Anti-Herpesvirus Activities of *Pseudomonas* sp. S-17 Rhamnolipid and its Complex with Alginate

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The rhamnolipid biosurfactant PS-17 and its complex with the polysaccharide alginate, both produced by the *Pseudomonas* sp. S-17 strain, were studied for their antiviral activity against herpes simplex virus (HSV) types 1 and 2. They significantly inhibited the herpesvirus cytopathic effect (CPE) in the Madin-Darby bovine kidney (MDBK) cell line. The investigations were carried out according to the CPE inhibition assay protocol. The suppressive effect of the compounds on HSV replication was dose-dependent and occurred at concentrations lower than the critical micelle concentration of the surfactant. The 50% inhibitory concentration (IC_{50}) of rhamnolipid PS-17 was 14.5 μg/ml against HSV-1 and 13 μg/ml against HSV-2. The IC_{50} values of the complex were 435 μg/ml for HSV-1 and 482 μg/ml for HSV-2. The inhibitory effects of the substances were confirmed by measuring the infectious virus yields with the multicycle virus growth experimental design as well: Δlog $CCID_{50}$ of 1.84–2.0 against the two types of herpes simplex viruses by rhamnolipid PS-17 (20 μg/ml), and a strong reduction of the HSV-2 virus yield under the effect of the alginate complex at a concentration of 450 μg/ml. The results indicate that rhamnolipid PS-17 and its alginate complex may be considered as promising substances for the development of anti-herpetic compounds.

Key words: Herpes Simplex Virus, Rhamnolipid, Alginate

Introduction

Human infectious diseases caused by the herpes simplex virus (HSV) have a high incidence rate and show various clinical pictures, many of them quite severe and occuring often in immunocompromised individuals. While orofacial blisters, keratitis, pneumonia and encephalitis are related with human HSV type 1 (HSV-1), HSV type 2 (HSV-2) generally causes skin and genital lesions and encephalitis. Anti-herpes activities of several groups of compounds have been demonstrated. Some drugs like acyclovir, ganciclovir, lobucavir, cidofovir and foscarnet are successfully used for the treatment of infections with HSV-1 and HSV-2 (De Clercq *et al*., 2001). The isolation of a great number of HSV drug-resistant mutants to antivirals with nucleoside nature prompted the search of new anti-herpesvirus substances with non-nucleoside structure and novel (original) modes of action.

Biosurfactants are a natural group of surface-active compounds which are synthesized by bacteria, yeasts and fungi extracellularly or as a part of the cell membrane. They are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition the interface between fluid phases with different degrees of polarity. Biosurfactant effects include an increase of the surface area and bioavailability of hydrophobic compounds, heavy metal binding, bacterial pathogenesis and biofilm formulation (Banat *et al*., 2000; Singh and Cameotra, 2004).

Research on biosurfactant applications is focused on their use in environmental protection and despite their advantages, such as lower toxicity, higher biodegradability and environmental compatibility, only a few studies concern their application in the biomedical field (Rodriguez *et al*., 2006).

Ramnose-containing glycolipids from *Pseudomonas* sp., rhamnolipids, are one of the most important microbial surfactants. Rhamnolipids are biosurfactants produced by a number of pathogenic and non-pathogenic microbial species (Cote and Krull, 1988). These compounds predominantly

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consist of one (mono-rhamnolipids) or two (di-rhamnolipids) rhamnose molecules and one or two β-hydroxy fatty acids (Benincasa *et al.*, 2004). The length of the carbon chains found in the β-hydroxyacyl portion of the rhamnolipid can vary significantly. Moreover, a wide variety of rhamnolipid homologues is described (Rendell *et al.*, 1990; Abalos *et al.*, 2001). Mixtures of two or four homologues are predominant. Rhamnolipid molecules have free carboxylic groups and behave as anions at pH values above 4.0.

Rhamnolipids are powerful natural emulsifiers capable of reducing the surface tension of water from roughly 76 mN/m to 25 to 30 mN/m (Fieshter, 1992). The biosurfactant activity of rhamnolipids makes them quite suitable for cleaning of oil spills. Rhamnolipids also demonstrate antibacterial and antifungal activity, which suggests a possible role in medical and agricultural domains (Singh and Cameotra, 2004). Since these biosurfactants originate from a natural source and have low toxicity levels, rhamnolipids represent an attractive alternative to synthetic compounds.

Recently, a novel biosurfactant from the bacterial strain *Pseudomonas* sp. S-17 was isolated – rhamnolipid PS-17 (Karpenko *et al.*, 1996; Shulga *et al.*, 2000). Rhamnolipid PS-17 (PS-17) represents a unique natural biocomplex with high surface and emulsifying activities (Table I). It consists of two homologous extracellular biosurfactants – glycolipids RL-1 and RL-2, which contain two rhamnose residues and residues of 1-β-hydroxydecanoic acid (two in RL-1 and one in RL-2) (Fig. 1). Previous studies showed that rhamnolipid PS-17 has a mild effect on yeast cells, concerning mainly the lipid and protein components of the cell surface. It was also demonstrated, that the biosurfactant rhamnolipid PS-17 may have a neutral or detrimental effect on the growth of Gram-positive strains which depends on the surfactant's concentration (Vasileva-Tonkova *et al.*, 2001).

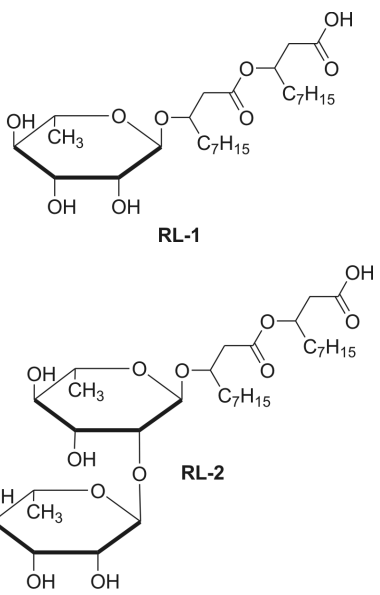

Fig. 1. Structure of rhamnolipid PS-17: RL-1 and RL-2.

Alginate (Fig. 2) is an acidic polysaccharide composed of 1,4-linked copolymers of β-D-mannuronic acid and α-L-glucoronic acid. It is produced by brown algae and bacteria belonging to the genera *Azotobacter* and *Pseudomonas* (Cote and Krull, 1988; Govan *et al.*, 1981). This polymer and its low-molecular weight polysaccharides are widely used in industry and biotechnology. Alginate-derived oligosaccharides exhibit a high cytostatic effect against solid Sarcoma 180 cells (Hu *et al.*, 2004).

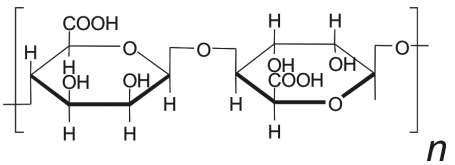

Fig. 2. Structure of alginate.

There are no available data concerning the antiviral effect of rhamnolipids, although their physicochemical activities have been studied extensively. The objective of this work was to investigate the anti-herpetic effects of rhamnolipids and their alginate complex *in vitro*.

Material and Methods

Compounds, microbial strain, virus and cell culture

The S-17 strain of *Pseudomonas* sp. was isolated from a sample of agricultural soil from the Western Ukraine. It was cultivated in mineral nutrient medium (g/l): $NaNO_3$ (4.0); $MgSO_4 \cdot 7H_2O$ (0.3); NaCl (0.3); KCl (0.3); $HOC(COONa)(CH_2COOH)$ (2.0); glycerol (30). The cultivation of the *Pseudomonas* strain was carried out in Erlenmeyer flasks (750 ml): 200 ml working volume on an orbital shaker (n = 200) during 120 h at 30 °C and pH 7.0.

Substance	CMC[a] [mg/l]	Surface tension [mN/m]	Interfusion tension (*n*-heptane) [mN/m]	Index of emulgation E_{24} (%)
Rhamnolipid PS-17	20–50	28.8	0.02	70
Rhamnolipid PS-17 + alginate complex	–	29.5	0.17	85

Table I. Physicochemical properties of rhamnolipid PS-17 and its complex with alginate produced by *Pseudomonas* sp. S-17.

[a] Critical micelle concentration.

Rhamnolipid PS-17 (Table I) and the complex of rhamnolipid PS-17 and alginate were prepared in the Department of Physical-Organic Chemistry, National Academy of Sciences of Ukraine, Lviv, Ukraine.

For isolation of the complex (rhamnolipid PS-17 + alginate) supernatants (cell-free cultural broth) were acidified to pH 3.0 with an aqueous 10% HCl solution and purified by reprecipitation. Rhamnolipid biosurfactant was obtained via extraction of the complex with Folch mixture (chloroform/methanol 2:1 v/v). After removal of the solvent, the residue was analyzed using thin layer chromatography (Karpenko *et al.*, 1996; Shulga *et al.*, 2000).

Three variants were used in the antiviral assay: (i) culture filtrate from *Pseudomonas* sp. S-17 containing 3 g/l of both rhamnolipid PS-17 and alginate; (ii) purified rhamnolipid PS-17; (iii) ramnolipid PS-17 (32 g/l) and alginate (8 g/l) complex.

Acyclovir provided from Burrough Welcome Co., Research Triangle Park, NC, was used as a reference anti-herpesvirus compound.

HSV-1 strain DA and HSV-2 strain BIA were received from Prof. S. Dundarov, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria. Both viruses were cultivated in a cell culture of Madin-Darby bovine kidney (MDBK) cells. The stock virus infectious titers were 10^7 $CCID_{50}$/ml (50% cell culture inhibitory dose) for HSV-1 and 10^6 $CCID_{50}$/ml for HSV-2.

MDBK cells were grown in DMEM containing 10% fetal bovine serum (Gibco BRL, Paisley, UK) supplemented with 10 mM HEPES buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (Merck, Darmstadt, Germany) and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin) in a CO_2 incubator (5% CO_2, HERA cell 150, Heraeus, Hanau, Germany) at 37 °C.

Cytotoxicity assay

The cytotoxicity of the test compounds was assayed in monolayer MDBK cell cultures. The compounds were diluted in PBS buffer. Their effects were traced microscopically on uninfected cell monolayers (in the stationary growth phase) in 96-well microplates (Costar, Corning, NY, USA). The cell monolayer state and cellular morphology were monitored for over signs of cytotoxicity during 48 h and the maximal tolerated concentration (MTC) value was determined.

Cytopathic effect (CPE) inhibition assay

MDBK cells were cultured in DMEM (Gibco BRL) supplemented with 2% fetal bovine serum and antibiotics in 96-well plates. The monolayers were infected with 100 $CCID_{50}$ of herpes simplex virus in a volume of 0.1 ml per well. After 60 min of virus adsorption at room temperature, the compounds were added at different concentrations (in one-half $\log_{10}$ dilutions in 0.1 ml/well). The plates were incubated at 37 °C/5% CO_2 for 48 h and the viral CPE was evaluated daily with an invert light microscope at 125× magnification. The IC_{50} values of the substances were determined as the lowest concentration reducing the virus-induced CPE by 50%.

Antiviral selectivity evaluation

The selectivity of the antiviral effect was expressed as selectivity index (SI) value, calculated by the ratio cytotoxicity on the cell monolayer (MTC) to IC_{50}.

Antiviral effect in multicycle virus growth assay

A confluent monolayer of MDBK cells in 24-well plates (Costar) was inoculated with 100 $CCID_{50}$ herpes simplex virus (0.1 ml/well). After 60 min of virus adsorption, different concentrations of the compounds were added to the cell culture and incubated at 37 °C/5% CO_2 for 48 h. Infectious virus titer was determined by the endpoint dilution method in 96-well microplate cultures of MDBK cells and expressed in $CCID_{50}$. The inhibitory effect on virus replication was ex-

pressed in Δlog $CCID_{50}$ (the difference between virus titers of the drug-treated sample and the drug-free control sample) towards the untreated control.

Results

Effects of rhamnolipid PS-17 and the rhamnolipid PS-17 + alginate complex against HSV-1 and HSV-2 in the CPE inhibition test

The *Pseudomonas* sp. S-17 culture filtrate, enriched with rhamnolipid and alginate, was tested for antiviral activity against HSV type 1 in preliminary experiments. A marked effect in the CPE inhibition test was manifested. Then the activity of this product in parallel with purified rhamnolipid PS-17 and its alginate complex was tested against HSV-1 and HSV-2.

In vitro cytotoxicity tests showed the following MTC values for MDBK monolayer cultures: 15 μg/ml for the culture filtrate containing rhamnolipid and alginate, 64 μg/ml for the purified rhamnolipid PS-17, and 640 μg/ml for the rhamnolipid + alginate complex. Obviously, rhamnolipid PS-17 had a stronger cytotoxic effect on the monolayer MDBK cultures, as compared to its alginate complex.

The test results of the anti-herpesvirus activities in MDBK cells of the three substances are summarized in Table II. Rhamnolipid PS-17 and its alginate complex exerted a marked suppressive effect on HSV-1 and HSV-2. It must be noted that rhamnolipid PS-17 inhibited herpesviruses in concentrations lower than the critical micelle concentration (20–50 mg/l). These data are in accordance with the observation of Vollenbroich *et al.* (1997) with the bacterial lipopeptide surfactin. In our experiments purified rhamnolipid PS-17 showed a similar antiviral effect against the two types of herpes simplex virus: IC_{50} values of 14.5 μg/ml and 13 μg/ml against HSV-1 and HSV-2, respectively. Very close activity vs. HSV-1 was manifested by the *Pseudomonas* sp. culture filtrates, containing rhamnolipid and alginate. The complex of rhamnolipid PS-17 + alginate exerted also a similar effect vs. HSV-1 and HSV-2: IC_{50} values of 435 μg/ml and 482 μg/ml, respectively.

Our results showed that the selectivity of rhamnolipid PS-17's anti-herpesvirus effects was significantly higher as compared to that of the rhamnolipid + alginate complex.

Effect of rhamnolipid PS-17 and the rhamnolipid PS-17 + alginate complex against HSV-1 and HSV-2 replication

As a next step, the effects of the purified rhamnolipid PS-17 and the rhamnolipid + alginate complex on the HSV-1 and HSV-2 replication in MDBK cells was studied by the multicycle virus growth experimental design. Acyclovir was used as a reference anti-herpesvirus compound. The results summarized in Tables III and IV show distinct inhibitory effects of PS-17 and its complex on HSV-1 and HSV-2 replication, expressed by reduced infectious virus yields, as measured 48 h post virus inoculation.

Rhamnolipid PS-17 manifested a similar activity against the two types of herpes simplex virus: marked inhibitory effects (Δlog $CCID_{50}$ = 1.84–2.0, see Tables III and IV) at a concentration of 20 μg/ml ($IC_{2\,log}$, SI = 3.2) and moderate ones (Δlog $CCID_{50}$ = 1.0–1.33, Tables III and IV) at concentrations of 10 and 16 μg/ml. The concentra-

Table II. Anti-HSV-1 and 2 effects of *Pseudomonas* sp. culture filtrate, purified rhamnolipid PS-17 and its complex with alginate in Madin-Darby bovine kidney cells.

Compound	IC_{50}[a] [μg/ml]		SI[b] MTC/IC_{50} [μg/ml]	
	HSV-1	HSV-2	HSV-1	HSV-2
Pseudomonas sp. culture filtrate	10.0 ± 0.14	n.d.	1.5 ± 0.38	n.d.
Rhamnolipid PS-17	14.5 ± 0.23	13.0 ± 0.17	4.41 ± 0.25	4.92 ± 0.29
Rhamnolipid PS-17 + alginate complex	435.0 ± 0.35	482.0 ± 0.29	1.47 ± 0.19	1.33 ± 0.15

[a] Half maximal inhibitory concentration, concentration reducing the viral cytopathic effect in MDBK cells by 50%.
[b] Selectivity of antiviral effect determined by calculation of selectivity index (SI) values: MTC/IC_{50}; MTC, maximal tolerated concentration.
Data are mean values of three independent determinations ± SD.

Drug	Concentration [μg/ml]	Virus titer log $CCID_{50}$/0.1 ± SD[a]	$\triangle$log[b]
Control	–	6.0 ± 0.26	–
Acyclovir	16	1.5 ± 0.41	4.5
	10	3.5 ± 0.14	2.5
	3.2	4.0 ± 0.23	2.0
Rhamnolipid PS-17	20	4.16 ± 0.19	1.84
	16	4.67 ± 0.54	1.33
	10	5.0 ± 0.28	1.0
	3.2	6.0 ± 0.50	0
	1	6.0 ± 0.22	0
Rhamnolipid PS-17 + alginate complex	500	2.58 ± 0.12	3.42
	450	3.91 ± 0.56	2.09
	400	4.58 ± 0.48	1.42
	300	5.33 ± 0.43	0.67

Table III. Effect of rhamnolipid PS-17 and its complex (rhamnolipid + alginate) on HSV-1 replication in MDBK cells (multicycle virus growth experimental setup).

[a] Data are mean values of three independent determinations ± SD.
[b] Difference between virus titers of the drug-treated sample and the drug-free control sample.

Drug	Concentration [µg/ml]	Virus titer log $CCID_{50}$/0.1 ± SD[a]	$\triangle$log[b]
Control	–	5.5 ± 0.23	–
Acyclovir	16	0	>5.5
	10	2.33 ± 0.16	3.17
	3.2	3.5 ± 0.55	2.0
Rhamnolipid PS-17	20	3.5 ± 0.19	2.0
	16	4.33 ± 0.38	1.17
	10	4.5 ± 0.56	1.0
	3.2	5 ± 0.62	0.5
Rhamnolipid PS-17 + alginate complex	500	0	>5.5
	450	0	>5.5
	400	2.5 ± 0.22	3.0
	350	3.5 ± 0.13	2.0
	300	4.33 ± 0.48	1.17
	250	4.33 ± 0.18	1.17
	200	4.5 ± 0.25	1.0
	150	4.5 ± 0.38	1.0
	100	5.33 ± 0.43	0.17

Table IV. Effect of rhamnolipid PS-17 and its complex (rhamnolipid + alginate) on HSV-2 replication in MDBK cells (multicycle virus growth experimental setup).

[a] Data are mean values of three independent determinations ± SD.
[b] Difference between virus titers of the drug-treated sample and the drug-free control sample.

tion of 10 μg/ml could be considered as a minimal inhibitory concentration ($IC_{1\ log}$, SI = 6.4). The antiviral effect of rhamnolipid PS-17 registered is significantly weaker than that of acyclovir, the $IC_{2\ log}$ value of which was 5 times lower (3.2 μg/ml). The selectivity ratio values evaluated in this test were comparable with those found by the CPE inhibition test (Table II).

The complex of rhamnolipid with alginate showed a significant inhibitory effect on herpesvirus replication. As seen in Tables III and IV, the HSV-1 and 2 virus yield was reduced according to the increased concentration of the complex. The antivirus effect was more pronounced against HSV-2 than against HSV-1. The $IC_{2\ log}$ was 350 μg/ml versus HSV-2, and 450 μg/ml against HSV-1, attaining > 5.5 log at 450–500 μg/ml against HSV-2 and only 2.09–3.42 against HSV-1. A $MIC_{1\ log}$ value of 150 μg/ml versus HSV-2 (SI = 4.27) and a value more than 2 times higher (between 300 and 400 μg/ml) against HSV-1 were observed.

Discussion

The results in this study show that rhamnolipid PS-17 and its complex with alginate demonstrate a marked antiviral activity against HSV-1 and HSV-2 replication in MDBK cells which was determined by the CPE inhibition test and by meas-

uring the infectious virus yields in a multicycle virus growth experimental setup.

As the culture filtrate of the *Pseudomonas* sp. S-17 strain, containing rhamnolipid PS-17 and alginate, was relatively toxic to a monolayer MDBK cell culture, the *in vitro* anti-HSV-1 activity could be due to a non-specific inhibitory effect of some components of the culture filtrate.

The anti-herpesvirus activities of the purified rhamnolipid PS-17 was manifested at non-cytotoxic concentrations, lower than those necessary for micelle formation (below the critical micelle concentration). Antiviral effects of rhamnolipid PS-17 on the replication of HSV-1 and HSV-2 were similar in the two tests used.

Only a few studies on the antiviral activity of biosurfactants have been carried out so far. Surfactin, a cyclic lipoprotein antibiotic, and the biosurfactant produced by *Bacillus subtilis* showed an inhibitory activity against enveloped viruses such as herpes simplex viruses (HSV-1, HSV-2), suid herpes virus (SHV-1), vesicular stomatitis virus (VZV), Semliki Forest virus (SFV) and simian immunodeficiency virus (SIV). It was shown that the antiviral action depends on a physicochemical interaction of the membrane-active surfactant and the virus lipid membrane (Vollenbroich *et al.*, 1997). An anti-HSV activity was reported for the surfactin analogue pumilacidin (Naruse *et al.*, 1990). It has recently been demonstrated that an anionic surfactant, sodium lauryl sulfate, suppresses the infectivity of HSV-1 strain F and HSV-2 strain 333 *in vitro* (Piret *et al.*, 2002).

In our study the complex of rhamnolipid PS-17 with the polysaccharide alginate manifested a marked antiviral activity in MDBK cells. Moreover, the inhibitory effect of this complex was much stronger against HSV-2 than against HSV-1.

Pancheva (1993) reported that the polysaccharide dextran sulfate inhibited the HSV attachment to the target cells. The efficacy of the combination of the polysaccharide alginate and rhamnolipid PS-17 against HSV replication could be explained with their dual action on HSV replication.

Fatty acids and fatty acid-containing molecules were found to inactivate enveloped viruses, HSV-1 included, by disintegrating the virus lipid envelope (Horowitz *et al.*, 1988; Kohn *et al.*, 1980; Thormar *et al.*, 1987). On the other hand, Hilmarsson *et al.* (2005) observed that the acidic environment makes HSV more sensitive to certain virucidal lipids (fatty alcohols and lipids), possibly by ionic changes in the envelope proteins. These data could be assigned to the anti-HSV activity of the rhamnolipid PS-17, as a result of a complex physicochemical interaction of the biosurfactant with the virus lipoprotein envelope. We can assume that the anti-HSV activity of rhamnolipid PS-17 is probably due to the influence of β-hydroxy fatty acids present in PS-17′s structure and to anionic properties of the surfactant on the lipids and/or proteins in the HSV lipoprotein envelope.

Presumably, the rhamnolipid PS-17-induced inhibition of HSV replication could be a result of the direct effect on *de novo* synthesized virions, thus preventing virus dissemination. An analogous hypothesis has been formulated by Piret *et al.* (2000) concerning the mode of action of sodium lauryl sulfate on HSV-1. While rhamnolipid could affect the structure of the lipoprotein membrane of HSV, alginate can prevent the adsorption of HSV to the host cells.

This is the first description of the antiviral activity of the rhamnolipid biosurfactant and its alginate complex. Our findings pave the way to a more extensive search for substances with analogous chemical structure and biophysical properties as antivirals.

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