhibitors, plaque reduction assays of HSV and CMV were performed as follows. Confluent monolayers of HEF were infected with approximately 100 PFU of virus in 35-mm culture dishes. After a 1 h-adsorption period at 37°C, cultures were overlaid with 2 ml of 0.5% agarose in MEM containing various concentrations of inhibitors. When distinct plaques were observed in the drug-free control cultures, cells were fixed with 5% formalin and stained with 0.7% crystal violet. The plaques were then counted using a dissecting microscope. The plaque assays of VSV, Sindbis and parainfluenza-2 were carried out in VERO cells as described previously (Shimokata et al., 1981).

Yield reduction assays

Monolayers of HEF were infected with HSV-2 or CMV at a multiplicity of about 10 PFU per cell and treated with various concentrations of citrusinine-I after 1 h virus adsorption at 37°C. HSV-2 and CMV were harvested at 24 h and 5 days post-infection, respectively. After freezing-thawing followed by low speed centrifugation, the supernatant was assayed for virus infectivity.

Polyacrylamide gel electrophoresis (PAGE)

PAGE was carried out by the method of Laemmli (1970). Samples labeled with [^{35}S]methionine were dissociated in 0.0625 M Tris-HCl (pH 6.8) containing 5% SDS, 2% 2-mercaptoethanol (2-ME), 10% glycerol and 0.001% bromophenol blue, followed by heating at 100°C for 1 min. The acrylamide concentrations were 8.5% for the separating gel and 3% for the stacking gel. After electrophoresis, the gels were fixed, dried and then exposed to Kodak Royal X-Omat films at −80°C.

Dot blot hybridization

Infected cells were collected by centrifugation, washed with phosphate-buffered saline (PBS), and suspended in a buffer solution (pH 8.1) containing 10 mM Tris-HCl, 0.1 mM EDTA and 150 mM NaCl. Infected cells were lysed by addition of 1.0% SDS, and digested with pronase at a concentration of 500 μg/ml at 37°C for 3 h. The solution was extracted three times with phenol plus chloroform-isoamyl-alcohol (24:1), and dialyzed against 2 l of TE solution (10 mM Tris-HCl, 1 mM EDTA) with several changes. The sample was treated with 100 μg/ml of DNase-free RNase at 37°C for 2 h, and extracted with organic solvents as described above. After dialysis, DNA was precipitated with ethanol, dissolved in TE solution, and denatured by heating. Each sample was serially diluted with 15×SSC (0.15 M NaCl, 0.015 M sodium citrate) to yield a final volume of 100 μl, and each dilution was applied with suction to a 4-mm diameter spot on 'gene screen plus' membrane purchased from New England Nuclear. Prehybridization and hybridization were performed following the protocol of the supplier. Briefly, the membrane was prehy-

bridized by treating in 10 ml of the following solution; 50% formamide (deionized), 1% SDS, 1 M sodium chloride and 10% dextran sulfate. The solution was added to a sealable plastic bag containing the membrane. The plastic bag was sealed and incubated with constant agitation for at least 6 h at 42°C. The solution containing denatured salmon sperm DNA (>100 μg/ml) and denatured radioactive probe (HSV-2 *Hin*dIII digested D fragment or CMV *Xba*I digested N fragment) was added to the bag containing the prehybridization buffer and the membrane. The plastic bag was resealed and incubated with constant agitation for 24 h at 42°C. The membrane was removed from hybridization solution, washed with SSC, allowed to dry at room temperature, and used for autoradiography.

TABLE 1

Antiherpetic and anticellular activity of acridon alkaloids

Compound[a]	Antiviral activity[b] EC_{50} (μg/ml)	Anticellular activity[c] IC_{50} (μg/ml)
1. Synthetic	7.3	10
2. Grandicine-II	>20	>10
3. Glycocitrine-II	10[d]	10
4. Grandicine-I	>20	>10
5. Citpressine-I	0.59	4.3
6. Citpressine-II	>20	>10
7. *O*-Methylglycocitrine-II	>20	9.3
8. Glycocitrine-I	5[d]	4.8
9. Grandisinine	10[d]	10
10. Citrusinine-I	0.74	>10
11. *N*-Methylatalaphilline	8.4	2.8
12. Glyfoline	>20	2.5
13. 1,3-*O*-Methyl-*N*-methylacridone	4.9	4.6
14. 1,3,5,6-*O*-Methyl-*N*-methylacridone	>20	13.6
15. (synthetic)	6.5	7.5
16. (synthetic)	8.4	5.5
1. Des *N*-methylnoracronycine	>20	2.0
2. Atalaphillidine	0.73	3.1
3. Noracronycine	>20	>10
4. 5-Hydroxynoracronycine	5[d]	2.1
5. Citracridone-I	1.3	4.8
6. Citracridone-II	>20	>10
7. Severifoline	>20	>10
8. Atalaphillinine	0.82	1.3
9. *N*-Methylseverifoline	>20	>10
10. 5-Hydroxy-*N*-methylseverifoline	2.0	1.0
11. Acronycine	3.3	4.0
12. 5-Methoxyacronycine	5.5	3.7
13. Dimethoxyacronycine	6.5	5.3

[a]The structures of compounds are shown in Fig. 1.
[b]The EC_{50} values were determined by plaque reduction assays with HSV-2 and HEF monolayers.
[c]The IC_{50} values were determined by growth inhibition assays against mouse leukemia L1210 cells.
[d]The EC_{50} values could not be precisely measured because of the cytotoxicity.

TABLE 2

Antiviral activity of citrusinine-I against various viruses[a]

Viruses	EC_{50} (μg/ml)
HSV-1	0.56
HSV-2	0.74
HSV-2 (TK^-)[b]	0.92
HSV-2 (PAA^r)[b]	0.63
HSV-2 (Aph^r)[b]	0.70
HCMV	1.5
Sindbis	>20
Parainfluenza-2	>20
VSV	>20

[a]The plaque reduction assays of herpesviruses (HSV-1,2 and HCMV) were performed in HEF monolayers, and those of RNA viruses (sindbis, parainfluenza-2 and VSV) in VERO cells.
[b]The mutants were all derived from the same parental clone of HSV-2 strain 186.

Enzyme assays

HSV and CMV DNA polymerases were partially purified as described previously (Nishiyama et al., 1984). The standard reaction mixture (50 μl) for viral DNA polymerase contained 50 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 100 mM ammonium sulfate, 80 μM each of dATP, dGTP, dCTP and [methyl-^{3}H]dTTP (0.5 μCi/nmol), 1.25 μg activated DNA, and enzyme. Incubation was carried out at 37°C for 15 min, and the reaction was stopped by the addition of 10% trichloroacetic acid (TCA). Acid-insoluble material was then collected by filtration onto glass fiber GF/C discs and washed sequentially with 5% TCA, 50% ethanol and 100% ethanol. Discs were then dried and radioactivity was counted in a liquid scintillation spectrophotometer.

Results

Antiviral and anticellular activities of acridone alkaloids in vitro

Table 1 and Fig. 1 present the chemical structures and the antiherpetic and anticellular activities of acridone alkaloids. Of 29 compounds, four had an ED_{50} less than 1.0 μg/ml for HSV-2 plaque formation. However, these compounds except for citrusinine-I also exhibited potent anticellular activity. Citrusinine-I did not show any significant inhibition of growth against mouse leukemia L1210 cells at concentrations at which HSV-2 plaque formation was inhibited by more than 95%. We therefore studied the antiviral activity and the mode of action of citrusinine-I in the following experiments.

Table 2 shows the ED_{50} of citrusinine-I for various viruses. The compound showed potent inhibitory effect against both HSV-1 and HSV-2 (ED_{50} ranging from 0.5 to 1 μg/ml), there was no significant difference among TK^-, PAA^r, Aph^r and

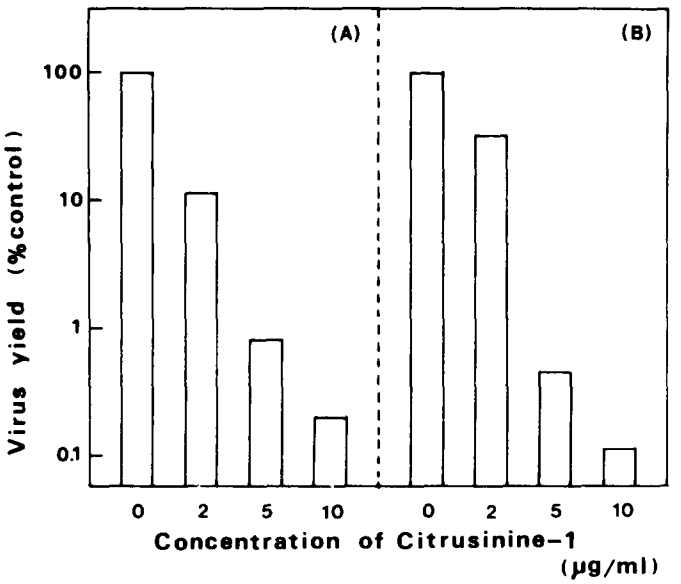


Fig. 2. Effect of citrusinine-I on the production of HSV-2 (A) and CMV (B). HEF cells were infected with either HSV-2 or CMV at a multiplicity of infection of approximately 10 PFU/cell, as explained in Materials and Methods. The titers were the average values for duplicate samples.

parental wild-type virus strains in their sensitivity to the inhibitor. The compound also exhibited anti-CMV potency, but no inhibitory effect was observed on the multiplication of RNA viruses such as VSV, Sindbis virus and parainfluenza virus type 2. Even when cells were infected with HSV-2 or CMV at a relatively high multiplicity (10 PFU/cell), treatment with citrusinine-I at 5 μg/ml reduced virus production by more than 99% (Fig. 2). We further evaluated the inhibitory effect of the compound on the growth of VERO cells and HEF. Cells were seeded at a concentration of about 1×10^5 cells/ml in the presence or absence of the drug, and cultured at 37°C. The number of cells was counted at day 3. The IC_{50} of citrusinine-I was 18 and 11 μg/ml for the growth of VERO cells and HEF, respectively (data not shown). With VERO cells, a 3.5-fold growth of cells was observed even in the presence of 20 μg/ml of citrusinine-I, compared to 7.5-fold growth in drug-free control cultures.

Effect of citrusinine-I on the synthesis of HSV-2- and CMV-induced proteins and DNA

To determine the effect of citrusinine-I on HSV-2- and CMV-induced protein synthesis, SDS-PAGE analysis was done. Confluent monolayers of HEF were infected with HSV-2 or CMV at a multiplicity of 10 PFU/cell. After 1 h virus adsorption period, cells were washed with PBS and incubated with maintenance medium containing various concentrations of citrusinine-I. In HSV-2-infected cells,

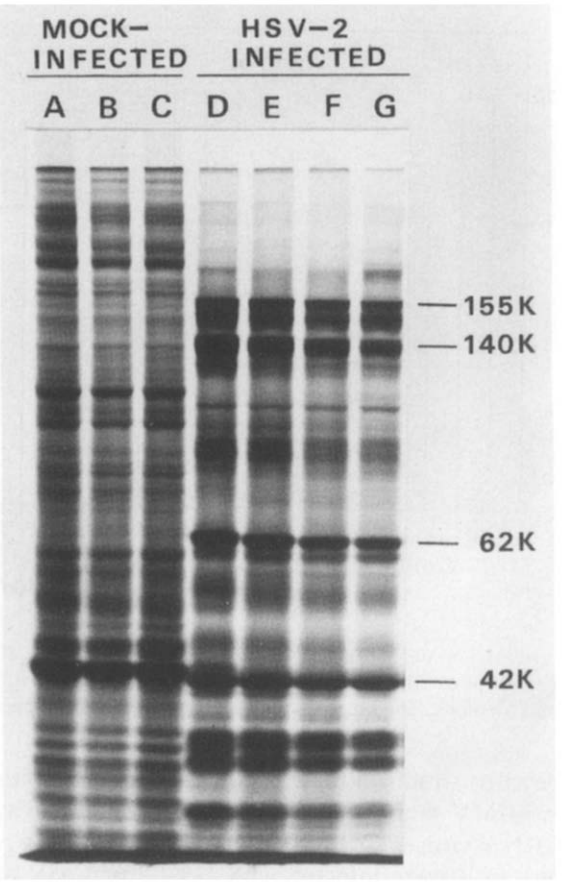


Fig. 3. Effect of citrusinine-I on HSV-induced protein synthesis. Mock-infected (A–C) or HSV-infected (D–G) HEF monolayers (about 10 PFU/cell) were incubated at 37°C in the absence (A,D) or presence of 2 (E), 5 (B,F) and 10I C,G) μg/ml of citrusinine-I, and labeled with[^{35}S]methionine (10 μCi/ml) from 3 to 4 h postinfection. The proteins were subjected to SDS-PAGE, followed by autoradiography.

cultures were labeled with [^{35}S]methionine (10 μCi/ml) from 3 to 4 h postinfection in the presence of the drug. As shown in Fig. 3, we did not observe an inhibition of the synthesis of HSV-2-induced early proteins at a concentration of 5 μg/ml. However, as shown in Fig. 4, citrusinine-I strongly inhibited the synthesis of CMV-induced late proteins such as the major capsid (150 kDa) and the major matrix (68 kDA) proteins, when the compound was added to the infected cultures at the early stage of infection, while the compound, when added at the late stage of infection, did not inhibit the synthesis of these late proteins. The effect of the drug on viral DNA synthesis was determined by dot blot hybridization technique with ^{32}P-labeled viral DNA fragments as a probe. The compound was added 1 h postinfection, and DNA was extracted at 6 h postinfection from HSV-2-infected cells or 72 h postinfection from CMV-infected cells. HSV-2 DNA synthesis was reduced to

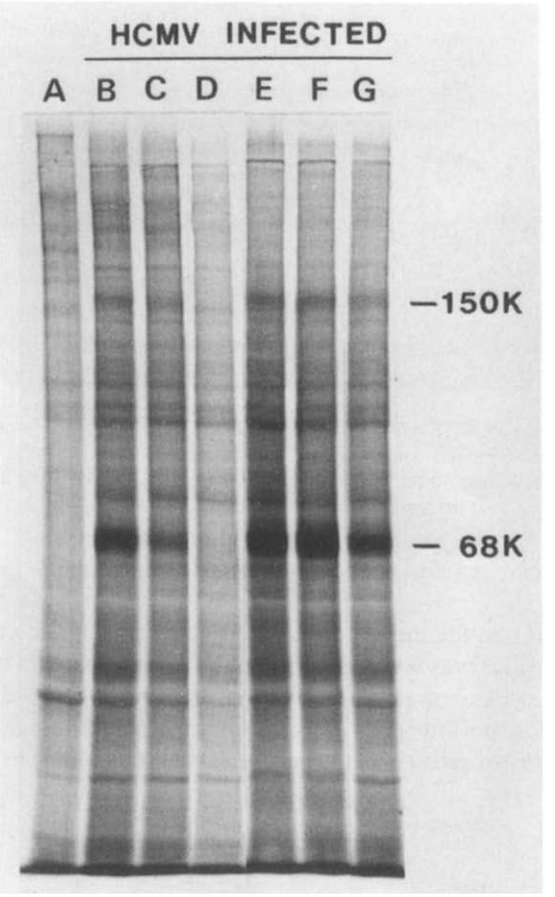


Fig. 4. Effect of citrusinine-I on CMV-induced protein synthesis. Mock-infected (A) or CMV-infected (B–G) HEF monolayers (about 10 PFU/cell) were incubated at 37°C in the absence (A,B,E) or presence of 1 (C,F), 5 (D) and 10 (G) μg/ml of citrusinine-I. The drug was added at 1 h postinfection (C,D) or 71 h postinfection (F,G). Cultures were labeled with [^{35}S]methionine from 72 to 73 h postinfection.

approximately 25% by the addition of 1 μg/ml of citrusinine-I. At a concentration of 10 μg/ml, viral DNA synthesis was not detectable (Fig. 5). Similar results were obtained with CMV-infected cultures treated with this drug: CMV DNA was not detectable at a concentration of 5 μg/ml citrusinine-I (Fig. 6).

These results indicated that citrusinine-I may interfere with viral DNA synthesis. We thus examined to determine whether the drug had any inhibitory effect on the activity of HSV or CMV DNA polymerase. The viral DNA polymerase assays were performed as described in Materials and Methods. The compound had no inhibitory effect on the activity of HSV or CMV DNA polymerase up to a concentration of 20 μg/ml (data not shown).

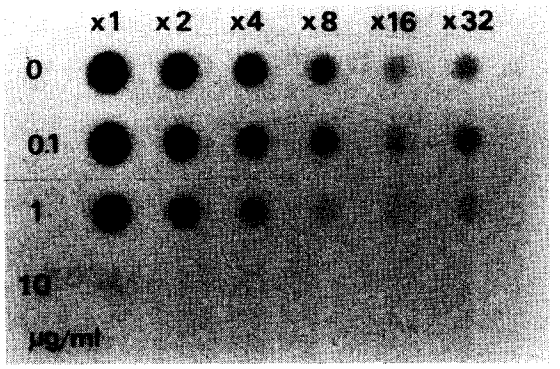


Fig. 5. Effect of citrusinine-I on HSV DNA synthesis. HSV-2-infected cells (about 10 PFU/cell) were incubated in the presence of various concentrations of citrusinine-I. Infected cells were collected at 6 h postinfection. DNA was extracted and diluted serially twofold. Dot-blot hybridization was carried out as described in Materials and Methods.

Reversal of antiherpetic activity of citrusinine-I by exogenous nucleosides

To further characterize the mode of action of citrusinine-I, the effect of addition of exogenous nucleosides was investigated. Confluent monolayers of HEF were infected with about 100 PFU of HSV-2 or CMV in 35-mm culture dishes, and after a 1 h virus adsorption period, cells were overlaid with 0.5% agarose containing various concentrations of citrusinine-I and nucleosides. As shown in Table 3, the

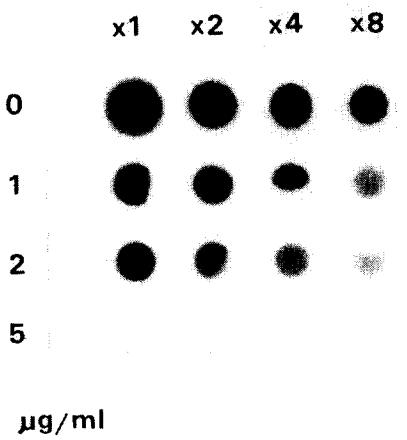


Fig. 6. Effect of citrusinine-I on CMV synthesis. CMV-infected cells (about 10 PFU/cell) were incubated in the presence of various concentrations of citrusinine-I. Infected cells were collected at 72 h postinfection. DNA was extracted and diluted serially twofold. Dot-blot hybridization was carried out as described in Materials and Methods.

TABLE 3

Reversal effect of deoxyribonucleosides on the plaque formation of HSV-2 and HCMV

Deoxyribonucleoside added	Concentration[b]	Plaque number[a] (% control) Citrusinine-I (μg/ml)		
		0.5	1	2
HSV-2				
None	0	69	45	16
4 deoxyribonucleosides[c]	60	82	67	46
4 deoxyribonucleosides[c]	120	96	83	61
deoxyadenosine + deoxyguanosine	120	67	44	10
deoxycytidine + deoxythymidine	120	70	30	14
		2	4	6
HCMV				
None	0	47	4	0
4 deoxyribonucleosides[c]	60	88	68	63
4 deoxyribonucleosided[c]	120	113	97	101
deoxyadenosine + deoxyguanosine	120	ND	4	ND
deoxycytidine + deoxythymidine	120	ND	1	ND

[a]The plaque reduction assays were performed in duplicate as described in the text. Plaque counts were expressed as a percentage of the number obtained in non-treated control cultures.
[b]Each deoxyribonucleoside was added to make final concentration 60 μM or 120 μM.
[c]Deoxyadenosin, deoxyguanosine, deoxycytidine and deoxythymidine were added.

antiviral activity of citrusinine-I could be reversed by the addition of the four deoxyribonucleosides. The addition of either deoxyadenosine plus deoxyguanosine or deoxycytidine plus deoxythymidine produced no significant reversal of the inhibitory effect of citrusinine-I.

Isolation of citrusinine-I-resistant mutants

If citrusinine-I specifically interacts with a viral protein, it should be possible to isolate drug-resistant virus mutants. To test this possibility, we tried to obtain HSV-2 mutants resistant to citrusinine-I. A plaque-purified wild type clone was mutagenized by UV-irradiation (3600 ergs/mm^2), and HEF monolayers were infected with UV-irradiated viruses, incubated for 48 h and then harvested. Viruses thus obtained were serially passaged in HEF exposed to concentrations of citrusinine-I which ranged from 1 to 5 μg/ml. After five to seven passages, viruses were plaque-purified and examined for their sensitivity to the drug. Fig. 7 represents a dose-response experiment for the effect of citrusinine-I on the plaque formation of an isolate and wild-type parental virus. Wild type virus plaque formation was inhibited by about 75% at a concentration of 1 μg/ml and almost completely at a concentration of 2 μg/ml of citrusinine-I. In contrast, plaque formation of the isolate was completely resistant to 1 μg/ml of citrusinine-I. The plating efficiency of the mutant in the presence of 2 μg/ml of citrusinine-I was more than 20-fold higher

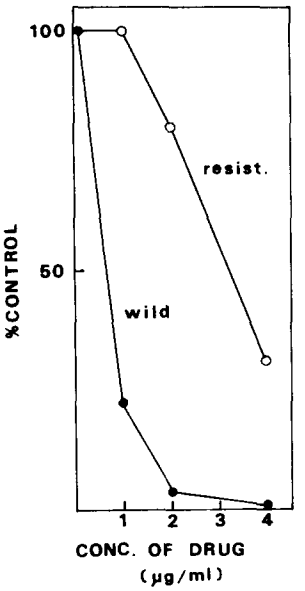


Fig. 7. Effect of citrusinine-I on the plating efficiency of wild-type HSV-2 and a citrusinine-I-resistant mutant. The sensitivity of viruses to the drug was measured by plaque reduction assays in monolayers of HEF. The plaque counts are expressed as the percentage of the number obtained in drug-free control cultures.

than that of the wild-type. These results suggest that the target for the action of citrusinine-I may be a virus-encoded protein.

Potentiation of antiherpetic activity of acyclovir (ACV) and ganciclovir (DHPG) by citrusinine-I

The above results indicated that citrusinine-I inhibited viral replication by impairing viral DNA synthesis, but also suggested that the mechanism of action of the compound was different from that of antiviral nucleoside analogues such as ACV and DHPG. The following experiments were done to determine whether citrusinine-I potentiated the antiviral activity of ACV and DHPG. Infected cells were treated with ACV alone, DHPG alone, or combinations of ACV or DHPG with citrusinine-I, and virus production was measured at 24 h postinfection in HSV-2-infected cells or at 5 days postinfection in CMV-infected cells. When the combined inhibitory effect of the compounds was assessed by the formula of Spector et al. (1982), a synergistic effect could be clearly demonstrated (Table 4).

TABLE 4

Potentiation of antiherpetic activity of ACV and DHPG by citrusinine-I

Virus	Citrusinine-I (μg/ml)	ACV or DHPG	Virus titer[a] (PFU/ml)	Expected titer[b] (PFU/ml)	Degree of synergy[b]
HSV-2		ACV			
	0	0	5.1×10^6		
		0.25	1.2×10^6		
		0.5	7.1×10^5		
		1.0	3.4×10^5		
	0.25	0	4.5×10^6		
		0.25	4.0×10^5	1.1×10^6	2.8
		0.5	1.1×10^5	6.3×10^5	5.7
		1.0	5.2×10^4	3.0×10^5	5.8
	0.5	0	3.9×10^6		
		0.25	3.0×10^5	9.2×10^5	3.1
		0.5	8.9×10^4	5.4×10^5	6.1
		1.0	2.5×10^4	2.6×10^5	10.4
HCMV		DHPG			
	0	0	4.5×10^5		
		0.25	2.5×10^5		
		0.5	1.5×10^5		
		1.9	4.4×10^4		
	0.25	0	4.0×10^5		
		0.25	1.5×10^5	2.2×10^5	1.5
		0.5	6.2×10^4	1.3×10^5	2.1
		1.0	8.0×10^3	3.9×10^4	4.9
	0.5	0	3.5×10^5		
		0.25	9.0×10^4	1.9×10^5	2.1
		0.5	3.4×10^4	1.2×10^5	3.5
		1.0	5.6×10^3	3.4×10^4	6.1

[a]Confluent monolayers of HEF were infected with HSV-2 or HCMV at a multiplicity of approximately 10 PFU/cell, and incubated with maintenance medium in the pressence or absence of appreprate concentrations of antiviral agents. The virus titers of HSV-2-infected and HCMV-infected cultures were assayed at 24 h and 5 days postinfection, respectively. The titers were the average of duplicate samples.
[b]Expected titer and degree of synergy were calculated by the formula of Spector et al. (1982).

Discussion

The present study demonstrates that citrusinine-I, a new acridone alkaloid isolated from the root bark of a rutaceous plant, had potent antiviral activity against HSV-1 and HSV-2 at concentrations well below the cytotoxicity threshold. Citrusinine-I was also active against CMV and three different HSV mutants which either encoded an altered viral DNA polymerase or did not induce viral thymidine kinase. Citrusinine-I had no inhibitory effect on the replication of RNA viruses such as VSV or Sindbis virus. The compound strongly inhibited viral DNA synthesis at

concentrations that did not affect the synthesis of virus-induced early polypeptides, whereas it did not inhibit the activity of HSV or CMV DNA polymerase in vitro. Taken these observations together, it is suggested that, although citrusinine-I interferes with the synthesis of viral DNA, its mode of action is different from that of the well-known antiherpetic nucleoside analogues acyclovir and BVDU. Since HSV mutants that were relatively resistant to citrusinine-I could be derived from the wild-type virus, the target for the antiviral action of the compound may well be a virus-encoded protein involved in HSV DNA synthesis. Although the mapping of the mutation responsible for citrusinine-I resistance is necessary to determine the target protein, one of the possible candidates is herpesvirus-encoded ribonucleotide reductase because the antiviral activity of citrusinine-I could be reversed by the addition of the four deoxyribonucleosides.

Recently, Spector et al. (1985) have shown that compound A 723U, a 2-acetylpyridine thiosemicarbazone, which is an inhibitor of HSV-induced ribonucleotide reductase, significantly potentiates the antiviral activity of ACV at subinhibitory concentrations. Citrusinine-I, when tested in combination with ACV and DHPG, also exhibited the synergistic effects against HSV-2 and CMV.

Of 29 acridone alkaloids tested, 4 compounds (citpressine-I, citracridone-I, atalaphillinine and atalaphillidine) exhibited an antiherpetic activity that was comparable to that of citrusinine-I. All these compounds, including citrusinine-I, possess OH groups at either the C-5 (R_6) or C-6 (R_7) position of the acridone moiety; the importance of the OH group at C-5 (R_6) for the antiherpetic activity could be easily recognized when compared to the pair atalaphillidine/DesN-methylnoracronycine or the pair atalaphillinine/severiforine. The presence of the *O*-alkyl moiety at C-3 (R_3: CH_3 or pyran ring) instead of an OH group, and the NH group in the acridones having a C-substituent at C-4 (R_4: prenyl or pyran ring) also appeared to be important for antiviral activity. However, all these compounds, except for citrusinine-I, were highly cytotoxic. Based on these observations, we are now trying to develop more active and/or more selective acridones than citrusinine-I.

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