Antiherpes Virus Effect of the Red Marine Alga *Polysiphonia denudata*

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The water extract from the red marine alga *Polysiphonia denudata* (Dillwyn) Kütz. from the Bulgarian Black Sea coast selectively inhibited the reproduction of herpes virus type 1 and type 2 in cell cultures (EC_{50} = 8.7 to 47.7 mg/ml) as shown by the reduction of virus-induced cytopathic effect and viral infectivity. The virus-inhibitory effect was dose-related, strain-specific and depended on virus inoculum. The inhibition affected adsorption as well as the intracellular stages of viral replication. The presence of the extract throughout the whole replicative cycle was necessary for the full expression of the antiviral effect. In higher concentrations (MIC_{90} = 6.5 mg/ml) the extract exhibited strong extracellular virus inactivating activity.

Introduction

Acute and recurrent herpes simplex virus (HSV) infections are still a problem. The search for selective antiviral agents has been vigorous in recent years but the need for new antiviral therapies still exists. Recent biochemical and pharmaceutical investigations on marine algae evaluate them as an interesting source of potential antiviral compounds (Che, 1991; Kerr and Kerr, 1999). As a part of a project on the antiinfective activities of marine organisms we evaluated the antiinfluenza virus activity of 70 products. 20% showed significant antiviral effect (Serkedjieva *et al.*, 2000). Most effective was the water extract of the red marine alga *Polysiphonia denudata*, var. Fragiris, endemic at the Bulgarian Black Sea sea coast. It inhibited selectively the reproduction of influenza virus in cell cultures and in fertile hen's eggs and protected mice against experimental influenza infection.

The present study was undertaken to evaluate the antiherpes virus effect of the preparation in cell cultures with respect to the selectivity and specificity of inhibition.

Materials and Methods

Plant material and extraction

The alga *Polysiphonia denudata* (Dillwyn) Kütz. (Rhodophyta – Florideophyceae) was collected along the South Bulgarian sea coast of the Black Sea near the Varvara village in summer at a depth of 2–10 m. The fresh organism was cleaned of epiphytes and dead parts, washed with fresh water, dipped in ethanol and transported to the laboratory. The alga was identified by Dr. Stefka Dimitrova – Konaklieva, Faculty of Pharmacy, Medical University, Sofia. About 100–120 g of the fresh material, corresponding to 10–15 g of dry material, was homogenized with chloroform – methanol (500 ml, 1:1, v/v) and refluxed for a few to inactivate the enzymes. The extraction was repeated three times, the combined extracts were concentrated to about 200 ml and water (200 ml) was added. Two layers were formed, the upper one – water – contained polar compounds. In the upper phase there was about 2–3 g material. It was evaporated to dryness (*Polysiphonia* extract). The *Polysiphonia* extract was prepared and kindly provided by Dr. Monika Konaklieva, Institute of Organic Chemistry with Centre of Phytochemistry, Bulg. Acad. Sci. For the antiviral experiments a 10% stock solution of the preparation was made with distilled sterile water and the subsequent working dilutions were made with cell culture medium *ex tempore.*

Cell cultures and media

Permanent cell lines from African green monkey kidney (Vero) cells (from the Institute of Antiviral Chemotherapy, Erfurt, Germany) and human embryonic skin-muscle (E_6SM) cells (from the Rega Institute of Antiviral Research, Leuven,

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Belgium) were cultivated in Eagle's minimal essential medium (MEM) (Sigma-Aldrich GmbH, Deisenhofen, Germany), supplemented with 10% foetal calf serum (FCS) and antibiotics (benzylpenicillin-100 IU/ml and streptomycin-100 μg/ml) and L-glutamine (growth medium) at 37 °C in the presence of 5% CO_2 till the formation of confluent monolayers. In cell toxicity and antiviral experiments 1.5% FCS was added to MEM (maintenance medium).

Viruses

Herpes simplex virus type 1 (HSV-1), strain Kupka was from the collection of the Institute of Antiviral Chemotherapy, Erfurt, Germany. The virus was propagated in Vero cells. The experiments with this virus were performed in the laboratory of Prof. R. Klocking in the same institute.

HSV-1, strain KOS and HSV type 2 (HSV-2), str. GC25927 were from the collection of the Rega Institute of Antiviral Research, Leuven, Belgium. The viruses were propagated in E_6SM cells. The experiments with these viruses were performed in the laboratory of Prof. E. De Clercq in the same institute.

Cell toxicity

Cell toxicity was monitored by determining the effect of the preparations on cell morphology and viability. The cytopathic effect (CPE) was scored on day 2–7 of incubation as described in Serkedjieva and Hay (1998). The concentration required to cause visible changes in 50% of intact cells, 50% toxic concentration (TC_{50}), was estimated from graphic plots. The cytotoxicity for Vero cells was determined also by a colorimetric method based on the reduction of the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyltetrazolium by viable cells (MTT assay) (Mosmann, 1983). The optical density ($OD_{540,690}$) was determined by an Anthos Microplate Reader. The percentage of viable treated cells was calculated in relation to untreated controls (% of cell control = $OD_{exp}/OD_{cell\ control} \times 100$). TC_{50} was the concentration required to reduce the OD by 50%.

Virucidal activity

The direct virus inactivating effect of the preparation was tested in the contact assay as described before (Serkedjieva and Hay, 1998). The minimal concentration required to reduce the infectious titre, expressed in 50% tissue culture infectious doses per ml ($TCID_{50}$/ml) by 1 $\log_{10}$ (by 90%) was estimated from graphic plots (MIC_{90}).

Antiviral assays

The virus-inhibitory effect of the preparation was studied by:

1. *Cytopathic effect reduction assay (CPER).* Quadruplicate confluent monolayers in 96-well plates were overlaid with serial two-fold drug-containing medium (0.05 ml) and equal volume of virus suspension (10^2–10^4 $TCID_{50}$/ml). The virus induced CPE was scored on day 3–4 post infection (p.i.) for E_6SM and on day 5–7 p.i. for Vero by an inverted microscope as described by Serkedjieva and Hay (1998). The reduction of virus multiplication was calculated as % of virus control (% virus control = $CPE_{exp}/CPE_{virus\ control} \times 100$). The concentration reducing CPE by 50% in respect to virus control was defined as 50% effective concentration (EC_{50}) and was estimated from graphic plots. The selectivity index (SI) was calculated from the ratio TC_{50}/EC_{50}.

2. *Infectious virus yield reduction assay (IVYR).* This was performed after Serkedjieva and Hay (1998). The concentrations that reduced virus infectivity by 90% (1 $\log_{10}TCID_{50}$/ml; EC_{90}) was determined.

3. *MTT assay.* We used a modification of an MTT-assay developed for screening of anti-HSV compounds by Takeuchi *et al.* (1991). In short, quadruplicate confluent monolayers in 96-well plates were overlaid with serial two-fold drug-containing medium (0.05 ml) and an equal volume of virus suspension (10^2–10^4 $TCID_{50}$/ml). On day 7 p.i. the supernatants were discarded and 20 μl of MTT, 5 mg/ml in PBS, was added to each well; the monolayers were incubated for 3–6 h at 37 °C. The resulting formazan precipitate was dissolved in iso-propanol with the addition of DMSO and the OD were determined at 492 nm (test) and 690 nm (reference). The % of protection was calculated by the following formula

$$(OD_{exp})\text{-}(OD_{virus\ control})/(OD_{cell\ control})\text{-}(OD_{virus\ control}) \times 100\ (\%),$$

where (OD_{exp}), $(OD_{virus\ control})$, $(OD_{cell\ control})$ in-

dicate the absorbancies of the test sample, the virus control and the cell control, respectively. The EC_{50} was estimated from graphic plots as the concentration achieving 50% protection of infected cells according to the above formula. The SI for each compound was determined as described above.

4. *EZ4U assay.* The assay was performed with a commercial kit from Biomedica GmbH, Germany. It is an improved and simplified version of the MTT assay, on the base of another tetrazolium salt – 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT). The experiments were performed as described above, there was no need of organic solvent as the precipitate was water-soluble, the absorbancies were read at wavelengths 450 and 620 nm ($OD_{450,620}$). % of protection, EC_{50} and SI were estimated as described above.

Timing of addition studies

Confluent monolayers were incubated with plain or drug-containing medium for 1 h (effect of cell pre-treatment), washed twice with PBS and challenged with serial ten-fold dilutions of infectious virus. Adsorption was carried out for 1 h at 4 °C to prevent virus internalisation in the presence or in the absence of 2× drug-containing medium (effect on adsorption). The cells were washed twice with PBS and overlaid with plain or drug-containing medium for 1 h to allow viral particles to penetrate into cells (effect on penetration). Then plain or drug-containing medium was added and cells were cultivated for 24 h at 37 °C (effect on replication). At the end of cultivation the cells were frozen and after one freeze-thaw cycle infectious titres were determined. A viral titre reduction by 1 $\log_{10}TCID_{50}$/ml (90%) was considered to indicate a significant antiviral effect. The significance of the difference in infectious titres was estimated (Student's *t*-test).

Drug-susceptibility testing

The inhibition of virus replication, followed by CPER assay was used to determine the inhibitory effect of the extract on selected HSV strains.

In all antiviral experiments non-drug treated, mock-infected cells were used as cell control and non-drug treated, virus-infected cells – as virus control. The selective inhibitor of HSV reproduction (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) was used as a positive control.

Results

Cell toxic activity of *Polysiphonia* extract was monitored by two different assays – morphological (CPE) and colorimetric (MTT). The results for both assays were similar and the effect was dose-related (Table I).

Table I. Inhibitory effect of *Polysiphonia* extract on the reproduction of Herpes simplex viruses.

Virus strain	Cell cultures	TC_{50} [mg/ml]	Antiviral effect		
			Assay	EC_{50} [mg/ml]	SI[d]
HSV-1					
str. McIntyre*	Vero	8.2[a]	IVYR[c]	0.23	37.4
str. Kupka	Vero	8.6[b]	CPER	0.18	47.7
			MTT	0.31	27.7
			EZ4U	0.34	25.3
str. KOS	E_6SM	10.4[b]	CPER	0.42	24.8
HSV-2 str. GC25927	E_6SM		CPER	1.2	8.7

IVYR, Infectious virus yield reduction assay; CPER, Cytopathic effect reduction assay; MTT, a modification of the MTT-assay, described in Mosmann (1983); EZ4U, a modification of the MTT-assay (Biomedica GmbH, Germany).
* The experiments were done by Prof. I. Zgorniak-Nowosielska, Institute of Microbiology, Krakow.
[a] MTT assay, [b] CPE assay, [c] EC_{90}, [d] $SI = TC_{50}{}^{b}/EC_{50}$.

As a first experimental approach for the evaluation of the antiviral activity of the preparation we studied its ability to produce a direct inactivating effect on extracellular virus. The results are presented in Fig. 1. In concentrations <2 mg/ml the extract did not show any virucidal activity. The effect was dose-dependent. MIC_{90} was 6.5 mg/ml.

Polysiphonia extract inhibited significantly the reproduction of a range of herpes simplex type 1 and type 2 viruses in cell cultures (Table I, Fig. 2). The inhibitory effect was selective, strain-specific and dose-dependent; selectivity indices ranged from 0.18 to 1.2. Virus inhibition depended also on virus inoculum (data not shown). The susceptibility of three different strains to *Polysiphonia* extract was compared in the CPER assay. The results are presented in Fig. 2. Most susceptible was HSV-1, str. Kupka (SI=47.7). There were ob-

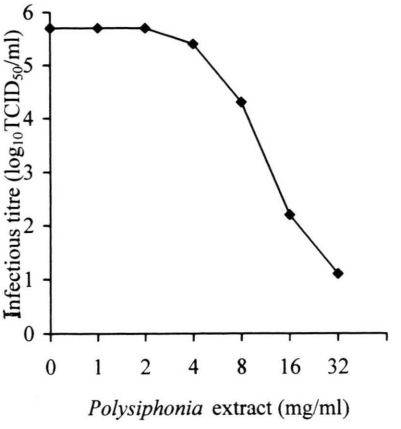

Fig. 1. Virus-inactivating effect of *Polysiphonia* extract on HSV-1, str. KOS.

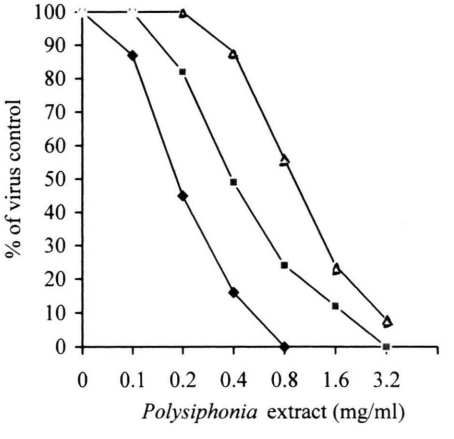

Fig. 2. Drug-susceptibility of HSV-1 and HSV-2 viruses to *Polysiphonia* extract inhibition.
– ◆ – Kupka
– ▨ – KOS
– △ – GC25927

served differences in the degree of viral inhibition depending on the assay used. Though the number of tested viruses was limited the observed strain-specificity of inhibition was indicative of selective antiviral action. *Polysiphonia* extract inhibited significantly the reproduction of HSV-1, str. McIntyre in Vero with doses >0.23 mg/ml (Table I). In the IVYR assay at 24 h of incubation with 0.4 and 0.8 mg/ml of the extract no infectious virus was detected; however at 48 h after infection a great reduction of infectivity was observed ($\delta log_{10}TCID_{50}$/ml = 2.8–3.2).

To examine the effect of the *Polysiphonia* extract on different steps of viral replication the preparation was added at varying times relative to viral infection. The results are presented in Table II. The pre-treatment of cells (A) with 1 mg/ml of the extract as well as the addition at the time of penetration (C) did not reduce virus infectivity. Virus replication was considerably reduced when the extract was added at the time of adsorption (B) and when the preparation was inoculated after viral infection (D) – $\delta log_{10}TCID_{50}$/ml = 2.39.

Table II. Inhibition of HSV-1, str. KOS reproduction in E_6SM cells by *Polysiphonia* extract: timing of addition.

Inoculation of *Polysiphonia* extract 1 mg/ml		Virus-inhibitory effect		
		log_{10} $TCID_{50}$/ml	δlog_{10} $TCID_{50}$/ml	Significance P<0.05
A. Pre-treatment of cells	1 h, 37 °C	5.66	0.1	–
B. Adsorption	1 h, 4 °C	3.23	2.44	+
C. Penetration	1 h, 37 °C	5.13	0.54	–
D. After infection	48 h, 37 °C	3.28	2.39	+
A+B+C+D		2.63	3.04	+

Discussion

A large number of new biologically active substances of interesting chemical structure and of favourable pharmacological activities have been found among marine natural products (Rinehart, 1989). The marine organisms represent also a promising source of antiviral compounds (for review see Che, 1991; Kerr and Kerr, 1999). A number of studies have been reported on the inhibitory effects of algal extracts and isolated biologically active constituents on the replication of herpes simplex viruses. Deig *et al.* (1974) reported that aqueous extracts from *Farlowia mollis* and *Cryptosiphonia woodii* interfered significantly with HSV-1 reproduction. Ehresmann *et al.* (1977) studied the virus-inhibitory activity of 28 extracts from marine algae; ten species of Rhodophyta contained substances which caused marked reduction of HSV-1 infectivity. Andersson *et al.* (1983) examined extracts from 25 marine organisms collected along the Swedish coast for biological activities, including the plaque formation with HSV-1. The chloroform extract from *Fucus seratus* and petroleum ether extract from *Laminaria digitata* ex-

hibited suppressive effects at concentrations of 100 μg/ml without showing toxicity. Girre *et al.* (1987) reported that a water extract from the marine alga *Ascophyllum nodosum* inhibited the replication of HSV-1. Hayashi *et al.* (1996) assayed 49 extracts from algae for anti-HSV and anti-HIV activities. Twenty five aqueous extracts showed anti-HSV activity, four of which were most potent inhibitors.

The herpes virus inhibitory effect of the water extract from *Polysiphonia denudata* was evaluated against the reproduction of HSV-1 and HSV-2 strains in two lines of cell cultures by a number of different assays. Virus-induced cytopathic effect and infectious virus yields were reduced at non-toxic concentrations of the extract. High concentrations of the preparation (>10 mg/ml) exhibited a strong inactivating effect on extracellular virus. The virus-inhibitory effect however could not be explained with the direct virucidal activity alone as MIC_{90} was 9.6 mg/ml while EC_{50} was 0.42 mg/ml. The selectivity of antiviral action was confirmed by the variation in sensitivity of different herpes viruses to *Polysiphonia* extract.

To investigate on the specificity of the inhibitory effect the preparation was tested for inhibitory action on the reproduction of poliovirus 1, str. Mahony in FL (Fogh-Lund human amniotic cell line) cell cultures and no reduction of infectivity was observed (unpubl.). The extract did not reduce the growth of *Staphylococcus aureus* and *Escherichia coli* (Serkedjieva *et al.*, 1998). Thus the virus-inhibitory effect seemed to be selective and specific.

Although adsorption was recognised as one of the targets of virus inhibition, the effect was directed also against some intracellular stages of viral replication; the process directly affected was not identified. At this stage of our investigations we can not discuss in detail the mechanism of the inhibitory effect of the water extract from *Polysiphonia denudata.* Ehresmann *et al.* (1977) and Richards *et al.* (1978) suggested that herpes virus inhibition by algal extracts was due to a block at viral adsorption. Al algal extract inhibited HSV-1 penetration into cells but did not inhibit virus attachment (Hayashi *et al.*, 1993). Virus-specific protein synthesis in HeLa cells was inhibited selectively as well (Hayashi *et al.*, 1993). The *Polysiphonia* extract reduced significantly the reproduction of influenza virus in cell cultures; an inhibition of adsorption and reduction of viral antigen expression on the surface of infected cells was observed (unpubl.). In connection with the presumed interference of the preparation with the early steps of viral replication – adsorption, fusion and/or entry – it would be interesting to study the interaction between the *Polysiphonia* extract and the herpes virus glycoproteins.

The dry *Polysiphonia* extract was fractionated with solvents with increasing polarity – petroleum ether, dichlorethane, ethyl acetate, butanol and water. Only the ethyl acetate fraction exhibited a significant antiviral effect. At this stage of investigation we cannot define the biologically active components of the preparation. However the current results support the view that marine organisms present an interesting source of potential antiviral compounds (Che, 1991; Kerr and Kerr, 1999).

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