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Review**Biohydrometallurgy in Bulgaria - Achievements and Perspectives**

Stoyan Groudev

*University of Mining and Geology "Saint Ivan Rilski", Sofia, Bulgaria***Abstract**

The past and current research and industrial activities in the field of biohydrometallurgy in Bulgaria cover a large number of problems: microflora of different mineral deposits (such as of ores of non-ferrous metals, uranium and gold; non-metallic mineral raw materials such as coal, oil, kaolins, quartz sands, etc.); physiology, biochemistry and genetics of the microorganisms participating in the transformations of the above-mentioned mineral substrates; bioleaching of copper and other non-ferrous metals and uranium by means of *in situ*, dump, heap and reactor techniques; pretreatment of gold and silver-bearing sulphide concentrates and ores by means of chemolithotrophic bacteria to expose these precious metals; combined microbial and chemical leaching of precious metals from oxide ores; microbial removal of iron from quartz sands and kaolins, of sulphur from coal, of silicon from low-grade bauxites, of phosphorus from iron ores; improvement of the ceramic properties of kaolins; microbial enhanced oil recovery; electricity production from organic wastes by means of microbial fuel cells.

Several of the above-mentioned activities are connected with the solution of essential environmental problems: prevention of the generation and/or treatment of toxic waters polluted by heavy metals, radionuclides, arsenic, oil, organochemicals, etc. by means of active and/or passive systems, reactive zones, rock filters, etc.; treatment of soils polluted with the above-mentioned pollutants by different reactors, heap and *in situ* techniques; bioremediation of post-mining landscapes and wastes.

Keywords: biohydrometallurgy, bioleaching, bioremediation, chemolithotrophic bacteria, mineral biotechnology

Резюме

Научните и промишлени дейности в областта на биохидрометалургията в миналото и понастоящем в България обхващат голям брой проблеми: микрофлора на различни минерални находища (на руди на цветни метали, уран и злато; неметални минерални суровини като въглища, нефт, каолини, кварцови пясъци и т.н.); физиология биохимия и генетика на микроорганизми, участващи в трансформациите на посочените минерални субстрати; биологично излугване на мед и други цветни метали и уран посредством техники, приложени *in situ*, в насипища и реактори; предварително третиране на златни и сребърни сулфидни концентрати и руди чрез хемолитотрофни бактерии за разкриване на тези благородни метали от минералните структури; комбинирано микробно и химично излугване на благородни метали от окисни руди; микробно отстраняване на желязо от кварцови пясъци и каолини, на сяра от въглища, на силиций от нискокачествени боксити, на фосфор от железни руди; подобряване на керамичните свойства на каолини; микробно повишаване на добива на нефт; получаване на електричество от органични отпадъци чрез микробни горивни клетки.

Редица от горепосочените дейности са свързани с решаването на съществени екологични проблеми: предотвратяване на генерирането и/или третиране на токсични води, замърсени от тежки метали, радионуклиди, арсен, нефт, органични химикали и т.н., посредством активни и/или пасивни системи, действащи *in situ* зони, скални филтри и т.н.; третиране на почви, замърсени с горепосочените замърсители, чрез техники, приложени в реактори, конструирани насипища и *in situ*; биоремедиация на ландшафти и отпадъци след рудодобив.

Introduction

The first studies in the field of biohydrometallurgy in Bulgaria were started in 1967 by a joint group of investigators and students from the University of Mining and Geology “Saint Ivan Rilski” - Sofia and the University of Sofia “Saint Kliment Ohridski”. In the course of time, engineers from the then-existing institute NIPRORUDA and from some industrial enterprises were involved in these activities.

The realization of efficient large-scale biotechnologies for processing different mineral raw materials and recovery of the relevant valuable components required preliminary studies on the chemical and mineral composition and structure of the raw materials, the composition of the indigenous microflora of the relevant deposits, as well as on the real participation and potential of this microflora in the processes of mineral transformations and leaching of different components. Initially, special attention was paid to the oxidation of sulphide minerals, ferrous iron and elemental sulphur by different acidophilic chemolithotrophic bacteria, the mechanisms and the optimum conditions for these processes, which were essential for the leaching of copper, other non-ferrous metals and uranium from the relevant mineral raw materials (Gaidarjiev *et al.*, 1975; Genchev *et al.*, 1978; Groudev *et al.*,

1978; Karavaiko and Groudev, 1985; Karavaiko *et al.*, 1988). The data from these studies made possible the efficient application of the relevant processes under real commercial-scale conditions.

Until now, there were five commercial-scale operations for dump bioleaching of copper from low-grade ores and mining wastes. One of them, located near to the Vlaikov Vrah copper mine, for a long period of time (1972 - 2003) was the largest operation of this type in Europe. More than forty million tons of ore with an average copper content of about 0.15 % were leached by indigenous populations of acidophilic chemolithotrophic bacteria, mainly of the species *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*. The growth and activity of these bacteria were stimulated by suitable changes in the levels of some essential environmental factors such as humidity, pH and content of air and nutrients (mainly of ammonium and phosphate ions) in the ore dumps. Initially, copper was recovered from the pregnant leach solutions by cementation with metallic iron (Fe⁰) but later solvent extraction followed by electrolysis was used for this purpose (Groudev and Genchev, 1976; Groudev *et al.*, 1978; Groudev and Groudeva, 1993; Groudeva *et al.*, 2009; 2011) (Tables 1 and 2, and Figures 1 - 5).

Table 1. Data about the recirculating solutions at the Vlaikov Vrah copper dump leaching operation

Year	pH	Cu, g/l	Fe ²⁺ , g/l	Fe ³⁺ , g/l
	Solution to dump			
1968 - 1971	Rainfall			
1972 - 1975	3.0 - 4.3	0.05 - 0.24	2.4 - 3.9	0.05 - 0.2
1976 - 1985	3.1 - 4.5	0.04 - 0.12	2.6 - 4.2	0.07 - 0.3
1986 - 1990	3.4 - 4.6	0.01 - 0.04	2.3 - 4.1	0.10 - 0.3
	Solution from dump			
1968 - 1971	2.1 - 4.4	0.3 - 0.9	0.03 - 0.2	1.6 - 2.3
1972 - 1975	1.9 - 3.0	1.0 - 3.2	0.01 - 0.2	1.7 - 3.0
1976 - 1985	2.0 - 3.4	0.5 - 1.5	0.01 - 0.3	1.8 - 3.2
1986 - 1990	2.6 - 3.5	0.2 - 0.4	0.01 - 0.3	1.4 - 2.8

Table 2. Microorganisms in the dump effluents in industrial copper dump leaching operations in Bulgaria

Microorganisms, cells/ml	Bioleaching operations			
	Vlaikov Vrah	Tzar Asen	Assarel	Medet
<i>Thiobacillus ferrooxidans</i>	10^2 - 10^8	10^1 - 10^6	10^2 - 10^7	10^2 - 10^6
<i>Thiobacillus thiooxidans</i>	10 - 10^5	1 - 10^3	1 - 10^4	1 - 10^4
<i>Leptospirillum ferrooxidans</i>	10^1 - 10^5	10^1 - 10^4	1 - 10^3	1 - 10^2
Bacteria capable of oxidizing S ⁰ at neutral pH (<i>T. thioparus</i> , <i>T. neapolitanus</i> , <i>T. denitrificans</i>)	0 - 10^3	10^2	0 - 10^3	0 - 10^3
Moderately thermophilic chemolithotrophic bacteria (<i>Thiobacillus caldus</i> , <i>Sulfobacillus thermosulphidooxidans</i>)	0 - 10^3	0	0 - 10^1	0 - 10^1
Extremely thermophilic chemolithotrophic archaea (<i>Sulfolobus</i> , <i>Acidianus</i>)	0 - 10^2	0	0	0
Saprophytic bacteria (<i>Acidiphilium</i> , <i>Pseudomonas</i> , <i>Bacillus</i> , <i>Aerobacter</i> , <i>Caulobacter</i> , etc.)	1 - 10^3	10^1 - 10^3	1 - 10^4	10^1 - 10^3
Fungi and yeasts (<i>Cladosporium</i> , <i>Penicillium</i> , <i>Trichosporon</i> , <i>Rhodotorula</i> , etc.)	0 - 10^3	1 - 10^3	0 - 10^3	1 - 10^3
Algae	0 - 10^2	0 - 10^1	0 - 10^1	0 - 10^1
Protozoa	0 - 10^2	0 - 10^1	0 - 10^1	0 - 10^1

Note: The genus *Thiobacillus* for the acidophilic bacteria was later transformed in the genus *Acidithiobacillus*.

**Fig. 1.** A general view of the operation for copper dump bioleaching at Vlaikov Vrah mine



Fig. 2 - 3. A general view of the operation for copper dump bioleaching at Vlaikovo Vrah mine

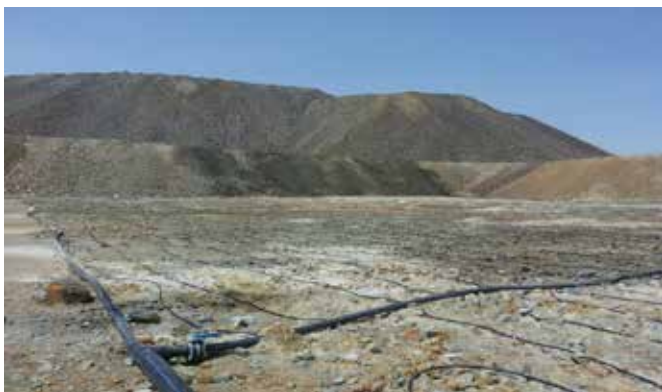


Fig. 4 - 5. The bioleaching of dumps of low-grade copper ores near Radovich, Macedonia

Three commercial-scale operations for in situ underground bioleaching of uranium existed until 1991 (Fig. 6 and 7). The largest of these operations was put into action in 1984 near the town of Simitli. Leach solutions containing sulphuric acid, ferric ions and chemolithotrophic Fe^{2+} -oxidizing bacteria (*Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*) were grown in aerated bioreactors by the well-known BACFOX (BACterial Film OXidation) process, in which biofilms of the above-mentioned bacteria formed on inert plastic surfaces together with a matrix of jarosites oxidized ferrous ions to the ferric state in diluted sulphuric acid (at pH of about 1.7 - 1.9). The leach solutions prepared in this way were injected through a large number of boreholes to a depth greater than 100 meters below the earth surface to reach the uranium-bearing ore layers. The sulphuric acid dissolved the hexavalent uranium present in the ore to the soluble uranyl sulphate, and bacteria and ferric ions oxidized the tetravalent uranium in the ore to the hexavalent state, which dissolved in the sulphuric acid. The pregnant leach solutions containing the dissolved uranium, the ferrous ions generated by the reduction of the ferric ions at the oxidation of uranium, as well as the viable bacteria, were recovered by a set of production boreholes and directed to the surface for uranium extraction by ion exchange resins. After the removal of uranium, the leach solutions were regenerated in the BACFOX reactors and were recycled to the uranium ores. The uranium retained by the ion exchange resins was solubilized and then recovered as a primary uranium-bearing concentrate (the so called “yellow cake”).

Several pilot-scale operations for bacterial pretreatment of gold-bearing sulphide ores to liberate the gold (and silver) encapsulated in the sulphide matrix were constructed and tested by the heap leaching technique using mixed cultures of acidophilic chemolithotrophic bacteria (Figure 8). The amount of ores tested in the relevant heaps largely varied (from 8 to about 4600 tons). The treatment was carried out until the sulphide oxidation reached the extent at which almost the whole quantity of gold was exposed and liberated from the sulphides. The ores pretreated in this way were washed by water to remove the residual acidity and then were leached by means of thiosulphate at a slightly alkaline pH (usually within the range of 8 - 9) to solubilize gold and silver. A real commercial-scale operation of this type was constructed and efficiently used at the Elshitzza mine in the period 1993-96. Some oxide ores containing main-



Fig. 6 - 7. *In situ* and heap bioleaching of uranium ores in Curilo deposit

ly well exposed free gold were subjected directly to leaching by thiosulphate (Groudev, 1987; 1999; Groudev *et al.*, 1999; Spasova *et al.*, 2015).

It must be noted, however, that much higher extractions of gold (in some cases even about 95%) were achieved by microbial pretreatment and subsequent leaching (by thiosulphate or cyanide) of concentrates obtained by dressing (usually by flotation) of the relevant gold-bearing ores. The microbial pretreatment was carried out by continuous leaching in systems consisting usually of three or four aerated and stirred bioreactors with acidified leach solutions containing nutrients, chemolithotropic microorganisms and concentrate at initial pulp densities of 10 - 20 %. Different microbial cultures at different temperatures were tested to establish the



Fig. 8. Pilot-scale operation for microbial pretreatment and leaching of gold-bearing sulphide ores

optimum pretreatment conditions: mixed mesophilic cultures, containing mainly *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*, at 30 - 37 °C; mixed cultures of moderately thermophilic bacteria, mainly of *Sulfobacillus spp.* and *Acidithiobacillus caldus* at 45 - 55 °C; mixed cultures of extremely thermophilic archaea, containing mainly species of the genera *Sulfolobus*, *Acidianus* and *Metallosphaera*, at 68 - 80 °C (Spasova *et al.*, 2015) (Tables 3 and 4).

It is possible to say that samples from practically all Bulgarian gold deposits (more than 10 in numbers) were tested by the above-mentioned methods for gold (and silver) extraction.

Pilot-scale operations were also constructed and efficiently used for removal of iron impurities from quartz sands, kaolins and clays by leaching in stirred reactors with culture solutions containing oxalic and citric acids produced by *Aspergillus niger* grown on a nutrient medium with sucrose as a source of carbon and energy; for production of high-quality kaolins by treatment with "silicate" bacteria of the species *Bacillus mucilaginosus* and

Bacillus circulans, (Table 5) and the operation for microbial enhanced oil recovery in Tulenovo, located near the Black Sea coast (Groudev *et al.*, 1985, 1989; Groudev, 1987, 1999; Karavaiko *et al.*, 1988).

Several activities in the field of biohydro-metallurgy were connected with relevant environmental problems: prevention of the generation of toxic acid drainage; treatment of waters polluted by heavy metals, radionuclides, arsenic, oil, organochemicals, etc. by means of active and/or passive systems (Table 6 and Figures 9 and 10); treatment of soils polluted with the above-mentioned pollutants by different reactor, heap and in situ techniques (Tables 7 - 9); bioremediation of post-mining landscapes (Georgiev *et al.*, 2013; 2014; Groudev *et al.*, 2005, 2010; Nicolova *et al.*, 2014) and recovery of valuable components from technological wastes (Spasova *et al.*, 2007).

The largest operations of this type were constructed and used in the uranium deposit Kurilo located within a relatively short distance of Sofia (Table 8). Uranium was recovered for a long period of time from this deposit and after the end of the recovery acid drainage waters containing radionuclides, heavy metals (copper, zinc, cadmium, lead, nickel, cobalt, iron, manganese), arsenic and sulphates were generated in concentrations usually much higher than the relevant permissible levels for waters intended for use in agriculture and/or industry. Different passive systems such as natural and constructed wetlands (Fig. 9), alkalizing limestone drains, permeable reactive multibarriers (Table 6 and Fig. 10), reactive in situ zones, rock filters, were used separately or in different combinations to treat the polluted waters. The most efficient in this respect was a multicomponent system consisting of a permeable multibarrier (with an alkalizing lime-

Table 3. Effect of the prior bacterial oxidation of sulphides on the extraction of precious metals during the subsequent leaching

Content of sulphidic sulphur, g	Degree of sulphide oxidation, %	Extraction of precious metals, %	
		Au	Ag
42.8	0	23.0	19.4
38.5	10	41.0	32.5
33.8	21	62.2	50.3
25.3	41	81.1	70.7
20.1	53	90.5	79.0
16.3	62	93.2	83.3
12.4	71	94.1	85.1

Table 4. Data about the continuous - flow microbial leaching of a gold - bearing pyrite concentrate

Parameters	Values
Pulp density, %	10
Residence time, h	768
Dilution rate, h ⁻¹	0.00595
Sulphidic sulphur oxidation, %	62
Sulphidic sulphur oxidation rate, g/l	0.1577
Sulphate ions generated during the leaching, g/l	79.5
Sulphate generation rate, mg/l.h	473.21
Iron content in the effluent, g/l	23.90
Iron extraction rate, mg/l.h	142.26
Solid residue after leaching, g	62.8
Weight losses, %	37.2

Table 5. Effect of the way of microbial treatment on the ceramic properties of kaolin

Parameters	Before treatment	After treatment	
		By contact in humid medium	By leaching with stirring
Al ₂ O ₃ , %	32.7	32.3	25.0
SiO ₂ , %	51.2	50.7	40.1
Fe ₂ O ₃ , %	1.16	0.82	0.78
TiO ₂ , %	0.24	0.23	0.15
CaO, %	0.82	0.77	0.24
Loss on drying, %	0.35	0.91	1.25
Bending strength, MPa	1.4	3.5	2.1
Plasticity, %	32.3	36.9	33.6
Drying shrinkage, %	3.5	4.2	3.9
Firing shrinkage at 960 °C, %	4.2	4.6	3.9
Water saturation capacity at 960 °C, %	28.0	30.2	27.5
Formation water, %	32.1	34.1	32.5
Whiteness on drying, %	76.5	82.0	82.8
Size fractions, % :			
>60 µm	0.02	0.02	0.02
10-60 µm	1.25	1.14	1.07
5-10 µm	14.1	11.32	14.01
1-5 µm	33.95	31.12	33.7
<1 µm	50.68	56.4	51.2

stone drain and an anoxic section for microbial sulphate reduction, biosorption and additional chemical neutralization) and a constructed wetland. Efficient removal of pollutants was achieved by means of this system during a period of about ten years and during the different climatic seasons, even during the cold winter months at water and ambient temperatures close to 0 °C (Groudev *et al.*, 2008).

A very efficient operation for cleaning acid-leached cinnamonic forest soil heavily contaminated with radionuclides (mainly uranium and radium) and non-ferrous metals (mainly copper, zinc and cadmium) was constructed and tested in situ under real field conditions using the activity of the indigenous soil microflora. This activity was enhanced by suitable changes of some essential environmental factors such as pH and water, oxygen and nutrient contents of the soil (Figure 11). The treatment was connected with solubilization and removal of contaminants from the top soil layer (horizon A) due to the joint action of the soil mi-

croorganisms (mainly of the acidophilic chemolithotrophic bacteria) and leach solutions (diluted sulphuric acid) used to irrigate the soil. The dissolved contaminants were transferred through the drainage soil effluents to the deeply located soil subhorizon B₂ where they precipitated as the relevant insoluble forms (uranium as uraninite, and the non-ferrous metals as the relevant sulphides) under the action of the sulphate-reducing bacteria inhabiting that soil subhorizon. The effluents from the subhorizon B₂ containing much lower concentrations of inorganic contaminants, but enriched in dissolved biodegradable organics, were subjected to additional treatment in a constructed wetland for removing these residual contaminants. However, it was demonstrated that an efficient treatment of the effluents from the subhorizon B₂ could be achieved by a microbial fuel cell in which the water clean-up was connected with electricity generation (Groudev *et al.*, 2010; 2014) (Table 10).

Some other essential problems in the field of

Table 6. Data about the acid mine drainages in the uranium deposit Curilo before and after their treatment by means of the permeable reactive barrier

Parameters	Acid mine drainage	Multibarrier effluents	Permissible levels
Temperature, °C	(+1.2) - (+25.1)	(+1.4) - (+27.5)	-
pH	2.42 - 4.25	6.22 - 7.83	6 - 9
Eh,mV	(+290) - (+597)	(-140) - (-280)	-
Dissolved O ₂ , mg/l	1.7 - 6.0	0.2 - 0.4	2
TDS, mg/l	930 - 2972	545 - 1827	1500
Solids, mg/l	41 - 159	32 - 104	100
DOC, mg/l	0.5 - 2.1	51 - 159	20
SO ₄ ²⁻ , mg/l	532 - 2057	275 - 1225	400
U, mg/l	0.10 - 2.75	< 0.05	0.6
Ra, Bq/l	0.05 - 0.5	< 0.03	0.15
Cu, mg/l	0.79 - 5.04	< 0.2	0.5
Zn, mg/l	0.59 - 59.8	< 0.2	10
Cd, mg/l	< 0.01 - 0.1	< 0.004	0.02
Pb, mg/l	0.08 - 0.55	< 0.02	0.2
Ni, mg/l	0.17 - 1.49	< 0.03 - 0.1	0.5
Co, mg/l	0.12 - 1.22	< 0.03 - 0.1	0.5
Fe, mg/l	37 - 671	0.5 - 9.5	5
Mn, mg/l	2.8 - 79.4	0.5 - 5.2	0.8
As, mg/l	0.15 - 0.59	< 0.01	0.2

Table 7. Bioremediation in situ of contaminated soils (horizon A) in uranium deposits in Bulgaria

Parameters	U	Ra	Cu	Zn	Pb	Cd
	Contents of contaminants, ppm					
<i>Vromos Bay deposit</i>						
Before treatment pH - 7.5	41	280	611	251	268	7.3
After treatment pH - 7.3	7.1	55	242	99	109	1.7
Permissible levels (pH>7.0)	10	60	280	370	80	3.0
<i>Kurilo deposit</i>						
Before treatment pH - 4.40	68	510	190	215	122	4.5
After treatment pH - 3.21	8.0	65	35	48	55	0.4
Permissible levels (pH<4.3)	10	65	20	30	60	0.5

**Fig. 9.** Treatment of acid mine drainage by constructed wetland in the uranium deposit Kurilo

biohydrometallurgy which are currently developed in Bulgaria can be also mentioned:

- Test systems of the “push-pull” and “push-through” types for studying the possibilities for efficient *in situ* underground bacterial leaching of non-ferrous metals and uranium from ores were developed. Such systems were prepared for application in the deposit located near Radovich, Macedonia, in connection with the possible future copper bioleaching operation, which will be the largest operation of this type in Europe. These test systems can be also applied in other ore deposits intended

**Fig. 10.** Treatment of acid mine drainage by means of permeable reactive multibarrier in the Kurilo deposit

for underground *in situ* bioleaching.

- Characterization of the processes of dump and heap bioleaching of copper in the commercial-scale operation near Radovich, Macedonia, the largest operation of its type in Europe, was carried out with recommendations for stimulation of the *in situ* bacterial activity and improvement of the processing of the copper-bearing pregnant solutions.

- Systems connecting the bioremediation of polluted waters and soils with electricity or hydrogen generation were developed on the basis of specific microbial fuel (or electrolytic) cells. Elec-

Table 8. Toxicity of the soils in uranium deposits before and after the treatment and remediation

Test - organisms	Toxicity		
	Before treatment (pH 7.52)		After treatment and remediation (pH 7.81)
<i>Soil in the Vromos Bay area</i>			
<i>Bacillus cereus</i>	40		NOEC at 100
<i>Pseudomas putida</i>	40		100
<i>Lactuca sativa</i>	50		NOEC at 100
<i>Trifolium repens</i>	50		NOEC at 100
<i>Avena sativa</i>	30		90
<i>Lumbricus terrestris</i>	30		90
<i>Soil in the Kurilo deposit</i>			
	pH 4.4	pH 3.2	pH 4.4 (by lime)
<i>Bacillus cereus</i>	40	40	80
<i>Pseudomas putida</i>	30	40	100
<i>Lactuca sativa</i>	40	40	NOEC at 100
<i>Trifolium repens</i>	40	50	NOEC at 100
<i>Avena sativa</i>	30	40	90
<i>Lumbricus terrestris</i>	20	10	60

Notes: The toxicity was expressed as the lowest observed effect concentration (LOEC) at different contents (in wt. %) of contaminated soil in a mixture with clean soil at the relevant type: NOEC - Not observed effect concentration.

Table 9. Bioremediation in situ of soil in the Tulenovo oil deposit polluted by oil and toxic heavy metals

Pollutants	Content in the soil		Pollutant removal, %
	Before treatment	After treatment	
Hydrocarbons, g/kg dry soil			
Parafins	3.8	0.01	99.7
Naphthens	10.6	0.08	99.3
Aromatics + polars	3.6	1.61	55.3
Total hydrocarbons 18.0	18.0	1.7	90.6
Heavy metals and arsenic, mg/kg dry soil			
Lead	185	64.0	65.4
Cadmium	5.1	2.3	54.9
Copper	262	140.0	46.6
Zinc	212	95.0	55.2
Arsenic	32	18.0	43.7

Table 10. Data about the electricity generation from wastewater by means of microbial fuel cell (MFC)

Parameters	Values
Influents in the MFC	
Chemical oxygen demand, mg/l.day	2500 - 3000
pH	6.8 - 7.1
Eh	(-210) - (-230)
Temperature, °C	32
COD used in the MFC, mg/l.day	2300 - 2700
% from the initial	90 - 92
Current density, mA/cm ²	0.08 - 0.18
Voltage of the open circuit, mV	86 - 170
External resistance	20 - 80
Power, mW/m ²	770 - 980



Fig. 11. *In situ* treatment of soil polluted with heavy metals

trochemically active bacteria able to transfer electrons directly to the anode electrode were selected and used in some of these systems (Groudev *et al.*, 2010; 2014).

- Experimental leaching of certain precious and rare elements (such as the platinum group of metals, gold, silver, rhenium) from different ores, mineral processing wastes, and pyrometallurgy, by combined chemical and biological treatments using different heterotrophic and chemolithotrophic microorganisms.

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Review**Enteroviruses and Perspectives for Etiotropic Therapy of Enteroviral Infections**

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Abstract

Millions of people, mostly children, are infected annually by enteroviruses with a wide variety of clinical manifestations. Most enteroviral infections are inapparent (Modlin *et al.*, 2001), i.e. these viruses circulate in the human population without being “spotted”. In a number of cases they cause clinically manifested illnesses of varying severity, including diseases affecting the central nervous system, heart, endocrine pancreas, etc. The high morbidity, the mortality rates in children and the high-risk groups (immunocompromised patients and newborns), as well as the lack of effective vaccines (excluding polioviruses) determine chemotherapy as the primary means of control of enteroviral infections.

Up to now, no effective chemotherapeutic agents for specific treatment of such infections have been registered. A multitude of compounds have been tested, but only few of them are active in vivo. The substances that have reached clinical trial stages have adverse side effects, low selectivity, high toxicity or unfavorable pharmacokinetic properties, and are, therefore, not suitable for use in clinical practice.

The main reason for the failure of the anti-enteroviral therapy is that enteroviruses become drug-resistant and even drug-dependent. This is due to the extremely high levels of mutation and recombination in these viruses, leading to heterogeneous mutant populations. One way to combat resistance is to develop approaches for combined application of new or already existing substances with different mechanisms of action. Our team was the first to introduce analysis of the combined effects of selective inhibitors of enterovirus replication with different mechanisms of action (Nikolaeva and Galabov, 1999, 2000, 2011). Furthermore, the process of development of resistance to the most effective enterovirus inhibitors - the WIN compounds - was studied. A panel of phenotype markers was also presented for characterization of drug-dependent mutants (Nikolova and Galabov, 2003; Nikolova *et al.*, 2011). In addition, a new highly effective approach has been developed for combined administration of anti-enteroviral substances for chemotherapy of coxsackievirus neuroinfection in newborn mice, which could serve as the basis for new therapeutic strategies. It consists in consecutive, alternating, not concomitant, application of the compounds in the combination (consecutive alternating administration treatment course, CAA course). Double, triple and quadruple regimens with some of the best studied anti-enteroviral inhibitors were tested. The highest activity was manifested by the triple combinations in consecutive administration of inhibitors with different mechanisms of action, applied in a strict order (Vassileva-Pencheva and Galabov, 2010; Stoyanova *et al.*, 2015).

Key words: enterovirus, classification, replication, antivirals, drug-resistance

Резюме

Ентеровирусите инфектират милиони хора всяка година и имат разнообразни клинични прояви, като засягат най-вече децата. По-голямата част от ентеровирусните инфекции протичат инапаратно (Modlin *et al.*, 2001), т.е. тези вируси циркулират в човешката популация без да бъдат откривани. В редица случаи причиняват клинично изяви заболявания с различна тежест, включително поразяващи ЦНС, сърцето, ендокринния панкреас и други. Високата заболяемост и смъртността при децата и високо-рисковите групи (имунодефицитни пациенти и новородени), както и липсата на ефективни ваксини (изкл. полиовирусите) определят химиотерапията като основно средство за контрол на ентеровирусните инфекции.

До този момент няма регистрирани ефективни химиотерапевтици за специфично лечение на тези инфекции. Изпитани са множество съединения, но само малка част от тях са активни *in vivo*. Тези от веществата, достигнали клинични изпитания показват нежелани странични ефекти, ниска избиращелност, висока токсичност или неблагоприятна фармакокинетика и поради това не могат да се използват в клиничната практика.

Главната причина за неуспеха на анти-ентеровирусната терапия е развитието на лекарствена резистентност и дори лекарствена зависимост от ентеровирусите. Това се дължи на изключително високите нива на мутации и рекомбинации при тези вируси, водещи до хетерогенни мутантни популации. Един от начините за борба с резистентността е разработването на подходи за комбинирано прилагане на нови или вече известни вещества с различен механизъм на действие. Нашият екип за първи път въведе изследвания върху комбинираните ефекти на селективни инхибитори на ентеровирусната репликация с различен механизъм на действие (Nikolaeva and Galabov, 1999, 2000, 2011). Наред с това бе изследван процесът на развитие на резистентност към най-ефективните инхибитори на ентеровирусите - WIN съединенията. Представен е и панел от фенотипни маркери с цел характеризиране на лекарствените мутанти (Nikolova and Galabov, 2003; Nikolova *et al.*, 2011). Също така е разработен нов високоефективен подход за комбинирано прилагане на анти-ентеровирусни вещества за химиотерапия на коксакивирусна невроинфекция в новородени мишки, който може да послужи като основа за нови терапевтични стратегии. Той се състои в последователно, редуващо се, а не едновременно, прилагане на веществата в комбинацията (consecutive alternating administration treatment course, САА course). По тази схема на прилагане бяха изпитани двойни, тройни и четворни комбинации с едни от най-добре проучените анти-ентеровирусни инхибитори. Най-висока активност показаха тройните комбинации при последователно прилагане на инхибитори с различен механизъм на действие подавани в точно определен ред (Vassileva-Pencheva and Galabov, 2010; Stoyanova *et al.*, 2015).

Importance of enteroviruses for human pathology

Human enteroviruses are ubiquitous and can be isolated from sewage, water bodies, as well as from contaminated food. They are primarily transmitted via the fecal-oral route, airborne droplet (respiratory) route, objects and belongings in the patient's immediate environment, contaminated hands, the source of infection being the sick person or the carrier. Enteroviruses normally replicate in the mucous membrane of the gastrointestinal tract or the upper respiratory tract (rhinovirus), wherein the infection may be subclinical or manifest itself as a mild illness. Virus particles are excreted in the feces or saliva even before symptoms appear. The incubation period is 3-10 days, during which time the viruses migrate to the regional lymphoid tissue causing a mild viraemia. They subsequently spread to target organs where they replicate and in some of the cases cause illnesses of various severity. During their replication in the gastrointestinal tract, enteroviruses are subject to high mutation rates, which can lead to long-term excretion and neurovirulence. After a viral illness, long-lasting type-specific immunity develops. Children who have gone through an inapparent form of infection acquire such immunity but remain virus carriers.

Enteroviral infections affect millions of in-

dividuals annually, with a high level of hospitalization even in the developed countries (Pallansch, 2011), even though the majority of infections are asymptomatic (Morens and Pallansch, 1995; Pallansch and Roos, 2006; Strauss and Strauss, 2008). They are characterized by high contagiousness, diverse clinical symptoms, and are extremely stable in the environment, often causing outbreaks of nosocomial infections that are particularly severe in neonatal units and nurseries. The main clinical manifestations of the enteroviral infection are fever with or without rashes, inflammation of the upper respiratory tract and nasopharynx (rhinitis, sinusitis, summer flu, acute lymphonodular pharyngitis), rarely gastrointestinal disorders. Rhinoviruses, which are highly similar to enteroviruses, have been shown to be the causative agents of the common cold that contributes to the development of chronic pulmonary diseases (Tan, 2005; Mallia *et al.*, 2007). Representatives of the genus can also cause: meningitis, encephalitis, neonatal sepsis, myocarditis, dilated cardiomyopathy, pericarditis, pleurodynia (Bornholm disease), polio-like illnesses, acute flaccid paralysis, pancreatitis, with subsequent complications or chronic course, such as insulin-dependent diabetes mellitus type I (Hyoty and Taylor, 2002; Galabov and Angelova, 2006), hand-foot-and-mouth disease (HFMD), nonspecific my-

algias and severe multi-organ disease in newborns (Morens and Pallansch, 1995), herpangina, acute haemorrhagic conjunctivitis, uveitis, pneumonia, hepatitis, chronic fatigue syndrome, etc.

Different enteroviral serotypes can present with different symptoms and vice versa, identical clinical manifestations can be caused by different enteroviral serotypes (Strikes *et al.*, 1986). Enteroviral infections occur seasonally during the summer - with peaks from early to late summer (Khesuriarni *et al.*, 2006). Enteroviral infections are rarely fatal, but there is a high risk of complications in newborns and immunocompromised individuals. Persons of all ages can become ill, especially vulnerable children. An epidemiological peculiarity of enteroviruses is their high contagiousness. Part of the affected individuals develop mild non-specific symptoms and syndromes. Clinically ill patients constitute a very small part of the individuals actually infected with enteroviruses. It is therefore considered that both persons with clinical manifestations and those with asymptomatic infection should be treated with effective chemotherapeutic agents (Galabov and Angelova, 2006).

As there is no specific therapy for these infections, the treatment is often symptomatic. In some cases of severe enterovirus infections, immunoglobulins and pleconaril have been used.

Classification and structure of enteroviruses

Enteroviruses belong to the family *Picornaviridae*, which comprises 29 genera of viruses that infect a wide range of vertebrate hosts. The genus *Enterovirus* is subdivided into 12 species on the basis of differences among hosts and the pathogenic potential. Each subtype contains a different number of unique serotypes, which differ in their ability to be neutralized by specific antisera. Hu-

man enteroviruses include the three types of poliovirus, coxsackie A and B viruses, rhinoviruses, ECHO viruses and enteroviruses types 68-71.

Enteroviruses are some of the most extensively studied viruses. They are small particles (27-30 nm in diameter) with a relatively simple structure. The virions consist of a capsid without envelope, enclosing positive-sense single-stranded RNA. The capsid has icosahedral symmetric and is composed of 60 capsomers, each of which consists of one copy of the four viral proteins: VP1, VP2, VP3 and VP4 (Hogle *et al.*, 1985).

Proteins VP1, VP2 and VP3 constitute the outer surface of the capsid, whose thickness is about 5 nm (Smyth and Martin, 2002). Protein VP4 lies below them and is part of the inner surface of the capsid. The differences in amino acid sequences of VP1, VP2 and VP3 confer certain morphology and antigenicity to each picornavirus.

The icosahedral structure of virions is associated with the minimum energy and maximum stability of the capsid. An icosahedron is composed of 20 equilateral triangles and 12 vertices. VP1 protein surrounds each of the 12 fivefold-symmetry axes, whereas VP2 and VP3 proteins surround each three-fold axis. Viral uncoating during replication is accomplished by destabilization of VP4.

The virion surface is not smooth, but has star-shaped protrusions, or "plateaus", located around the five-fold axes of symmetry and surrounded by deep valleys, or "canyons", which are protrusions around the three-fold axes forming the walls of the icosahedron (Racaniello, 2001).

The canyon is a receptor-binding site, where hydrophobic amino acid residues are located (Hogle *et al.*, 1985; Hendry *et al.*, 1999). It surrounds each of the vertices of the icosahedral structure and is located between VP1 on the one side, and

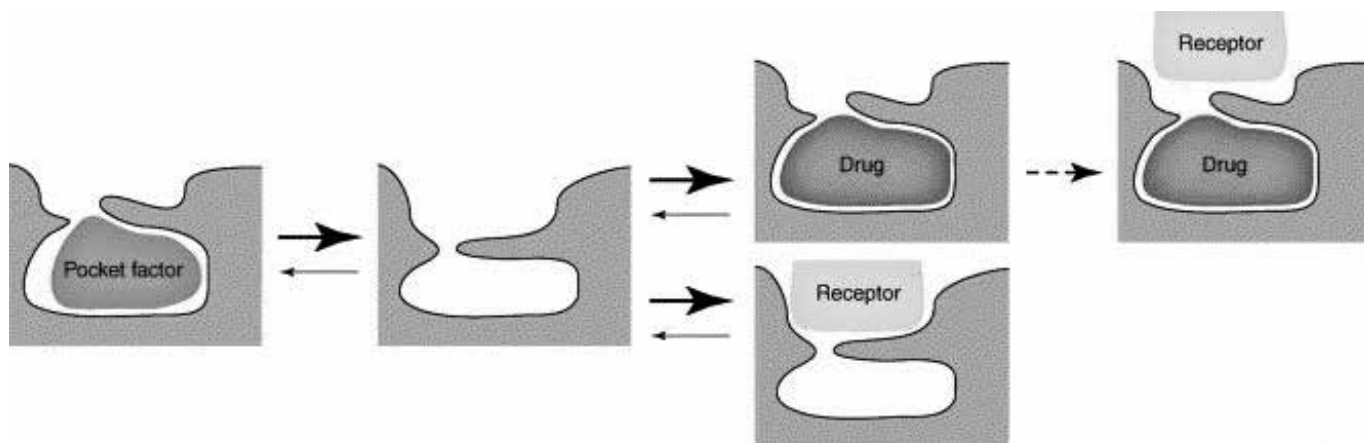


Fig. 1. Diagrammatic representation of the competition between binding of pocket factor or drug into the VP1 binding pocket and receptor into the canyon. (Rossmann *et al.*, 2002)

VP2 and VP3 on the other, and its depth is 25Å. Mutations in the amino acid sequence of the proteins that make up the canyon in the poliovirus and rhinovirus may cause a change in the interaction with the cellular receptors. Enteroviruses possess a so-called “hydrophobic pocket” under the base of the canyon (Fig. 1). It is occupied by a higher fatty acid, or so-called pocket factor. It is suggested that the pocket factor regulates the stability of the capsid in the transmission of the virus between hosts. The virus is released from the pocket factor upon binding to its receptor molecule (Rossman and Oliveira, 1993), triggering the uncoating process (Filman *et al.*, 1989; Rossmann, 1994). Certain antiviral agents such as the WIN compounds displace the pocket factor and bind tightly to the hydrophobic pocket, thereby stabilizing the capsid and inhibiting the binding of the virus to the receptor and the subsequent uncoating. According to the so-called “canyon hypothesis”, this depression is a strategy to evade immune response. The position of the receptor-binding site in the canyon hampers the recognition of the virus by the antibodies of the host, while binding to the cellular receptor remains possible, because it is narrower than the antibod-

ies (Rossmann *et al.*, 1985; Rossmann и Johnson, 1989; Reisdorph *et al.*, 2003).

Organization of enterovirus genome

The enteroviral genome is monopartite, linear ssRNA(+) of 7.2-8.5 kb in length. Viral genomic RNA has a viral protein (VPg) at its 5' end instead of a methylated nucleotide cap structure. A single reading frame begins several hundred nucleotides from the 5' end and terminates several dozen nucleotides from the 3' end, just upstream from a poly-A tail. The long UTR at the 5' end contains a type I internal ribosome entry site (IRES). The untranslated sequences at both the 5' and 3' ends are involved in viral regulatory activities such as translation and replication. A single polypeptide is translated from the open reading frame (ORF). The P1 region encodes the structural polypeptides. The P2 and P3 regions encode the nonstructural proteins associated with replication. Post-translational modification is accomplished by virus-encoded proteases and results in the generation of the four capsid proteins, as well as the enzymes necessary for replication and translation (Hellen and Wimmer, 1995). Within hours of infecting a host cell, all host cell function

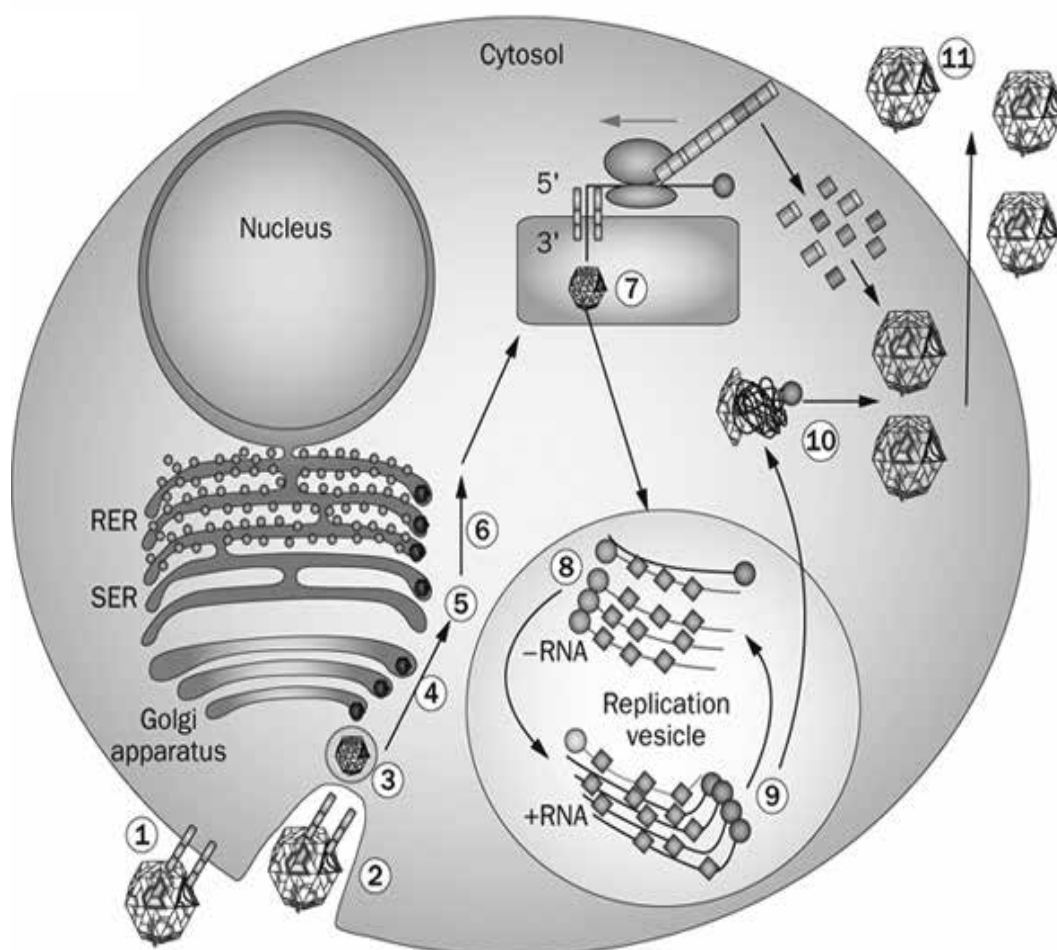


Fig. 2. Enterovirus replication (Hober and Sauter, 2010)

is aborted and the cell becomes a factory for viral replication. Cell death is by lysis, with release of progeny virus into the immediate environment of the cell and the resumption of the viral infection cycle with attachment of new virus particles to neighboring cells (Rotbart, 2000).

Enterovirus replication

The replication cycle of the enteroviruses occurs entirely in the cytoplasm (Fig. 2). The viral particle binds to a specific receptor (1) and triggers its own endocytosis (2). The particle is transported by endocytic vesicles (3) to the Golgi apparatus (4), and then through the smooth endoplasmic reticulum (SER) (5) and rough endoplasmic reticulum (RER) (6). Uncoating occurs and viral RNA is translated at the surface of the RER into a viral polyprotein autocleaved into structural and nonstructural proteins (7). In replication vesicles, positively and negatively stranded viral RNAs are produced with the help of nonstructural proteins (8, 9). Viral RNA encapsidates with structural proteins (10) and new viral particles are extruded (11). A replicative cycle of enteroviruses occurs between 5 and 10 hours depending on the virus, the temperature, pH, multiplicity of infection, and the host cell (Hober and Sauter, 2010).

Inhibitors of enterovirus replication

The combat against enteroviruses follows two main directions. The first them is the development and implementation of effective vaccines. The second direction is the discovery and development of chemotherapeutic agents that selectively inhibit viral replication.

The historical Salk and Sabin vaccines against polio are at present the only ones applied in clinical practice. In 2014, China completed Phase III clinical trials of inactivated EV71 whole-virus vaccines. The results of these tests reveal a high level of protection and good immunogenicity of EV71 vaccine. The protective effect exceeds 90% in EV71-associated HFMD, and over 80% in other EV71-associated diseases, but has no effect on infections caused by other enteroviruses (Li *et al.*, 2014; Liang and Wang, 2014). Phase IV clinical trials and registration of the vaccine for clinical use are forthcoming. Despite the breakthrough in the development of a vaccine for EV71, there is still lack of effective vaccines and medications for other non-polio enteroviruses. This requires the search for specific anti-enterovirus chemotherapeutics.

A major obstacle is the extremely high genetic variability of these viruses, due to the high error

rate of the viral RNA polymerase and the absence of a repair mechanism. This feature of the RNA viruses underlies the rapid development of drug resistance. Another obstacle to the chemotherapy of enteroviral infections is the high recombination frequency, which also favours the acquisition of drug resistance.

Several groups of anti-enterovirus inhibitors have been described to date. They show good antiviral effects *in vitro*, but unfortunately most fail to manifest a promising effect *in vivo*. This is due to the extremely rapid development of resistance. Nevertheless, a few chemotherapeutic agents have reached clinical trials, while others, though not so promising from a clinical point of view, have contributed to a more detailed understanding of enterovirus replication. Current efforts are directed towards finding highly selective chemotherapeutics and shortening the time of their impact, the aim being to avoid the selection of resistant mutants.

Modern antiviral chemotherapy is based on the specific interaction of test substances with viral targets, involved in certain stages of viral replication. There are several steps in the replication cycle of the enteroviruses that are potential targets in antiviral therapy (Rotbart, 2002). Cell susceptibility, viral attachment, viral uncoating, viral RNA replication, viral protein synthesis and host cell factors have all been studied as targets of anti-enteroviral compounds (Table 1).

Inhibitors of attachment, entry, and uncoating

Receptor binding, uncoating and release of the viral RNA into the host cell are the primary steps in the viral life cycle. Hence, antiviral drugs that interfere with any of these processes block the subsequent steps and thus inhibit viral replication. The most extensively studied class of molecules targeting the early events of attachment, entry, and uncoating, are the so-called “WIN compounds” (De Palma *et al.*, 2008).

Inhibitors of virus-specific RNA synthesis

The replication of enteroviral RNA occurs within the replication complex, which is associated with the membrane of virus-induced vesicles in the cytoplasm of infected cells. The viral RNA replication complex comprises a variety of viral proteins including 2B, 2C, 2BC, 3A, 3B (VPg), 3AB, 3CD and 3D. The inhibitors of virus-specific RNA synthesis interacting with components of the viral replicative complex such as viral 2C protein (may have two functions during viral RNA synthesis: as

an NTPase and directing replication complexes to cell membranes), 3A protein (ability to serve as a membrane anchor), viral RNA polymerase complex, etc. These drugs preventing the production of the infectious viral RNA of susceptible viruses.

Virus-specific protease inhibitors

The initial step in the translation of the positive stranded genome of enteroviruses is the formation of a single large polyprotein of about 250 kDa. This polyprotein is rapidly processed into mature viral proteins by co- and posttranslational cleavages (Kitamura, 1981), which are executed by the viral 2A and 3C protease. In view of the fact that the 3C^{pro} and 2A^{pro} proteases are highly conserved and because of its important role, these proteases may be an interesting target for antiviral drug design.

Inhibitors of host cell factors

This group of antiviral drugs includes compounds that interfere with or modulate the function of host factors that play a critical role in the virus replication cycle. Since host factors are unlikely to mutate and develop resistance in response to therapy, they are attractive targets for antiviral drugs. A potential disadvantage of host-targeting compounds is that interference with a cellular target may be associated with toxic side effects (Van der Schaar *et al.*, 2013).

Antivirals with other mechanism of action

This group comprises compounds that inhibit viral assembly and release, increase the nucleotide incorporation error rate during RNA viral replication, as well as antiviral agents that target viral RNA in the 5'UTR end, etc.

Table 1. Antivirals Active vs. Enteroviruses (adapted, Rotbart, 2002; Galabov and Angelova, 2006; De Palma *et al.*, 2008; Fechen *et al.*, 2011; Tan *et al.*, 2014; Van der Linden *et al.*, 2015)

Cell susceptibility
- Interferons (IFN-α, Betaferon[®]) - Immunoglobulins
Receptor antagonists
- Anti-SCARB2 antibodies - Anti-PSGL-1 antibodies - Anti-heparan sulfate peptide - Bovine lactoferrin - Human lactoferrin - SP40 peptide
Viral attachment and binding to host
Antibodies
Soluble receptor analogues (SRA)
- soluble cells ICAM - soluble DAF-IgG Fc fusion proteins (sDAF-Fc) - sCAR-Fc - sCAR-Fc/sDAF-Fc
Molecular decoys
- Recombinant SCARB2 - PSGL-1 - Heparin mimetics (Heparin, Heparan sulphate, Pentosan polysulfate, Dextran sulphate) - Suramin/NF449 - Kappa carrageenan
Viral uncoating/capsid function
WIN compounds
- Arildone (WIN38020) - Disoxaril (WIN51711) - WIN52452 - WIN52084 - WIN54954 - WIN56291

- WIN58768
- WIN61893
- Pleconaril (WIN63843)

Pyridazinamine analogues (R compounds)

- R77975 (Pirodavisir)
- R61837
- R78206
- BTA-188
- BTA-39
- BTA-798
- Compound 13

Isoxazole Derivatives

- compound VIa
- compounds 19, 20 and 21

Pyridyl Imidazolidinones

- BPROZ-194
- BPROZ-299
- BPROZ-284
- BPROZ-160
- BPROZ-112
- BPROZ-103
- BPROZ-101
- BPROZ-074
- BPROZ-033
- DBPR-103
- compound 28b

SCH compounds

- SCH 38057
- SCH 47802
- SCH 48973
- Pocapavir (V-073)

SDZ 35-682

SDZ 880-061

Phenoxy pyridine carbonitriles

- DEPC: 6-(3,4-dichlorophenoxy)-3-(ethylthio)-2-pyridine carbonitrile

Flavane derivatives

- BW 683C: 4',6-dichloroflavan
- BW 4294: 1-anilino-9-benzyl-2-chloropurine
- 3(2H)-isoflavene

Chalcones (Ro compounds)

- Ro 09-0410
- Ro 09-0179
- Ro 09-0696
- Ro 09-0881

Methylthiopyrimidines

- S-7: ethyl-2-methylthio-4-methyl-5-pyrimidine carboxylate

Rhodanine (2-thio-4-oxothiazolidine)

44 081 RP: 2-[(1,5,10,10a-tetrahydro-3H-thiazolo[3,4b]isoquinolin-3-ylidene)amino]-4-thiazoleacetic acid

Dibenzofuran

Dibenzosuberol derivatives

- 2-hydroxy-3-dibenzofuran carboxylic acid
- Dibenzosuberone

3,4-dihydro-2-phenyl-2H-pyrano[2,3-b]pyridines, the 6-substituted 2-(3',4'-dichlorophenoxy)-2H-pyrano[2,3-b]pyridines

- MDL 20,610

- MDL 20,646 - MDL 20,957
Pyridine (compound 71): 2-(3,4-dichlorophenoxy)-5-(methylsulfonyl)
Inhibitors of Virus-Specific RNA Synthesis
Guanidine hydrochloride PTU-23: N-phenyl-N'-2-hydroxyphenylthiourea MRL-1237: 1-(4-fluorophenyl)-2-(4-imino-1,4-dihydropyridin-1-yl)methylbenzimidazole hydrochloride Benzimidazoles - HBB: 2-α-hydroxybenzyl-benzimidazole - Enviroxime [2-amino-1-(isopropyl sulfonyl)-6-benzimidazole phenyl ketone oxime] TBZE-029 [1-(2,6-difluorophenyl)-6-trifluoromethyl-1H,3H-thiazolo[3,4-a]benzimidazole] Enviroxime-related derivatives (vinyl acetylene benzimidazoles) - Enviradene: (E)-1-[(1-methylethyl)sulfonyl]-6-(1-phenyl-1-propenyl)-1H-benzimidazole-2-amine - 2-amino-6-[(E)-1-phenyl-2-(N-methylcarbamoyl)vinyl]-imidazo[1,2-a]pyridines - AN-12-H5 - AN-23-F6 - TTP-8307 Oxoglucine OSW-1: (3β,16β,17α-trihydroxycholest-5-en-22-one 16-O- {O-(2-O-(4-methoxybenzoyl)-β-D-xylopyranosyl)-(1$\rightarrow$3)-2-O-acetylα-arabinopyranoside Thiomersal (2-FMC): 2-furylmercury chloride Phenoxybenzenes, Phenoxypyridines and Related Analogues - MDL-860 (DNB): 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile - Compound 13: (3,4-dichlorophenoxy)-(5-methylsulfonyl-2-pyridinyl)-methane - Compound 21: (2-(3,4-dichlorobenzylamino)-5-methylsulfonylpyridine) Isothiazole Derivatives - DID: 5,5'-diphenyl-3,3'-diisothiazole disulphide Flavonoids - Ro 09-0179 - Ro 09-0298 - 3-MQ: 3-methylquercetin - 3-methylkaempferol and 3,4'-dimethylkaempferol - Compound 4h Glutotoxin Nucleoside Analogues - 2'-C-methylcytidine - 5-nitro and 5-aminocytidine analogues Pyrrolidine Dithiocarbamate (PDTC) Quinacrine Amantadine Metrifudil N₆ benzyladenosine DTriP-22 Fluoxetine
Virus-Specific Protease Inhibitors
E-64: L-Trans-Epoxy succinyl-Leucylamido(4-Guanidine)Butane <i>3C PROTEASE INHIBITORS</i> Peptidic Inhibitors - Peptide Aldehydes - Michael Acceptor-Containing - Rupintrivir (AG7088): ethyl(2E,4S)-4-(((2R,5S)-2-(4-fluorobenzyl)-6-methyl-5-(((5-methylisoxazol-3-yl)carbonyl)amino)-4-oxoheptanoyl)amino)-5-((3S)-2-xopyrrolidin-3-yl)pentenoate

- Azapeptides
- Compound 1 (AG7404)
- Diazomethyl Ketones (DMK)
- Peptidyl Monofluoromethylketones
- Keto-Glutamine Analogues
- S-nitrosothiols
- Peptidyl N-iodoacetamides
- Tripeptidyl α -ketoamides

Non-Peptidic Inhibitors

- 2-Pyridone-Containing Peptidomimetics
- Substituted Benzamide Inhibitors (α,β -unsaturated etobenzamides; 5-substituted benzamides)
- Isatins
- Spiro-Indolinone β -Lactams
- Homophthalimides
- β -Lactones
- Pseudoxazolones
- Low Molecular Weight Non-Peptidic Inhibitors

Compound 10b

Fisetin

Rutin

Microbial Extracts

- naphthoquinonelactol, quinine-like citrinin, radicinin and triterpene sulfates

2A PROTEASE INHIBITORS

Thiol Alkylating Agents

- Iodoacetamide and N-ethylmaleimide

Classic Elastase-Specific Inhibitors

- MCPK: Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone
- Elastatinal

Homophthalimides

Peptide-Based Fluoromethyl Ketones

- zVAD.fmk (benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone)
- zIETD.fmk (benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone)

LVLQTM peptide

Nitric oxide (NO) donors (S-nitrosylation)

- GTN, ISDN
- NO-metoprolol

Inhibitors of Host Cell Factors

PI4KIII β vinhibitors (Phosphatidylinositol-4-Kinase III Beta)

- Enviroxime
- GW5074
- T-00127-HEV1
- AG7404 (compound 1)
- BF738735

OSBP inhibitors (Oxysterol-Binding Protein)

- Itraconazole
- OSW-1
- AN-12-H5
- T-00127-HEV2
- 25-hydroxycholesterol

Ubiquitin-proteasome system (UPS)

- Pyrrolidine dithiocarbamate (PDTC)
- Curcumin
- MG132
- Lactacystin

Assembly inhibitors

Chaperone Hsp90 Inhibitors

- Geldanamycin
- 17-AAG (17-allylamino-17-demethoxygeldanamycin)

BSO (buthionine sulfoximine) (glutathione synthesis inhibitor)

TP219 (glutathione scavenger)

Antivirals with Other Mechanisms of Action

Ribavirin (1-β-D-ribofuranozyl-1,2,4'-triazole-3-carboxamide)

Valopicitabine (2'-C-methylcytidine)

2'-C-methyladenosine

4'-azidocytidine

5-substituted cytidine analogues

- 5-nitrocytidine

N-6-substituted purine analogues

Amiloride

Pyrazolo[3,4-d]pyrimidine

Hygromycin B

Hydantoin (5-(3,4-dichlorophenyl) methylhydantoin)

Mycophenolic acid (MPA)

EnteroX

Aziridine and N-dansylaziridine

Antisense Oligonucleotides

Heat-shock protein 90 inhibitor

- Geldanamycin
- 17-AAG

RNA-based therapeutics

- siRNA
- shRNA

Drug resistance - an obstacle to effective chemotherapy

Drug resistance is reduced or absent virus sensitivity to a particular replication inhibitor and is characterized by significantly higher values of 50 (IC₅₀) and 90 (IC₉₀) inhibitory concentration. While drug resistance is a phenotypic feature, a phenotype is determined by the genotype and specific genotype mutations that cause changes in the viral protein targeted by the relevant inhibitor (Heinz and Vance, 1996).

Enterovirus populations display quasispecies dynamics, characterized by high rates of mutation and recombination, followed by competition, selection, and random drift acting on heterogeneous mutant spectra. Direct experimental evidence indicates that high mutation rates and complex mutant spectra can serve for the adaptation of enteroviruses to complex environments. Studies with the RNA-dependent RNA polymerase of picornaviruses suggest that multiple enzyme sites may influence the template-copying fidelity (incorporation of incorrect vs correct nucleotide) during RNA replication. Mutation and recombination are an unavoidable

consequence of the molecular mechanisms inherent to the process of viral genome replication and underlie the diversification of enterovirus genomes as they multiply in human and animal hosts.

Enteroviruses, as other RNA viruses, share a potential for adaptation and rapid evolution associated with two key features of their replication: high mutation rates and quasispecies dynamics. One of the first measurements of mutability of an RNA virus was carried out by Eggers and Tamm (Eggers and Tamm, 1965) who calculated a rate of 1×10^{-4} for the transition of coxsackievirus A9 from dependence to independence of 2-(a-hydroxybenzyl)-benzimidazole (HBB). The value is in line with measurements of mutation rates (the rate of occurrence of mutations during genome replication) and mutation frequencies (the frequency of mutations in a genome population) carried out with several RNA viruses (Drake and Holland, 1999). The genome size of RNA viruses is in the range of 3-33 kb. The combined mutation rate and genome size values imply that an average of 0.1-3 mutations per genome are expected to occur every time an RNA template is copied into a complementary RNA or DNA copy. Such template-copying

events may take place hundreds of times in each infected cell from an infected organism.

The diversity of disease manifestations associated with closely related enteroviruses is probably attributable to profound biological effects of some mutations that, because of their limited number, do not necessarily affect the phylogenetic position of the virus. The combination of highly dynamic mutant spectra with unpredictable alterations of biological behaviour by minimal genetic change defies classical classification schemes. The result is the need to update the grouping of enteroviruses quite frequently into genetic and serological types and subtypes.

The application of quasispecies theory to an understanding of the limits of viral genomes to accept mutations, together with an increasingly deeper understanding of the mechanisms of mutagenesis by nucleoside analogs, has paved the way for the application of lethal mutagenesis as a new antiviral strategy (Domingo *et al.*, 2007).

The probability of resistant mutants being generated and their population growing after treatment with antiviral agents is due to four major factors (Richman, 1996): (i) the mutation rate during genome replication; (ii) the inherent ability of the target site of inhibition to mutate; (iii) the selective pressure exerted by the viral inhibitor; (iv) the extent of viral replication.

The enterovirus progeny, resistant to a particular viral inhibitor, grow quite readily and rapidly as a result of the selection of pre-existing mutants with mutations in the ligand-binding domain (for the antiviral substance) of the virus-specific target protein. Such mutants always exist in the virus population, which consists of a large number pseudo-species (Loddo, 1980; Nikolova and Galabov, 2003). The selective pressure exerted by a certain antiviral agent leads to an increase in the population of mutant viruses (Cuevas *et al.*, 2005), since they are the fittest in the presence of this compound (Zambon and Pillay, 1998). The hypothesis accepted today is that of Herrmann and Herrmann (1977), according to which the selection of resistant viral progeny is an indicator of specific antiviral activity.

A great number of cases have been described of laboratory selection of viral strains resistant to guanidine (Melnick *et al.*, 1961), to HBB (Loddo, 1963), to arildon (Egger and Rosenwirth, 1988), to disoxaril (Nikolaeva and Galabov, 2003), to oxoglaucine (Nikolaeva-Glomb and Galabov, 2008), etc.

Combination chemotherapy

Although many substances have been developed and their effect has been studied both in animal models and in humans, to date there are no registered products for specific treatment of enteroviral diseases. More than 1 000 substances have been selected as enteroviruses inhibitors in experiments *in vitro*, but less than 20 of them have manifested activity *in vivo*. The practical implementation of inhibitors is limited by the fact that these viruses have a very short replicative cycle and undergo multiple such cycles in the presence of the substances under conditions of experimental infection *in vivo*, resulting in decreased sensitivity to the inhibitor and development of resistance. The huge serotype diversity in the family and the possible toxicity of the medications used also contribute to the limited practical use.

These disadvantages can be avoided using combination therapy for treatment of viral infections in clinical practice. When applied in appropriate combinations, viral inhibitors have a higher efficacy than when used independently. One of the advantages of combination chemotherapy is that it restricts the selection of resistant and dependent mutants. Even in the case of prior resistance of a particular strain to one of the partners in the combination, most likely it will be sensitive to the effect of the other partner. The possibility of dual resistance to both partners in the combination is minimal, especially if the partners in the combination have different mechanisms of action.

Another advantage of the combination chemotherapy is that by using synergistic combinations, a more potent antiviral effect can be achieved at lower concentrations of the relevant inhibitors as compared to their independent administration, thereby reducing the so-called “dosage pressure” favouring the selection of resistant mutants, and solving the problem of toxicity.

To be considered effective and clinically promising, antiviral combinations must meet one the following conditions (Schinazi, 1991): to have at least an additive effect; to suppress or at least restrict the possibility of emergence of dependent and resistant mutants to each of the partners in the combination; to possess a therapeutic index which is higher than that of the two substances taken separately, i.e. the combination should not have enhanced toxicity.

The study of combined effects of the replication inhibitors of picornavirus, enterovirus in particular, is necessary because of the need for ef-

fective chemotherapy of enteroviral infections and better understanding of the mechanism of action of the combination partners, as well as to clarify the different stages of the replicative cycle of viruses.

The Laboratory of Experimental Chemotherapy of enteroviral infections at the Stephan Angeloff Institute of Microbiology has designed a model scheme for implementation in experimental infections *in vivo*, in which the approach to the application of substances differs from the standard simultaneous administration of the partner substances in the combination. The new approach for combined administration of antivirals is by sequentially alternating, non-simultaneous, application of the combination (CAA) of enterovirus inhibitors with different mechanisms of action. Initially, this scheme tested various double, triple and quadruple combinations against an experimental neurotropic infection with coxsackieviruses B1 in newborn mice. In these pilot studies, the best effect was achieved with the triple combination disoxaril/guanidine hydrochloride /oxoglaucine (DGO) (Vassileva-Pencheva and Galabov, 2010). Later on, two new triple combinations were tested on the same experimental model: pleconaril/guanidine hydrochloride/oxoglaucine (PGO) and pleconaril/MDL-860/oxoglaucine (PMO), where two of the partner compounds were replaced with more active ones (Stoyanova *et al.*, 2015; unpublished data). All three combinations showed distinct activities - a decrease in lethality and prolonged mean survival time in the experimental animals. The course with the triple combinations administered simultaneously led to high lethality and resistance to each of the partners. In contrast to this result, CAA courses with DGO, PGO and PMO led to a significant reduction of the viral content in the brain of animals. The sensitivity to each partner inhibitor was not only preserved but even increased in all three combinations. The way the compounds are arranged in the combination is of key importance for its performance. This course of treatment has confirmed its efficacy in other experimental models of coxsackie B virus infection *in vivo* (Vassileva-Pencheva and Galabov, unpublished data). The effectiveness of this new approach has been proven and can serve as the basis for developing other combinations and novel therapeutic strategies (Vassileva-Pencheva and Galabov, 2008, 2010, Stoyanova *et al.*, 2015). The traceability of phenotypic markers has been introduced for characterization of drug mutants (resistant and dependent) as an essential stage in the study of enteroviral inhibitors (Nikolova and Galabov, 2003).

In recent years, the studies on the antiviral activity of small interfering RNAs (siRNAs) have been gaining more and more attention. Once they have been inserted into the cell, siRNAs induce RNA interference (RNAi). RNA interference is a mechanism in which, in the presence of a double-stranded RNA molecule homologous to a given mRNA (or viral genome), the expression of the latter ceases. Since a gene has been made inoperative, the mechanism is referred to as post-transcriptional gene silencing. This mechanism may be used with certain viruses, where the goal is to inhibit their replication. The possibility of producing siRNAs that are effective against many closely related enteroviruses makes this approach particularly attractive. A team of the Department of Virology has studied the *in vitro* effect of siRNAs on the replication of coxsackieviruses B1 and B3 by a new technology for generation of a pool of siRNAs targeting a specific genetic region, overlapping and silencing the target sequence, irrespective of whether mutation or recombination has occurred in it (Petrov and Galabov, 2012, 2015). The possibility of producing siRNAs that are effective against many closely related enteroviruses makes this approach particularly attractive. As with all approaches, further testing is necessary with different enterovirus infections first in animals, then in humans, including the development of an efficient and convenient system for delivery of siRNA.

Due to the rapid development of resistance in enteroviruses, successful chemotherapy and prophylaxis of infections caused by them will probably involve a combination of powerful antiviral medications or a combination of an antiviral drug and an immunomodulator. Another no less promising solution is the combination therapy through sequentially alternating administration of antivirals with different mechanisms of action, unlike the standard simultaneous administration of the partner substances in the combination.

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Anti-Influenza Virus Activity of 1-(4-Morpholinomethyl)-tetrahydro-2(1H)-pyrimidinone (Mopyridone)

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Abstract

Mopyridone inhibited the replication of influenza viruses A(H3N2), A(H2N2), and B in MDCK and in primary calf kidney (CK) cell cultures, a higher activity been found in CK cells. The effects against A(H1N1) and A(H7N7) subtypes strains were distinctly lower. Mopyridone at effective concentrations did not influence DNA, RNA and protein syntheses in intact cells. The compound-susceptible period in the influenza virus one-step growth cycle embraces the first 4 hours post virus adsorption. The high susceptibility of the A(H3N2) subtype to mopyridone was also manifested in embryonated eggs tests. Mopyridone was superior in comparison to rimantadine by its stronger and more selective *in ovo* effect. The compound demonstrated a marked anti-influenza activity in mice experimentally infected with influenza A(H3N2) and B viruses (even at massive virus inocula). This activity was similar to that of rimantadine by its protective rate, but a significantly higher by its selectivity: a selectivity (therapeutic) ratio value of 426 been recorded. Besides, mopyridone showed a week protective effect in the case of mouse infection with A/Puerto Rico/8/34 (H1N1) strain (drug-resistant in the *in vitro* experiments). The compound optimal treatment course was determined: 37.5 mg/kg orally daily (divided in two intakes) for 5 days from the day of infection.

Key words: influenza viruses, mopyridone, rimantadine, effect *in vitro*, activity in mice

Резюме

Мопиридон инхибира репликацията на грипни вируси А(Н3Н2), А(Н2Н2) и В в клетки МДСК и в първична култура от телешки бъбрек (СК), като по-висока активност бе установена в клетки СК. Ефектът спрямо щамове на подтипове А(Н1Н1) и А(Н7Н7) бе значително по-нисък. Мопиридон в ефективни концентрации не повлиява синтезите на ДНК и РНК, както и протеиновия синтез в интактни клетки. Чувствителният към съединението период в едностъпния репликативен цикъл на грипния вирус обхваща първите 4 часа след вирусната адсорбция. Високата чувствителност на щамове А(Н3Н2) към мопиридон се репродуцира и в тестове в кокоши ембриони. Мопиридон превъзхожда римантадин с по-силния си и по-избирателен ефект *in ovo*. Съединението показва също така отчетлива анти-грипна активност в мишки, експериментално заразени с грипни вируси А(Н3Н2) и В (даже при масивни вирусни инокулуми). Тази активност е подобна на активността на римантадин по индекса на протекция, но е значително по-висока по своята избирателност: селективният (терапевтичният) индекс на мопиридон е 426. Друга разлика на мопиридон от римантадин е неговия слаб протективен ефект при инфекция на мишки с щам А/Puerto Rico/8/34 (H1N1) (резистентен към мопиридон в експерименти *in vitro*). Определен бе оптималният терапевтичен курс с мопиридон: перорална дневна доза 37.5 мг/кг (разделена на два приема) в продължение на 5 дни от деня на заразяването.

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Introduction

At present two groups of antivirals effective on replication of influenza viruses are recommended for the treatment of flu: [i] M2 protein blockers rimantadine (α -kethyl-1-adamantane-methylamine hydrochloride), amantadine (1-aminoadamantane hydrochloride) and adapromine (ethyl-1-adamantylmethylamine hydrochloride) (Zlydnikov *et al.*, 1987; Kubar, 1988); [2] viral neuraminidase inhibitors oseltamivir, zanamivir and peramivir. The great problem for the M2 blockers is the relatively quick development of drug-resistance and the lack of effect against influenza B virus.

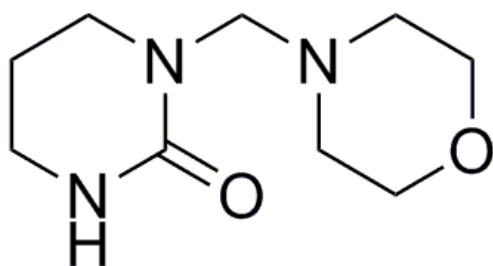


Fig. 1. 1-(4-morpholinomethyl)-tetrahydro-2(1H)-pyrimidinone (mopyridone)

The present paper deals with 1-(4-morpholinomethyl)-tetrahydro-2(1H)-pyrimidinone (mopyridone) (Fig. 1), a compound synthesized originally by Sidzhakova *et al.* (1982), and found to have anti-orthomyxovirus and anti-togavirus effects in a broad-scope antiviral screening carried out (Galabov *et al.*, 1984). Here we describe studies characterizing the *in vitro*, *in ovo*, and *in vivo* effects of mopyridone.

Materials and Methods

Compounds

Mopyridone (MMTHP, DD-13, MCU), with molecular weight 199.25, m.p. 143-146°C, represents white fine crystals, easily soluble in water. The compound was synthesized by Dorotea Sidzhakova (Faculty of Pharmacy, Medical University of Sofia; Medical University of Plovdiv, Bulgaria).

Rimantadine hydrochloride was kindly supplied by Georgii A. Galegov (The D.I. Ivanovskii Institute of Virology, Moscow, Russia) and Vera I. Ilyenko (Research Institute of Influenza, St. Petersburg, Russia).

Cells and media

Primary chick embryo fibroblast cultures (CEF) were prepared after Porterfield (1960) by seeding cell suspension, 1.1×10^6 cells/ml, in a growth medium Eagle's MEM (Difco) supplemented with 10% calf serum.

Primary calf kidney cultures (CK) were prepared by Bodians's (1956) method.

The cell suspension, $6-8 \times 10^5$ cells/ml, was grown in a medium of 10% calf serum, 0.2% egg hydrolisate and 0.5% lactalbumine hydrolysate in Hanks' saline.

Madin-Darby canine kidney (MDCK) cells were cultivated in a medium of 10% foetal bovine serum (Flow) in Eagle's MEM (Flow), pH 7.5.

Embryonated eggs

Nine-to-eleven-day old embryonated eggs of Leghorn hens were employed.

Mice

White mice of the randomly bred H line, 10 g body weight, were used. Mice of the ICR randomly bred line weighing 20 g were employed in experiments with influenza virus A/Victoria/35/72 (H3N2) only.

Viruses

The following influenza virus strains were used: A/chicken/Germany/27 (FPV, Weybridge) (H7N7), A/Puerto Rico/8/34 (H1N1), A/England/333/80 (H1N1), A/Chile/1/83 (H1N1), A/Sofia/1672/86 (H1N1) [a clinical isolate of Chile/1/83], A/Taiwan/1/86 (H1N1), A/Krasnodar/101/59 (H2N2), A/Hong Kong/1/68 (H3N2), A/Aichi/2/68 (H3N2), A/Victoria/35/72 (H3N2), A/Sofia/897/87 [a clinical isolate of A/Philippines/2/82 (H3N2)], A/Mississippi/1/85 (H3N2), A/Sofia/2541/87 (a clinical isolate of A/Mississippi/1/85), A/Leningrad/360/86 (H3N2), B/Lee/40 and B/Beijing/1/87. The influenza virus strains adapted to mice (A/Puerto Rico/8/34, A/Aichi/2/68, A/Victoria/35/72 and B/Lee/40) were passed serially by intranasal route in mice using lung extracts as inocula and by single alternative passages in embryonated eggs. These strains were received from the collections of the D.I. Ivanovskii Institute of Virology, Moscow (A/Aichi/2/68) and of the Research Institute of Influenza, St. Petersburg (A/Puerto Rico/8/34, A/Victoria/35/72, B/Lee/40). All other strains were cultivated through serial allantoic passages in chick embryos and were received through the courtesy of Dr Rossitsa Kotseva (National Influenza Center, National Institute of Infectious and Parasitic Diseases, Sofia).

Cytotoxicity test

The effect of the test compound on uninfected confluent cell monolayer and cellular morphology was traced for overt signs of cytotoxicity during 96-h incubation at 37°C and the maximum tolerated (nontoxic) concentration (MTC) value has being determined. In addition, quantitative assessment of

possible cytostatic effect was made by growing uninfected cells in the presence of the compound studied till reaching the stationary growth phase (48 h for CEF and MDCK cells, and 96-120 h for CK cells). The 50% cell growth inhibitory concentration (CGIC₅₀) value was evaluated on the basis of the average cell number counted.

Cellular DNA, RNA and protein synthesis

Uninfected monolayer CEF cultures (in the stationary phase), grown in 20-ml scintillation vials, were treated with the compound tested for 18 h. ³H-Thymidine or ³H-uridine (Amersham, both with specific activity 5 Ci/mM), 2.5 µCi/ml each, were added to treated or untreated (control) cells for 60 min after 17 h of incubation. For protein labeling a ³H-labelled amino-acid mixture, 1 µCi/ml each of l-leucine, l-valine and l-phenylalanine (UVVVI, Prague, Czech Republic, specific activity 150 mCi/mM), was applied after the same scheme. The acid-insoluble products of the DNA, RNA or protein synthesis were retained on nitrocellulose membrane filters (Synpore RuFS, Czech Republic) and washed with 5% trichloroacetic acid. The radioactivity was measured in a non-polar scintillation solution via an Intertechnique counter (Comef, France).

CPE inhibition assay procedure

Monolayer cell cultures grown in Flow 96-well plastic microplates were inoculated with serial 10-fold virus dilutions (1 - 1000 CCID₅₀), 0.01 ml per well, by 60 min adsorption at room temperature. Then, the compounds tested at 0.5 log₁₀ increasing consecutive concentrations were added to the maintenance medium (0.2 ml per well of 2% v/v 1M HEPES buffer in Eagle's MEM Flow medium containing 3 µg/ml trypsin, penicillin 100 U/ml, and streptomycin 100 µg/ml). The plates were incubated at 37°C for 4 days and viral CPE was followed every day by inverted light microscope at 125 x magnification. Four wells per test sample were used. CPE was scored on a 0 - 4 basis with 4 representing total cell destruction. These data were used to obtain dose-response curves at 10 - 100 CCID₅₀ viral doses. From these graphs the minimal inhibitory concentration causing a 50% reduction of CPE as compared to the untreated controls (MIC₅₀ value) was determined.

Plaque-inhibition test

It was carried out according to technique of Herrmann(1961)-Siminoff (1961). Monolayer CEF cultures in 70 mm diam. Petri dished (Anumbra, Czech Republic) were inoculated with 100-130 PFU of FPV per dish by 60 min adsorption at room temperature. The compounds tested were incorpo-

rated in the agar overlay (1% Bactoagar Difco in Eagle's MEM Difco medium with 10% heated calf serum, 1.65 mg/ml sodium bicarbonate, penicillin 100 U/ml, and streptomycin 100 µg/ml). The mean plaque number (3 dished per test sample) and the PFU percentage to the control, respectively, were checked after 72 h of incubation at 37°C.

Kinetic (timing of addition) studies

The one-step cycle design was followed. Monolayer cultures of CK cells were inoculated with influenza virus B/Lee/40 at multiplicity of infection (m.o.i.) 8-10. Mopyridone at MTC was added to the maintenance medium (3% calf serum in Eagle's MEM Difco medium) immediately after virus inoculation (0 h) or at the 2nd, 4th, 6th, 8th, 10th and 12th h. The infectious virus yields were recorded at the 6th, 8th, 10th, 12th and 15th h post virus inoculation (incubation at 36°C) in EID₅₀/ml (in an assay procedure of 72 h incubation at 33°C).

Toxicity testing in embryonated eggs

Substances tested at different doses (mopyridone 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 6, 12 or 24 mg per embryo; rimantadine 0.094, 0.187, 0.375, 0.75, 1.5, 3, 6 or 9 mg per embryo) as serial dilutions in Dulbecco's PBS were injected by allantoic route (in 0.2 ml volume) in 10-11-day-old embryos. The TD₅₀ (50% toxic dose) and MTD (maximum tolerated dose) for embryonated eggs were determined by checking-up both the viability and the hatching rate.

Antiviral action testing in embryonated eggs

Virus infection was performed in the allantoic sac (0.1 ml inoculum volume) and the substance tested was introduced (0.2 ml) also by allantoic route 60 min before virus inoculation. Virus and compound dilutions were done in Dulbecco's PBS. The experimental setup used represents parallel viral titrations in the presence or absence of the substance tested. The antiviral effect was measured on the basis of Δlog₁₀ EID₅₀ evaluation.

Compound toxicity determination in mice

Acute (single-dose) toxicity of mopyridone for white mice was determined after the routine procedure (a 7-day observation period, 10 animals per test group), oral and subcutaneous LD₅₀ been calculated using the Pershin method (cf. Kudrin and Ponomareva, 1967).

Short-term toxicity was assessed by twice daily oral or subcutaneous dosing for 8 days on groups of 10 mice per each dose. Changes in general condition, behavior and body weight of animals were followed for 15 days after treatment onset and the maximum tolerated dose (MTD) was determined.

Experimental influenza virus infections in white mice and criteria for assessment of the compound effect

White mice were infected intranasally with appropriate inoculation dose (LD₉₅ or higher) of influenza virus strains A/Puerto Rico/8/34, A/Aichi/2/68, A/Victoria/35/72 or B/Lee/40 (12-20 animals per test group). Compound and placebo treated animals were observed for 14-16 days post infection and the compound effect was determined on the basis of the following indices: (a) mortality rate with a calculation of the protection index (PI) = [(PC-1) / PC] x 100, where PC (protection coefficient) is the ratio between mortality percentage in placebo and mopyridone treated test groups at the end of the observation period; (b) mean survival time (MST). The maximum error (Δ_p) of the mortality rate relative portion (p) was evaluated by the Fisher's ϕ -method (the Van der Warden's method being used in cases of p = 0 or 100 %). The standard error of the quadratic deviation of the MST (σ_x) was calculated as described previously (Karparov *et al.*, 1985).

Results

In vitro cytotoxicity studies

Studies in three types of cell cultures (Table 1) found that (a) mopyridone toxicity is lowest in CEF cells; (b) mopyridone is less toxic than rimantadine. The mopyridone CGIC₅₀ value is higher than that of rimantadine, 4 times in CEF, 3 times in MDCK cells and approximately 2 times in CK cells, respectively.

The effects of the compound on the cellular DNA, RNA and protein synthesis were studied by using radioisotope methods. It was found that

18-h treatment of confluent CEF monolayers with mopyridone in the concentration range of 0 - 100 μ g/ml does not decrease the ³H-thymidine, ³H-uridine and ³H-amino acids incorporation rates as compared to the untreated controls (data not shown).

Susceptibility of influenza viruses to mopyridone

Mopyridone showed high activity against influenza virus A, subtypes H3N2 and H2N2, and influenza virus B by the CPE inhibition assay procedure (Table 2). A clearly higher (ten times and more) of the compound effect was revealed in CK cells as compared to MDCK cells when tested versus A(H3N2) strains. It was worth noting that mopyridone potency against influenza virus A (H2N2, H3N2) strains was similar to that of rimantadine. In the case of A(H3N2) strains in MDCK cells the rimantadine MIC₅₀ value was 2 - 10 times lower than that of mopyridone, but the selectivity index (SI = CGIC₅₀/MIC₅₀) values were comparable, and in CK cells the mopyridone SI index values were higher as compared to the rimantadine ones.

Influenza virus A/Puerto Rico/8/34 (H1N1) could be qualified as slightly sensitive or resistant (in analogy to rimantadine) to mopyridone (Table 2). A similar low susceptibility to mopyridone was observed when tested other influenza virus A(H1N1) strains: A/England/333/80 (SI = 35.1), A/Chile/1/83 (SI = 8.8) and Taiwan/1/86 (SI = 35.1).

A substantially lower activity mopyridone manifested also against influenza virus A(H7N7). The mopyridone effect on FPV/Weybridge replication in CEF cultures was one thousand fold less than that of rimantadine (Table 2). This was also demonstrated by the plaque-inhibition test in the

Table 1. Effect of mopyridone and rimantadine on the MDCK, CEF and CK cell growth and monolayer state

Cell culture	Mopiridone		Rimantadine	
	CGIC ₅₀ ^a μ g/ml	MTC ^b μ g/ml	CGIC ₅₀ ^a μ g/ml	MTC ^b μ g/ml
MDCK	54.8 (49.7,57.0,57.7)	60.0 (60.0,60.0)	18.0 (13.0, 22.6,18.5)	25.0 (20.0,30.0)
CEF	59.6 (57.5, 61.7)	60.0 (50.0, 50.0)	15.1 (16.7, 13.5)	10.0 (10.0, 10.0)
CK	40.2 (42.7, 37.7)	50.0 (50.0, 50.0)	23.7 (23.7, 23.7)	25.0 (25.0, 25.0)

^aCGIC₅₀ value is the mean from 2-3 experiments (in each experiments: 3 culture samples per drug concentration were recorded on the 24, 48 and 72 h after the cell seeding).

^bMTC value is the mean from 2 experiments (4 culture samples per drug concentration). The results of individual experiments are listed in brackets.

Table 2. Antiviral activity of mopyridone and rimantadine against influenza viruses A and B strains in cell cultures (CPE inhibition assay)

Influenza virus strain	Cell culture	Mopyridone		Rimantadin	
		MIC ₅₀ ^a , µg/ml	SI ^b	MIC ₅₀ , µg/ml	SI
A/PR/8/34	MDCK	11.8	4.6	12.6	1.4
A/Krasnodar/101/59	MDCK	≤0.1	≤548.0	≤0.1	≤180.0
A/Hong Kong/1/68	MDCK	0.32	171.2	0.05	360.0
A/Aichi/2/68	MDCK	0.27	203.0	0.13	138.5
	CK	≤0.03	≤1340.0	≤0.03	≤790.0
A/Victoria/35/72	MDCK	0.45	121.8	0.04	450.0
	CK	≤0.03	≤1340.0	0.06	395.0
FPV/Weybridge	CEC	14.5	4.1	≤0.01	≤1510.0
B/Lee/40	MDCK	0.05	1096.0	>20.0	<0.9
B/Beijing/1/87	MDCK	0.12	456.7	>10.0	<1.8

^aDetermined in 3-5 experiments.

^bCGIC50/MIC50.

same cell culture, in which the 50% plaque inhibitory concentration of mopyridone was 4507-fold higher than that of rimantadine (32.9 and 0.0073 µg/ml, respectively).

Timing-of-addition study

In order to determined the mopyridone-susceptible period in influenza virus (B/Lee/40) replication cycle the one-step growth cycle setup in CK cells was performed. The compound was applied at MTC (Fig. 2). A marked inhibition of infectious virus production ($\Delta \log_{10} \text{EID}_{50} = 2.0$) was established when mopyridone was applied to the maintenance medium within the period 0-4th h post virus inoculation. Its addition at the 8th h and later was without effect.

Toxicity study on embryonated eggs

MTD of mopyridone was more than twice higher than that of rimantadine, ≥ 6 mg and 3 mg per embryo, respectively. Obviously, mopyridone TD₅₀ lies between 9 and 12 mg per embryo, and rimantadine TD₅₀ - between 3 and 6 mg, respectively. These data for rimantadine coincide with Indulen *et al.* (1979) data: MTD and TD₅₀ values of 3 and 4 mg per embryo, respectively.

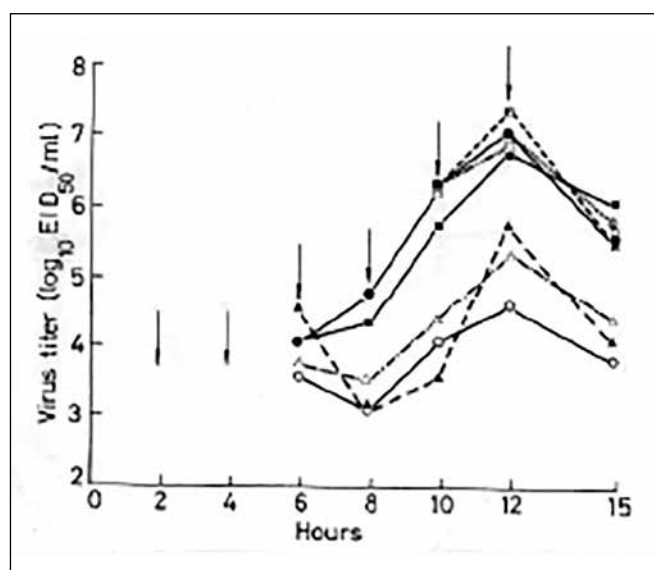


Fig. 2. Effect of mopyridone, added at different time after virus adsorption on influenza virus B/Lee/40 replication in CK cells (one-step growth cycle setup, m.o.i. = 8-10). The arrows indicate time of mopyridone (50 µg/ml) addition: immediately after virus inoculation (○---○), at the 2nd h (▲---▲), at the 4th h (△---△), at the 6th h (■---■), at the 8th h (□---□); at the 10th h (◇---◇), at the 12th h (○---○); control (no mopyridone, ●---●)

Effect of mopyridone on influenza viruses A(H3N2) and B growth in embryonated eggs

Mopyridone activity *in ovo* towards influenza virus A/Hong Kong/1/68 (H3N2) was established to be higher than that of rimantadine on the basis of four criteria: (a) lower minimal inhibitory dose value - <0.75 and 1.5 mg/embryo, respectively; (b) stronger antiviral effect at the optimal effective dose (6 mg/embryo for mopyridone, 1.5 mg for rimantadin) - an inhibition ($\Delta\log_{10} \text{EID}_{50}$) of 2.2 and 1.5, respectively; (c) lower toxicity - the 50% toxic dose value between 9 and 12 mg per embryo for mopyridone, and between 3 and 6 mg for rimantadine; (d) higher SI value - >12 and 2-4, respectively.

ly. A similar sensitivity to mopyridone *in ovo* was registered for a series of other influenza A(H3N2) strains (A/Philippines/2/82, A/Mississippi/1/85, A/Leningrad/360/860/86) and the Beijing/1/87 strain of influenza B virus, too (not illustrated).

Effect of mopyridone on experimental influenza A and B infections in white mice

Initially, mopyridone was administered subcutaneously in a daily dose of 300 mg/kg - 1/24 of the LD₅₀ (single dose toxicity value), 7200 mg/kg (Karparov *et al.*, 1985) - as a 8-days treatment course started on the day of virus inoculation.

Table 3. Effect of mopyridone on influenza A and B virus infections in white mice

Virus strain	Inoculum LD ₅₀ / mouse	Drug tested	Daily dose, mg/kg	Route	Treat- ment course, days	Morta- lity rate ^a	p±Δp			PI, %	MST±σ _x , days
B/Lee/40	40-50	Mopyridone	300.0	s.c.	1-8	2/10	0.024	< 0.2	< 0.488	80.0	13.1±1.4
		Placebo				10/10	0.679	< 0.917	< 1.0		4.5±0.5
	1-3	Mopyridone	150.0	oral	1-8	0/19	0	< 0.047	< 0.175	100.0	> 16.0
			75.0			1/19	0.0001	< 0.053	< 0.195	89.4	15.9±0.9
			37.5			2/20	0.011	< 0.1	< 0.265	80.0	15.1±0.8
			18.7			1/19	0.0001	< 0.053	< 0.195	89.4	15.7±0.8
			9.4			5/17	0.108	< 0.294	< 0.525	41.2	14.6±0.9
		Placebo				10/20	0.288	< 0.5	< 0.712		12.5±0.6
	3-5	Mopyridone	300.0	oral	1-8	0/12	0	< 0.071	< 0.283	100.0	> 16.0
			150.0			1/12	0.000	< 0.83	< 0.296	84.4	15.7±1.4
			75.0			0/12	0	< 0.071	< 0.283	100.0	> 16.0
			37.5			1/12	0.0001	< 0.083	< 0.296	84.4	15.7±1.4
18.7			3/12			0.057	< 0.25	< 0.521	53.0	13.9±1.2	
Placebo		300.0			8/15	0.287	< 0.533	< 0.771		12.5±0.9	
A/PR/8/34 (H1N1)	10-20	Mopyridone	300.0	s.c.	1-8	7/11	0.345	< 0.636	< 0.881	36.6	10.6±1.0
		Placebo				11/11	0.703	< 0.941	< 1.0		6.9±0.7
	10-20	Mopyridone	37.5	oral	1-8	7/13	0.275	< 0.538	< 0.79	46.2	11.6±0.9
			80.0	oral	1-8	5/15	0.125	< 0.333	< 0.582	66.7	13.0±0.9
		Placebo	20.0			8/14	0.314	< 0.571	< 0.809	42.9	9.9±0.7
							15/15	0.772	< 0.941	< 1.0	
A/Aichi/2/68 (H3N2)	33	Mopyridone	75.0	oral	1-8	4/15	0.081	< 0.267	< 0.51	73.3	13.3±0.9
			37.5	oral		2/15	0.015	< 0.133	< 0.343	86.7	14.3±1.0
		Placebo				15/15	0.772	< 0.941	< 1.0		5.3±0.4
	33	Mopyridone	37.5	oral	1-8	1/15	0.001	< 0.067	< 0.242	93.3	14.5±1.0
		Rimantadine	20.0	oral	1-8	2/15	0.015	< 0.133	< 0.343	86.7	13.7±0.9
		Placebo				15/15	0.772	< 0.941	< 1.0		5.5±0.4
	≥50	Mopyridone	37.5	oral	1-5	3/14	0.047	< 0.214	< 0.457	78.6	12.6±0.9
			18.7			6/15	0.175	< 0.4	< 0.65	60.0	11.3±0.8
		Rimantadine	20.0	oral	1-5	3/14	0.047	< 0.214	< 0.457	78.6	12.6±0.9
			10.0			6/15	0.175	< 0.4	< 0.65	60.0	10.9±0.7
		Placebo	5.0			7/15	0.229	< 0.466	< 0.712	53.3	9.9±0.7
							15/15	0.772	< 0.941	< 1.0	
	≥50	Mopyridone	9.4	oral	1-5	15/15	0.772	< 0.941	< 1.0		5.7±0.4
			20.0	oral	1-5	4/15	0.081	< 0.267	< 0.51	73.3	12.1±0.8
		Rimantadine	10.0			7/15	0.229	< 0.467	< 0.712	53.3	10.2±0.7
			5.0			7/15	0.229	< 0.467	< 0.712	53.3	10.2±0.7
Placebo					15/15	0.772	< 0.941	< 1.0		3.8±0.3	
A/Victoria/ 35/72 (H3N2)	5	Mopyridone	75.0	oral	1-5	4/14	0.089	< 0.286	< 0.541	57.1	13.1±1.0
			37.5			5/15	0.125	< 0.333	< 0.582	50.0	12.3±0.8
		Rimantadine	18.7	oral	1-5	7/14	0.25	< 0.5	< 0.75	30.1	13.1±1.0
						1/15	0.001	< 0.006	< 0.242	90.0	14.5±1.0
		Placebo				10/15	0.457	< 0.667	< 0.9		10.0±0.7

^aDied/total animal number (p = m/N); ^bP<0.01 at t>2.56; P<0.05 at t>1.96.

In the case of influenza virus B/Lee/40 infection a significant protection was recorded even at the extremely massive virus inoculation dose of 50 LD₅₀, whereas a borderline effect was found in the case of A/Puerto Rico/8/34 (H1N1) infection (Table 3).

Further experiments demonstrated that mopyridone administered orally exerted a marked protective effects in mice infected with influenza virus A/Aichi/2/68 (H3N2) and B/Lee/40. Toxicological studies done beforehand showed a very low LD₅₀ value, 8000 mg/kg of mopyridone for this route of administration. In the case of influenza virus B/Lee/40 infection (at a comparatively low virus inoculation dose of 1-5 LD₅₀ per mouse) the compound ED₅₀ (50% effective dose) varied within the dose range of 9.4-18.7 mg/kg daily in the individual experiments, PI values exceeded 80% within the compound dose range of 37.5 - 300 mg/kg (in a course Days 1 - 9 of infection) and a high SI (LD₅₀/ET₅₀) value of 426 was registered (Table 3).

In influenza virus A/Aichi/2/68 (H3N2) infection, mopyridone administered via a 5 - 8 days course with a 37.5 mg/kg daily dose manifested a high protective effect (PI > 80% at SI value of 426) even at massive virus inocula (more than 30 virus ID₅₀ per mouse). This effectivity was comparable to that of rimantadine applied at a daily dose of 20 mg/kg (the optimal effective dose). The ED₅₀ values of mopyridone and rimantadine were 18.7 mg/kg and 5-10 mg/kg, respectively.

Influenza virus infection in adult mice (20 g body weight) with another A(H3N2) strain, A/Victoria/35/72, was also susceptible to mopyridone treatment, but in this case the rimantadine activity was found to be superior (Table 3).

In the case of influenza virus A/Puerto Rico/8/34 (H1N1) infection, mopyridone administered orally at a dose of 37.5-75 mg/kg daily (in analogy to rimantadine, 20-40 mg/kg daily) showed a weak efficiency expressed by a lengthening of the mean survival time and a insignificant decrease of the mortality rate.

Discussion

The anti-influenza effect of mopyridone is apparently similar to that of rimantadine. These substances manifested a strong activity against subtype A(H3N2) strains which was of the same range both in *in vitro* and *in vivo* experiments. Furthermore, the activity was reached at almost equal compound concentrations or daily doses. The two compounds showed a weak protective effect in influenza virus

A/Puerto Rico/8/34 (H1N1) infected mice in contrast to their inefficiency towards subtype A(H1N1) strains in cell culture experiments. In the case of mopyridone this inconsistency could be explained with an immunomodulatory effect, i.e. a slight stimulation of alveolar macrophages and thymocyte proliferation, and also by a small augmentation of the antigen binding cells (Neychev *et al.*, personal communication). As for the rimantadine, the compound capability to interfere with the development of capillarotoxicity, one of the leading mechanisms in pathogenesis of influenza infection (Ilyenko *et al.*, 1982), could be taken in consideration.

At the same time some striking differences between the effects of the two substances were observed: (i) mopyridone was active against influenza B virus, unsusceptible to rimantadine; (ii) subtype A(H7N7) was significantly more sensitive to rimantadine as compared to mopyridone; (iii) mopyridone manifested a higher activity than rimantadine towards influenza virus A/Hong Kong/1/68 (H3N2) when tested in embryonated eggs; (iv) rimantadine is more toxic than mopyridone (*in vitro*, *in ovo*, *in vivo*); (v) the mopyridone effect was distinguished by its considerably higher selectivity *in vivo* (the oral SI values were 107-426 and 14-113 for mopyridone and rimantadine, respectively) (Galabov *et al.*, 1991).

These characteristics presume different mechanisms of action of the two influenza virus replication inhibitors. Actually, experimental evidence is available for two different targets, namely, the M2 protein for the close amantadine hydrochloride (Hay *et al.*, 1985; Wharton *et al.*, 1990), and the M1 protein for mopyridone (Tverdislov *et al.*, 1988; Galabov *et al.*, 1990, 1994; Wassilewa *et al.*, 1995). An initial study on the mechanism of anti-influenza virus action of mopyridone by using flat bilayer lipid membranes and purified influenza A virus structural proteins showed that this compound interacts directly with M1 protein, thus interfering with its adsorption and insertion into the bilayer (Tverdislov *et al.*, 1988). Significant changes were found in the antigenic structures (sites 1A, 2 and 3) of M1 protein of the mopyridone-resistant mutants of influenza virus A/Hong Kong/1/68 (H3N2) developed from the wild mopyridone-sensitive strain (Galabov *et al.*, 1990, 1994) as well as in the content of some polar amino acids in this protein. As a consequence, the virions of mopyridone-resistant progenies manifested a series of major deviations in their physico-chemical properties and M1 protein-lipid inter-

actions (Wassilewa *et al.*, 1995).

Literary data in the wide field of experimental chemotherapy shows that mopyridone is the first anti-influenza virus antiviral which targets M1 protein. Thus, the results of the timing-of-addition study merit special attention for understanding the M1 role in the influenza virus replication cycle.

In full agreement with these findings demonstrating different mode of anti-influenza virus action of mopyridone and rimantadine are the data showing a synergistic character of their combined effect (Galabov *et al.*, 1991), as well as an efficiency of mopyridone against *in vitro* replication of rimantadine-resistant influenza A(H3N2) virus mutants (V. Kalnina, personal communication).

All these studies characterize mopyridone as an effective antiviral against influenza viruses A (subtypes H3N2 and H2N2) and B.

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Reasons for Two Consecutive *Helicobacter pylori* Treatment Failures

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Abstract

The present case focuses on a symptomatic patient with two consecutive *Helicobacter pylori* treatment failures. *H. pylori* strain isolated from the patient was susceptible to amoxicillin, metronidazole, tetracycline and rifampin, but was resistant to clarithromycin and ciprofloxacin/levofloxacin. The first treatment failure could be the consequence of a suboptimal treatment regimen (esomeprazole, amoxicillin and tetracycline) despite *in vitro* susceptibility of the isolate to both antibiotics. The second eradication attempt (esomeprazole, amoxicillin, levofloxacin and colloidal bismuth subcitrate) was unsuccessful due to *in vitro* resistance of the isolate to quinolones. In conclusion, not all antibiotics can be successfully co-administered simultaneously for *H. pylori* eradication despite the *in vitro* susceptibility. The patients should inform clinicians about their previous clinical and paraclinical evaluations, including the bacterial susceptibility testing results to receive the most appropriate treatment regimen.

Key words: *Helicobacter pylori*, eradication, failure, suboptimal, regimen, resistance

Резюме

Настоящият случай фокусира симптоматичен пациент с две последователни неуспешни терапии за *Helicobacter pylori*. Щамът *H. pylori* изолиран от пациента беше чувствителен към амоксицилин, метронидазол, тетрациклин и рифампин, но резистентен към кларитромицин и ципрофлоксацин/левофлоксацин. Първият неуспех на терапията може да бъде следствие от субоптималния режим (esomeprazole, амоксицилин и тетрациклин) въпреки *in vitro* чувствителността на изолата на двата антибиотика. Вторият опит за ерадикация (esomeprazole, амоксицилин, левофлоксацин и колоиден висмут субцитрат) беше неуспешен поради *in vitro* резистентността на щамата към хинолоните. В заключение, не всички антибиотици могат да бъдат успешно комбинирани за ерадикация на *H. pylori* въпреки *in vitro* чувствителността. Пациентите трябва да информират клиницистите за техните предишни клинични и параклинични резултати, включително тези от тестовете за бактериалната чувствителност, за да получат най-подходящия режим на лечение.

Introduction

Helicobacter pylori eradication is difficult to achieve and the treatment success of first-line triple therapy has decreased over time (Smith *et al.*, 2014). Key reasons for treatment failure are poor patient compliance owing to complicated treatment regimens and *H. pylori* antibiotic resistance (Smith *et al.*, 2014), and an additional factor can be the use of inappropriate antibiotic regimens.

Case report

On 13 August 2014, a 46-year-old man with complaints of abdominal pain was admitted to the

Gastroenterology Unit of Department of Surgery, Medical University-Sofia for a gastroscopic evaluation. The gastroscopy showed the presence of reflux esophagitis, hiatal hernia and erosive gastroduodenitis. Gastric biopsy specimens were sent to the microbiology laboratory for *H. pylori* diagnostics.

H. pylori strain was isolated and identified as reported previously (Boyanova *et al.*, 2008) and the antibiotic susceptibility of the strain was tested by a breakpoint susceptibility testing method (Boyanova *et al.*, 2008) using Mueller-Hinton agar plates (Oxoid, Basingstoke, UK) with 5% sheep blood and one of the following antibiotic concentrations: amoxicillin 0.12, 0.25, 0.5, 1 or 2

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mg/L, metronidazole 4, 8 or 16 mg/L, clarithromycin 0.25, 0.5, 1, 2 or 4 mg/L, tetracycline 1 or 2 mg/L, and ciprofloxacin 1 or 10 mg/L. Susceptibility testing for ciprofloxacin was performed as a marker for the strain susceptibility to levofloxacin (Boyanova *et al.*, 2008). Rifampin susceptibility was tested by rifampin 5- μ g/disk (Oxoid, Basingstoke, UK). The antibiotics were obtained from Sigma-Aldrich, St. Louis, MO (amoxicillin, metronidazole, and tetracycline); Abbott Laboratories, Illinois (clarithromycin); and Actavis, Sofia, Bulgaria (ciprofloxacin).

The plates were incubated microaerophilically (CampyGen, Oxoid, UK) at 37° C for 2-3 days. Control strains were used for the susceptibility testing methods as previously described (Boyanova *et al.*, 2008). Resistance breakpoints were 8 mg/L metronidazole, 0.5 mg/L clarithromycin, 0.12 mg/L amoxicillin, 1 mg/L tetracycline and 1 mg/L levofloxacin (EUCAST, 2015). Strains with inhibition zone diameters of ≥ 21 mm were classified as rifampicin/rifabutin susceptible (Glocker *et al.*, 2007).

The isolated *H. pylori* strain was susceptible to amoxicillin, metronidazole, tetracycline and rifampin, but was resistant to clarithromycin and ciprofloxacin/levofloxacin.

Treatment was administered for 10 days with esomeprazole 20 mg bid, tetracycline 500 mg qid and amoxicillin 1000 mg bid.

On 18 December 2014, *H. pylori* eradication of the patient was assessed by a ^{13}C urea breath test (*Helicobacter* Test INFAI via mass spectrometric analysis) and the result was positive (difference 8.737) for *H. pylori* infection.

On 10 March 2015, due to absence of his treating gastroenterologist, the patient visited another physician and was administered esomeprazole 40 mg bid, amoxicillin 1000 mg bid, levofloxacin 250 mg bid and colloidal bismuth subcitrate 120 mg tid for 10 days.

On 29 April 2015, the patient underwent a stool antigen test for *H. pylori* (Premier Platinum HpSA PLUS, Meridian Bioscience, Cincinnati, OH, USA) and the result was positive (0.200 U/mL).

Discussion

In the present study, the first treatment regimen with esomeprazole, amoxicillin and tetracycline was unsuccessful. Similarly, Perri *et al.*, (2002) reported a low (35%) eradication rate by amoxicillin 1000 mg bid plus tetracycline 500 mg

qid and lansoprazole 30 mg bid for 14 days.

The second treatment regimen of the patient (esomeprazole, amoxicillin, levofloxacin and colloidal bismuth subcitrate) was also unsuccessful because the strain was *in vitro* resistant to quinolones, however, the patient did not report the susceptibility testing results to the physician. *H. pylori* fluoroquinolone resistance has been reported to decrease the success of the fluoroquinolone-based eradication of regimens by 41.7-66.7% in comparison with that of susceptible strains (Perna *et al.*, 2007; Nishizawa *et al.*, 2009).

An appropriate regimen for the patient can be the bismuth-containing quadruple therapy used with eradication success of 86% and 81.1% according to the intention-to-treat and per-protocol analyses, respectively (Gokcan *et al.*, 2015). The regimen includes bismuth salts, tetracycline, metronidazole and a proton pump inhibitor. Another suitable eradication regimen can be the rifabutin-based triple therapy, including a proton pump inhibitor, rifabutin and amoxicillin (Papastergiou *et al.*, 2014). In Korean patients (53 subjects), the eradication success of the regimen including lansoprazole (60 mg bid), amoxicillin (1000 mg tid) and rifabutin (150 mg bid) for 7 days was 96.3% according to the intention to treat analysis and 100% according to the per-protocol analysis (Lim *et al.*, 2014).

Conclusion

Two main conclusions can be drawn from the results of this case. Firstly, not all antibiotics to which the isolate is susceptible can be successfully co-administered simultaneously for *H. pylori* eradication. Secondly, the patients should inform the clinicians about their previous clinical and paraclinical evaluations, including the susceptibility testing results in order to obtain the optimal *H. pylori* eradication regimen.

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Monoclonal Antibody against Lipooligosaccharide of *Moraxella catarrhalis* Decreases Resistance to Aminopenicillins

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Abstract

The following *Moraxella catarrhalis* strains were used in the experiments: eight beta-lactamase-producing clinical isolates and four isolates including a control strain 353 CCUG without beta-lactamase. The other strains used as controls in this procedure were two isolates of *Staphylococcus aureus* and two of *Escherichia coli*: beta-lactamase producers and non-producers control strains of *S. aureus*: ATTC 29213 and *E. coli* ATCC 25922. All of the microbial strains were tested with ampicillin alone and with ampicillin in combination with murine monoclonal antibody (MAb) 219 against lipooligosaccharide of *M. catarrhalis* in a 96-well plate to determine the minimal inhibitory concentration (MIC). The testing was performed by broth dilution method and minimal bactericidal concentration (MBC) on brain heart infusion agar (BHI).

The effect of the combination of aminopenicillin with MAb219 was manifested as a fourfold reduction of the MIC and the MBC of the tested beta-lactamase producing *M. catarrhalis*. The effect over the control strains and moraxellae without beta-lactamase was either missing or there was a slight reduction of MIC and MBC.

The conclusion of the study is that MAb219 in combination with ampicillin in *in vitro* testing can decrease resistance to aminopenicillins in *M. catarrhalis* after the linking of MAb219 with lipooligosaccharide of the OM in the antigen-antibody complex.

Key words: *Moraxella catarrhalis*, beta-lactamase, lipooligosaccharide, monoclonal antibody

Резюме

В експериментите бяха използвани следните щамове *Moraxella catarrhalis*: осем бета-лактамаза продуциращи клинични изолата и четири, включително контролен щам 353 CCUG без бета-лактамаза. Другите щамове, използвани като контроли в тази процедура са два изолата на *Staphylococcus aureus* и два *Escherichia coli*: бета-лактамаза продуценти и непроизвеждащи бета-лактамази контролни щамове *S. aureus*: ATTC 29,213 и *E. coli* ATCC 25922. Всички микробни щамове бяха тествани с ампицилин самостоятелно и с ампицилин в комбинация с мише моноклонално анти тяло (MAb) 219 срещу липоолигозахарида на *M. catarrhalis* в 96-ямякова плака за определяне на минималната инхибираща концентрация (MIC). Тестването се извършва по метода на разреждане на бульон и минимална бактерицидна концентрация (MBC) на мозъчно-сърдечен инфуз (BHI) агар. Ефектът на комбинацията аминопеницин с MAb219 се проявява като четирикратно намаляване на MIC и MBC при изпитваните бета-лактамаза позитивни *M. catarrhalis*. Ефектът върху контролните щамове мораксели без бета-лактамаза беше липса или леко намаление на MIC и MBC. Заключение от проучването е, че MAb219 в комбинация с ампицилин в *in vitro* тестване може да се намали резистентността към аминопеницилините в *M. catarrhalis* след свързването на MAb219 с външномембрания липоолигозахарид в комплекс антиген-анти тяло.

Introduction

Moraxella catarrhalis is a Gram-negative diplococcus that colonizes the mucosa in healthy

preschool children and also is considered as an important cause of respiratory tract infections in children and elderly people (Gergova *et al.*, 2013; Hays 2009; Sethi *et al.*, 2007; Verduin *et al.*, 2002; Verhaegh *et al.*, 2011). The permanent expansion of infections caused by this microorganism in the last

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years is caused by two things: - on the one hand, the increased resistance to antimicrobial drugs by producing beta-lactamase (mainly aminopenicillins and first generation cephalosporins), and on the other hand, the resistance to the normal serum bactericidal effect. (Gergova *et al.*, 2009; Theoga *et al.*, 2014; Verhaegh *et al.*, 2011; Wirth *et al.* 2007; Zaleski *et al.* 2000). These interesting facts are a stimulus for new investigations into *M. catarrhalis* pathogenesis and its prevention with an appropriate vaccine (Luke-Marshall *et al.*, 2013; Gergova *et al.*, 2007; Mitov *et al.*, 2010; Ren, 2011; Tan *et al.*, 2007; Su, 2012). In previous studies, the murine monoclonal antibody MAb 219 IgM (Gergova *et al.* 2007) has been produced created and characterized as a bactericidal antibody against *M. catarrhalis*, *H. influenzae* and *H. parainfluenzae* in the presence of a complement.

The aim of this study is to determine the effect of the combination of MAb 219 (against lipooligosaccharide of *M. catarrhalis*) and aminopenicillin to decrease the resistance of beta-lactamase-producing moraxellae.

Material and methods

Eight clinical isolates of *M. catarrhalis*, previously identified as serotype A (Gergova *et al.* 2007) and BRO-1 producers, with *bro1* gene (Gergova *et al.* 2009) were randomly chosen. A control strain *M. catarrhalis* 353 CCUG and three other clinical serotype A isolates without *bro* gene were used.

Ampicillin (Sigma) was applied to determine the minimal inhibitory concentration (MIC) of *M. catarrhalis* strains by the broth dilution method and minimal bactericidal concentration (MBC) on brain heart infusion agar (BHI) using the agar dilution method, according to the Clinical and Laboratory Standards Institute (CLSI) 2014 criteria (CLSI, 2014).

Murine monoclonal antibody MAb 219 IgM, produced by hybridoma technology bactericidal with complement (C') only, (Gergova *et al.*, 2007) was used in undiluted and 1/4 to 1/64 diluted ascitic fluid to test the MIC and MBC of *M. catarrhalis* isolates for ampicillin in twofold dilutions by the broth dilution method (Mahon *et al.*, 2007). Two controls with ampicillin alone and with MAb 219 IgM alone for missing suppressing effect were used to each strain (0.5 ml PBS was added versus MAb with ampicillin). The synergistic action of MAb 219 IgM and ampicillin was tested three times.

The strains of *M. catarrhalis* (diluted in 0.5 ml PBS to 0,5 MF) with the antibacterial agent - 0.5ml and 0.5ml MAb 219 on a 96-well polystyrene microtiter plate (Nunc) were incubated at 35°C for 2 hours. After incubation, 20µl were dropped from each dilution to BHI. After incubation at 35°C/24 hours of the 96-well plates and petri dishes with BHI, the MIC in the plates and MBC on the agar were determined.

Table 1. Results of MICs and MBCs of tested *M. catarrhalis* and control strains

Microbial strain	Ampicillin alone		Amp with MAb219 undiluted		Amp with MAb219 diluted ½		Amp with MAb219 diluted ¼	
	MIC mg/L	MBC mg/L	MIC mg/L	MBC mg/L	MIC mg/L	MBC mg/L	MIC mg/L	MBC mg/L
<i>M. catarrhalis</i> bro 1 (n=4)	8	16	2	4	2	4	4	8
<i>M. catarrhalis</i> bro 1 (n=4)	16	32	4	8	4	8	8	16
<i>M. catarrhalis</i> without bro (n=3)	0.25	0.5	0.125	0.25	0.125	0.25	0.25	0.5
<i>M. catarrhalis</i> 353 CCUG	0.125	0.25	0.125	0.25	0.125	0.25	0.25	0.5
<i>S. aureus</i> (n=2)	16	32	16	32	16	32	16	32
<i>S. aureus</i> ATTC 29213	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5
<i>E. coli</i> (n=2)	64	128	64	128	64	128	64	128
<i>E. coli</i> ATCC 25922	0.5	1	0.5	1	0.5	1	0.5	1

Results

The MICs of ampicillin alone of the selected strains with *bro1* gene were in the range of 8-16 mg/L, and the MBCs were in the range of 16-32 mg/L (Table 1). When ampicillin was used in combination with MAb 219 undiluted, the MICs and the MBCs of the tested *M. catarrhalis* isolates decreased four times. After a ¼ dilution of the MAb 219, the effect of the combination decreased. The other strains (without *bro* gene) were sensitive to ampicillin with MIC - 0.25 mg/L and MBC - 0.5 mg/L, respectively. The missing or minor effect of the combination aminopenicillin with MAb was a reduction of the MIC and the MBC (one diluting dilution of the control strains).

Discussion

It is known that ampicillin, like other beta-lactam antimicrobial agents, must use penicillin binding proteins (PBPs) to pass through the bacterial cell wall (Mahon *et al.*, 2007). In *M. catarrhalis* an essential outer membrane porin M35 has already been established, a potential vaccine target that mediates the transport and susceptibility to aminopenicillins (Jetter *et al.*, 2009).

The role of LPS is still being researched as an attractive vaccine candidate such as antigen of the cell wall of *M. catarrhalis* with a unique structure of glycolipids (Gergova *et al.* 2007; Martin *et al.*, 2015). The contribution of the LPS - as a fundamental element of the OM of the Gram-negative bacteria, in the transport through the Gram-negative cell wall is still very unclear. During the last years that process has been studied extensively especially in *Escherichia coli*, *Salmonella enterica*, *Neisseria meningitidis* and *M. catarrhalis* as appropriate subjects (Bos *et al.*, 2007; Clements *et al.*, 2002; Martin *et al.*, 2015). The function of LPS is known to be a formidable barrier for hydrophobic molecules. The LPS also mediated rearrangements in the OM surface-exposed loops of few proteins. The so-called Lpt system consists of seven LPS transport proteins (LptA-G) (Bos *et al.*, 2007; Bowyer, 2011; Narita, 2009, Narita, 2011). Molecules participating in the biogenesis of LPS may represent new antimicrobial targets, as demonstrated by the development of antibiotics targeting LpxC, an enzyme involved LPS biogenesis and LptD, an OM protein involved in LPS transport. As already known, LpxC is a target for the bacteriostatic agent chloramphenicol. Mutations in the *lpxC* gene can lead to increased susceptibility to other antibiotics, due to the changed permeability in the gram-negative OM (Clements

et al., 2002). LPS with protection of OMP OprH is linked with aminoglycosides permeability in *P. aeruginosa*, but no data about the participation of LPS in beta-lactams transport (Moore and Flaws, 2011) has been proved.

The results in this study were provided to test the hypothesis that some antibodies against lipopolysaccharide (LPS) of *M. catarrhalis* in the complex with ampicillin reduce the resistance to aminopenicillins.

The cases of *M. catarrhalis* respiratory diseases will increase further in the next years after the introduction of vaccines against *Streptococcus pneumoniae* and *Haemophilus influenzae*, because of the biological niche they opened for the spread of *M. catarrhalis* (Gergova *et al.*, 2013; Hays, 2009). The features and the qualities of the LOS of *M. catarrhalis* therefore are examined and options are still being sought for obtaining a vaccine with a part of this immunogenic ingredient (Luke-Marshall *et al.*, 2013; Ren, 2011; Tan and Riesbeck, 2007; Su, 2012). It is known that MAb219 against the common epitope for the three serotypes of *M. catarrhalis* LOS is bactericidal in the presence of C' (Gergova *et al.*, 2007), but now it is established that MAb219 can increase susceptibility to aminopenicillins in vitro. This result supports the idea of common epitope from *M. catarrhalis* LOS to run as a prospective vaccine target, which stimulates the production of very useful antibodies. The resistance to aminopenicillins in clinical isolates of *M. catarrhalis* is increased and is now near 100%. The antibodies against A, B, C common epitope of LOS can recover the susceptibility of *M. catarrhalis* to the most adequate antimicrobial agents for the childhood - aminopenicillins.

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Promotion of the Synthesis of a Concanavalin A-reactive Polysaccharide Upon Growth of *Escherichia coli* O157:H(-) on Solid Medium at 37°C

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Abstract

The polysaccharides exposed at the bacterial surface are involved in many processes related with the interaction of microorganisms with their environments, outside or inside the mammalian host. The aim of this study was to test the effects of growth temperatures (20°C and 37°C) on some characteristics of the surface polysaccharides of *E. coli* O157:H(-). The glycoconjugates were isolated by hot phenol-water extraction. The tests included ELISA, ELLA, immunoelectrophoresis (classical, or modified for application with concanavalin A/ConA/) and Western blot. It was shown that the water-phase fraction of the strain cultivated at 37°C was distinctive among the compared samples. It was poorly recognized by specific anti-*E. coli* O157 antiserum but, contrary to all the other fractions, interacted well with the lectin, ConA. A similar ConA-reactivity was demonstrated for the purified cyclic enterobacterial common antigen (ECACYC4). This implies that the ConA reactivity may likely be due to ECACYC. The promotion of the release of this ConA-reactive entity at 37°C, i.e., the temperature condition characteristic for host body, indicates its importance in the host-invader interplay.

Key words: *E. coli* O157, polysaccharides, ConA, ECA_{CYC}

Резюме

Полизахаридите, локализирани по клетъчната повърхност на бактериите, играят роля при множество процеси, свързани с взаимодействието между микробите и обкръжаващата ги среда извън или вътре в техния гостоприемник. Целта на изследването е да се изясни ефектът на растежната температура (20°C и 37°C) върху някои характеристики на повърхностните полизахариди на *E. coli* O157:H(-). Изолирането се осъществи чрез екстракция с воден разтвор на фенол при 60°C. Проведени са тестове ELISA, ELLA, имуноелектрофореза (класическа или модифицирана за работа с конканавалин А /ConA/) и имуноблот. Показано е, че фракцията от водна фаза от щама, култивиран при 37°C, се отличава съществено от останалите проби. Тя се разпознава много слабо от специфичен анти-*E. coli* O157 антисерум, но за разлика от всички останали фракции, се свързва с лектина КонА. Подобна КонА-реактивност се демонстрира и за проба от пречистен цикличен ентеробактериален общ антиген (ECA_{CYC4}). Това сочи, че КонА-реактивността на фракцията е възможно да е дължи на ECA_{CYC}. Стимулираното освобождаване на КонА-реактивните молекули при 37°C, т.е., температура, характерна за тялото на гостоприемника, сочи тяхната важност при взаимоотношението бактерии-гостоприемник.

Introduction

Complex oligo- and polysaccharides located at the surfaces of bacterial, plant and animal cells can serve structural roles, mediate movement of glycoconjugates to the cell surface, act as markers

that mediate cell-cell and cell-matrix recognition (Slifkin and Doyle, 1990), etc. In Gram-negative bacteria, the cell surface glycome is represented by lipopolysaccharides (LPS), capsules (where available) and exopolysaccharides, although gly-

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coproteins may also contribute. In addition, the Enterobacteriaceae produce a surface polysaccharide known as “enterobacterial common antigen” (ECA) (Erbel *et al.*, 2003). The synthesis of these polysaccharides are regulated in response to environmental clues (Merino *et al.*, 1992, Lerouge and Vanderleyden, 2001), hence the heterogeneity and dynamics of the bacterial glycome presents a major challenge for analysts.

The present study examines polysaccharides isolated from *Escherichia coli* O157:H(-). Strains with this O-serotype are often the agents of food-borne infections that may sometimes cause severe, even lethal complications (Kaper *et al.*, 2004). The bacterial cell-surface polysaccharides are important participants in both the host-invader interplay and the survival of the bacteria in the environment (Greenfield and Whitfield, 2012). The transmission of *E. coli* O157:H(-) via its niches outside and inside the human body is accompanied with the respective temperature shifts. The question is: are these environmental shifts accompanied by changes in the glycome associated with the bacterial surface?

The present study combines the application of immunological approaches and glycan-binding proteins (lectins) to address the question whether the changes in cultivation temperature can influence the cell surface-associated glycome of the strain. The bacterial antigens were isolated by the most-widely applied method for LPS isolation, the method of Westphal and Jann (1965). It is based on the surface polysaccharide extraction with water phenol solution at 60°C followed by centrifugation upon which water and a phenol phases form. For most Gram-negative strains, the LPS is further isolated from the water-phase fraction. Unlike this, the O157 antigen is isolated from the phenol phase (Perry *et al.*, 1986; Vinogradov *et al.*, 1998). To our knowledge, water-phase polysaccharides of *E. coli* O157 have been examined earlier in two studies (Dodds *et al.*, 1987 a, b). Using the immunoblot technique, the authors have shown cross-reactivity of these samples with heterologous antisera from *Brucella abortus*, *Yersinia enterocolitica* O9 and *Vibrio cholerae*. Later on, the water, but not the phenol phase, of the water-phenol extract has been shown to contain two cyclic forms of ECA, a tetramer and a pentamer (Fregolino *et al.*, 2012). Bearing in mind that the growth temperature effects (if any) might be registered in either the water-, or the phenol-phase polysaccharides, or both, we compared these fractions isolated from bacteria grown at 20°C and 37°C.

Materials and Methods

Strain and cultivation

E. coli O157:H-, A2CK SS was used in the study. It is a Stx1⁻, Stx2⁻ strain. Overnight nutrient broth culture of the strain was used as inoculum. The bacteria were cultivated on nutrient agar for 24 h at 37°C or 48 h at 20°C.

Polysaccharides isolation

The method of Westphal and Jann (1965) was applied for isolation of the crude antigen fractions. The phenol-water extraction was performed for 1 h at 60°C with continuous stirring. The samples were centrifuged, the water and phenol phases were separated, dialysed extensively against running tap water for 4 days, and freeze-dried. ECACYC4 was purified as described earlier (Fregolino *et al.*, 2012). The polysaccharides were dissolved as 2 mg/ml stocks in double distilled, deionized water and kept frozen until use.

Reagents

Polyclonal rabbit diagnostic serum: anti-*E. coli* O157 (BulBio-NCIPD, Sofia, Bulgaria) was used as primary antibody. Anti-rabbit IgG-peroxidase (Sigma) was used as a secondary antibody. The following lectins were included: lectin from *Canavalia ensiformis* (concanavalin A, or ConA), lectin from *Triticum vulgaris*, or wheat-germ agglutinin (WGA), soybean agglutinin (SBA) *Ulex europaeus* agglutinin-I (UEA-I). Lectin-biotin and lectin-peroxidase conjugates were purchased from Vector Labs. Unlabeled ConA (Pharmacia) was applied for immunoelectrophoresis experiments. Avidin -peroxidase (Sigma) was used.

Dot blot

For dot-blot experiments, 1 mg/ml solutions of the polysaccharide samples were prepared in 0.2 M TBS (0.2 M Tris and 0.15 M NaCl), pH 7.3 with two further decimal dilutions. Samples of 5 µl were applied on nitrocellulose disks, allowed to dry, and the procedure was repeated so that the final amount of carbohydrate loaded to the disks was 10, 1, and 0.1 µg, respectively. Blocking of non-specific binding was by incubation for 1 hour in 5% BSA (Calbiochem). The disks were further incubated in ConA-peroxidase (1:100 in TBS containing 0.2 mM CaCl₂), or SBA-peroxidase, WGA-peroxidase and UEA-I-peroxidase (1:100 in TBS). Incubation in lectins was for 2 hours, followed by three washes in 0.05% Tween 20 in TBS, two washes in TBS, and one in distilled water. Controls included processing of disks devoid of antigen, or incubation in TBS instead of lectin. The peroxidase activity was demonstrated by short incubation in a mixture of

Na nitroprusside (0.4 g) and *o*-dianizidine dihydrochloride (0.04 g) in 100 ml of water to which 50 µl of 30% H₂O₂ was added.

Enzyme-linked immunosorbent assay (ELISA) and enzyme-linked lectin assay (ELLA)

For ELISA experiments, the aqueous stock solution of the polysaccharide samples was dissolved 1:10 in carbonate buffer, pH 9.6. The wells of 96-well flat-bottom microtitre plates were filled with 50 µl of the diluted antigen (final antigen amount of 5 µg) and loading was performed overnight at 4°C. Followed blocking with 2% BSA in PBS for 2 h at 37°C. Further protocols differed depending on the binding to be checked. To test the reactivity with the immune sera, 100 µl of serially diluted serum was applied to the wells and incubated for 1 h at 37°C. As a secondary antibody, peroxidase-conjugated anti-rabbit IgG was applied. To check for binding of the lectin ConA, ELLA test was applied. The wells were blocked with 5% BSA in PBS. Various amounts of ConA-biotin diluted in PBS containing 0.2 mM CaCl₂ were applied onto the wells and incubated for 2 h at room temperature. After thorough washes followed incubation in avidin-peroxidase for 1 h. In both protocols controls with the omission of each of the labelling molecules were included. The ELLA test included also inhibition control in the presence of 0.2 M methyl mannoside. For both ELISA and ELLA experiments, the peroxidase activity was determined using a substrate solution of *o*-phenylene diamine and H₂O₂. The measurements were performed on a plate reader at 492 nm.

Immunoelectrophoresis

Immunoelectrophoresis was performed on 2% agarose gel in veronal buffer, pH 8.6, with the addition of 0.2 mM CaCl₂ for the test with ConA. The polysaccharide stock solutions were applied into the pits and electrophoresis was done for 1 h at 120 V. Then troughs were cut into the gel and filled with either rabbit anti-*E. coli* O157 serum (1:100 in PBS), or 1 mg/ml ConA (Calbiochem), dissolved in TBS containing 0.2 mM CaCl₂. The gel was placed in wet chamber and diffusion proceeded for 24 hours at room temperature. After an overnight wash in 0.9% NaCl, the gel was dried and coloured for 15 min in Coomassie brilliant blue R 250.

Western blot

Western blot was performed on a BioRad mini-gel equipment. Initially, SDS-PAGE electrophoresis of the samples was done on 12% separating

acrylamide gel for 1 h at 180 V. Followed transfer onto nitrocellulose membrane for 1 h at 180 V. Blocking of non-specific binding was by incubation for 2 h in 1% BSA in PBS at room temperature. After extensive washes in PBS/BSA/0.01% Tween 20, the membrane was incubated overnight in rabbit anti-*E. coli* O157 serum (1:100 in PBS) at room temperature with continuous shaking. After three washes, followed 2 h incubation in the secondary antibody (anti-rabbit IgG-peroxidase, 1:400). Demonstration of the peroxidase activity was as in dot-blot.

Results

The dot-blot test applied with lectins showed positive reactivity only with ConA. This was registered for the water-phase fraction isolated from 37°C culture, as polysaccharide dose-dependent change in color intensity (data not shown). The other samples were negative. No reactivity with the other lectins was found.

The immunoreactivities of the fractions were examined by three approaches: ELISA, immunoelectrophoresis, and Western blot (Fig. 1). The three tests confirmed differences between the samples. Notably, the water-phase preparation of the bacteria cultivated at 37°C was characterized throughout by a low affinity for the polyclonal anti-*E. coli* O157 serum.

Bearing in mind the results from the dot-blot experiment, the further comparison between the fractions was performed with ConA. By both ELLA and immunoelectrophoresis, the water-phase fraction isolated from bacteria grown at 37°C was distinctive by its affinity for the lectin (Fig. 2). Upon immunoelectrophoresis, this sample produced two distinct precipitation bands with Con A. Some lower affinity for the lectin was registered for the water-phase fraction isolated from the strain grown at 20°C.

We have previously shown that ECA_{CYC} purified from the water phase phenol-water extract of the strain was non-reactive with anti-*E. coli* O157 specific antiserum, but reacted well with the C-type, mannan-binding lectin (Paunova-Krasteva *et al.*, 2014). Therefore, the next question we tried to answer here was whether the purified ECA_{CYC4} could react with the here tested C-type lectin, ConA. The ELLA test performed with purified ECA_{CYC4} showed a well-expressed ConA-binding affinity (Fig. 3) which implies a similarity of this purified antigen with the water-phase crude fraction, 37°C (Fig. 2A).

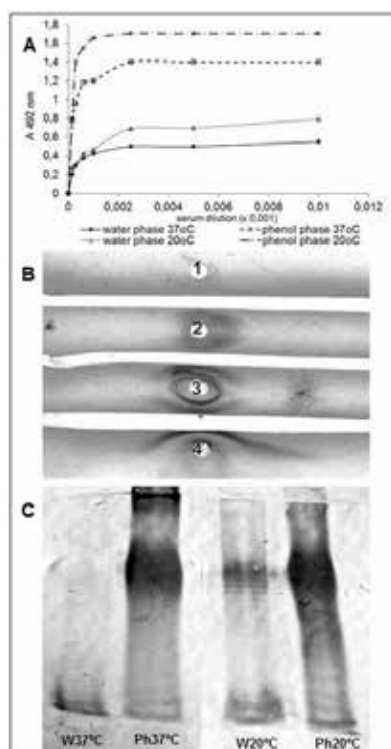


Fig. 1 Comparison of the reactivity of the water- and phenol-phase polysaccharides with polyclonal diagnostic rabbit anti-*E. coli* O157 serum. A, ELISA; B, immunoelectrophoresis: (1) water phase polysaccharide, 37°C, (2) water phase polysaccharide, 20°C, (3) phenol phase polysaccharide, 37°C, (4) phenol phase polysaccharide, 20°C; C, Western blot of the fractions.

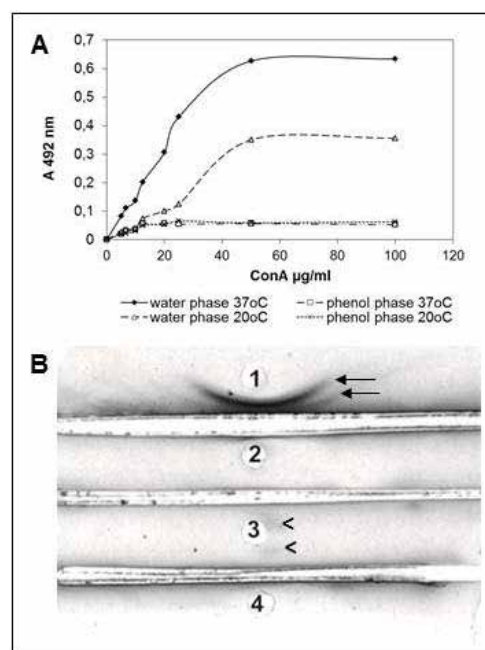


Fig. 2. Comparison of the reactivity of the water- and phenol-phase polysaccharides with ConA. A, ELLA; B, immunoelectrophoresis: (1) water phase polysaccharide, 37°C, (2) phenol phase polysaccharide, 37°C, (3) water phase polysaccharide, 20°C, (4) phenol phase polysaccharide, 20°C. In sample (1), two distinct precipitation bands are formed (arrows); in sample (3), more obscure areas slightly colored with Coomassie are registered (arrowheads).

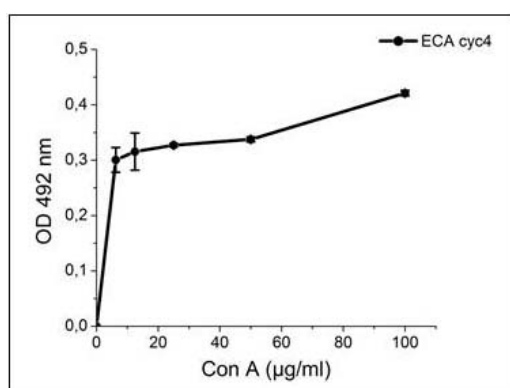


Fig. 3. ELLA test showing the ConA reactivity of purified ECA_{CYC4}.

Discussion

The present results show that the water-phase polysaccharide fraction of the strain is peculiar for its lack of immunoreactivity and the high affinity for ConA. In its low immunoreactivity, our data on this ConA - positive fraction is alike our previous results on ECA_{CYC} (Paunova-Krasteva *et al.*, 2014). What is more, here we demonstrated the affinity of

purified ECA_{CYC} for ConA. We can therefore conclude that the ConA reactivity of the water-phase crude extract from the strain grown at 37°C is due to ECA_{CYC}. Some, significantly less distinct ConA-reactivity was observed in the water-phase crude preparation of cells grown at 20°C. While this does not exclude the possibility that some amount of ECA_{CYC} may be produced also at this temperature, the results of the present study confirm a promotion of the synthesis of the ConA-reactive molecules when the strain was cultivated at 37°C.

ConA has long been known for its specific binding of mannosyl or glycosyl non-reducing termini in glycoconjugates (Doyle, 1994). More recently however the multivalency of this lectin has attracted attention, based on newly synthesized multivalency glycoligands (Wittmann and Pieters, 2013; Ponader *et al.*, 2014). The accommodation of such ligands is via the so-called “expanded binding site” which additionally strengthens the binding affinity via weak multi-locus molecular interactions (Moothoo and Naismith, 1998). Such type of reac-

tivity might expectedly be involved also in the system tested here.

The similarity in the ConA affinities of the crude water-phase fraction and the purified ECA_{CYC} is of special concern. We have previously shown that ECA_{CYC} may be considered as MAMP-ligands that interact with the host humoral mechanisms of non-self recognition (Paunova-Krasteva *et al.*, 2014). The promotion of the release of the ConA-reactive entity (most likely - ECA_{CYC}) at 37°C, i.e., temperature condition characteristic for host body, indicates its importance in the host-invader interplay.

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Antioxidant Activity of *Helix aspersa maxima* (Gastropod) Hemocyanin

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Abstract

Hemocyanins (Hcs) are oligomeric copper-containing proteins that function as oxygen carriers in the hemolymph of several mollusks and arthropods. In this study we present an investigation on the antioxidant activity of the Hc isolated from snails *Helix aspersa maxima* (HaH), using various experimental models.

The free radical scavenging activity was determined against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] free radical cation (ABTS). Cupric ion reducing antioxidant capacity (CUPRAC) and Ferric-reducing antioxidant power (FRAP) assay also were carried out. The results obtained show that HaH exhibits good radical scavenging activity by 59.54% reduction of the DPPH and 62.31% inhibition of ABTS radicals. Furthermore, HaH demonstrated a strong chelating effect on copper ions measured through CUPRAC method.

In conclusion, the present study revealed for the first time the antioxidant properties of molluscan Hc. The antioxidative activity of HaH could probably involve quenching of reactive oxygen species and metal ion chelation, thereby reducing the potential of prooxidants to attack cellular components.

Keywords: hemocyanin, *Helix aspersa*, mollusc, antioxidant activity

Резюме

Хемоцианините са олигомерни мед-съдържащи протеини, които функционират като кислородни носители в хемолимфата на повечето мекотели и ракообразни организми. Представено е изследване на антиоксидантната активност на хемоцианин, изолиран от охлюви *Helix aspersa maxima* (HaH), при използване на различни експериментални модели.

Радикал улавящата активност на хемоцианина е определена спрямо свободните радикали 1,1-diphenyl-2-picrylhydrazyl (DPPH) и [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS). Антиоксидантната активност на хемоцианина е анализирана и с методите CUPRAC и FRAP, които отчитат степента на електронен трансфер на антиоксиданта към радикала. Получените резултати показват, че HaH притежава добра радикал улавяща активност, изразена в 59,54% намаление на DPPH и 62,31% инхибиране на ABTS радикалите. Освен това, анализите с метода CUPRAC демонстрират, че HaH притежава силна способност да хелатира медни йони.

В заключение, настоящото изследване демонстрира за първи път антиоксидантните свойства на хемоцианин от молюски. Вероятно, антиоксидантната активност на HaH включва, както деактивиране на реактивни форми на кислорода, така и хелатиране на метални йони, като по този начин намалява потенциала на прооксидантите да атакуват клетъчни компоненти.

Introduction

Reactive oxygen species (ROS) are involved in normal and pathogenic oxygen metabolism. That is why, oxidative stress occurs when ROS (O_2 , OH , H_2O_2 and RO_2) exceed the organism's protective

capacity to remove or quench them, thus leading to cellular lipids, proteins and DNA damage resulting in aging and carcinogenesis. Therefore, it is of critical importance for the organism to possess well working mechanisms for protection against foreign

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and self-produced toxic substances, such as redox systems. Many investigations have proved the positive response to the oxidative stress of catalase, superoxide dismutase, glutathione/glutaredoxin and thioredoxine systems, devoting their activity to the presence of sulphydryl (-SH) groups (Aispuro-Hernandez *et al.*, 2008) or transition metals like copper, nickel and zinc (Shobana *et al.*, 2013). Copper is also a substantial catalytic co-factor in biological systems for a variety of metabolic reactions, electron transfer and oxygen transport proteins such as azurin, plastocyanin, laccase and hemocyanin. Copper acts as a reductant in the enzyme superoxide dismutase, cytochrome oxidase, lysyl oxidase, dopamine hydroxylase and several other oxidases which reduce molecular oxygen (Mistry *et al.*, 2003).

Hemocyanins (Hcs) are respiratory proteins of high molecular mass that use copper binding sites to bind and transport oxygen in the hemolymph of many arthropods and mollusks (van Holde and Miller, 1982). Molluscan Hcs are potent natural immunostimulants. When inoculated in mammals, they enhance the innate and adaptive immune response with beneficial clinical applications (Harris and Markl, 1999; Tchobanov *et al.*, 2008; Arancibia *et al.*, 2012; Gesheva *et al.*, 2015). Hcs have been used for therapy of superficial bladder cancer and murine melanoma models (Jurincic *et al.*, 1988; Rizvi *et al.*, 2007). Very recently, we demonstrated a potential anti-cancer effect of Hcs on a murine model of colon carcinoma suggesting their use for immunotherapy of different types of cancer (Gesheva *et al.*, 2014). Quite often good immunomodulators exhibit good antiradical and antioxidant properties. Till now, there has not been enough information about the antioxidant activity of Hcs. The only scientific report concerning an antioxidant activity of Hc is the one of Queinnec *et al.*, 1999. In order to determine the antioxidant activity of Hc, isolated from scorpion *Androctonus australis*, the authors have investigated the kinetics of superoxide anion decays using pulse radiolysis. Recently, a radioprotective effect of *Rapana thomasiana* hemocyanin (RtH) in gamma induced acute radiation syndrome has been established (Kindekov *et al.*, 2014). It was suggested that the radical-scavenging properties of this Hc are at the basis of the mitigation of radiation injuries.

In this study, we present for a first time a detailed investigation on the antioxidant activity of a molluscan Hc, namely the hemocyanin isolated from garden snails *Helix aspersa* maxima (HaH),

using various experimental models.

Material and Methods

Hemocyanin preparation

HaH was isolated from the hemolymph of garden snails *Helix aspersa* maxima and purified additionally by gel filtration chromatography as described in Raynova *et al.*, 2013. The protein concentration was determined spectrophotometrically using the specific absorption coefficient $A_{278}^{0.1\%} = 1.42 \text{ mg}^{-1} \text{ ml}^{-1} \text{ cm}^{-1}$ (20°C).

Antioxidant activity determination

DPPH assay

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma) scavenging activity assay was performed according to the procedure described by Thaipong *et al.*, 2006, with slight modifications. A solution of 0.1 mmol DPPH in methanol was prepared and 2.85 mL of the solution were mixed with a 0.15 mL sample. A blank sample was prepared in the same way, but replacing the sample with the same amount of water. The reaction mixture was incubated at 37°C in darkness and the decrease in the absorbance was measured spectrophotometrically after 15 min at 517 nm against methanol. The antioxidant activity was expressed as mmol Trolox equivalents (TE) per gram protein, using calibration curve (absorption vs. concentration) of Trolox dissolved in methanol at different concentrations (0.1; 0.2; 0.3; 0.4; 0.5 mmol).

ABTS assay

The procedure was previously described by Re *et al.*, 1999, and some modifications were applied. A stable (for 2-3days) ABTS radical was generated after 16 h at ambient temperature in darkness by mixing in equal quantities of two stock solutions: 7.0 mmol 2,2'-azinobis (3)-ethylbenzthiazoline-6-sulfonic acid (Sigma) in dd H₂O and 2.45 mmol K₂S₂O₈ (Sigma) in dd H₂O. Prior the analysis, 2.0 mL of the ABTS^{•+} solution were diluted to 60 mL with methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm. 0.15 mL of the samples were allowed to react with 2.85 mL freshly prepared ABTS^{•+} solution and after 15 min at 37°C the decolorization of ABTS^{•+} was recorded at 734 nm against methanol. A blank sample was prepared in the same way, but replacing the sample with the same amount of water and measured against methanol as well. Results were expressed in terms of Trolox equivalent antioxidant capacity (mmol Trolox equivalents per gram protein), using calibration curve (absorption vs. concentration) of Trolox dissolved in methanol at different concen-

trations (0.1; 0.2; 0.3; 0.4; 0.5 mmol).

Ferric reducing antioxidant power (FRAP)

FRAP assay was conducted by following the modified method of Benzie and Strain (Benzie and Strain, 1996). Fresh FRAP reagent was prepared by mixing the following stock solutions: 10 parts of 300 mmol sodium acetate buffer with pH 3.6; 1 part of 10 mmol 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma) solution in 40 mmol HCl and 1 part of 20 mmol $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Sigma) solution in dd H_2O . The working sample was prepared by mixing 3.0 mL of FRAP reagent with 0.1 mL of the investigated sample. The blank sample was prepared in the same way, but the sample was replaced with water. After 4 min at 37°C, the absorption was read against the blank sample at 593 nm. The results were expressed as mmol Trolox equivalents per gram protein, using calibration curve (absorption vs. concentration) of Trolox dissolved in methanol at different concentrations (0.1; 0.2; 0.3; 0.4; 0.5 mmol).

Cupric reducing antioxidant capacity (CUPRAC) assay

The method was adapted according to Apak *et al.*, 2004. Stock solutions of 10 mmol $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (Sigma) in dd H_2O ; 1.0 M ammonium acetate buffer in dd H_2O with pH 7.0; 7.5 mmol neocuproine (Sigma) in 96% ethanol were prepared. The reaction was performed in the following order: 1.0 mL 10 mmol $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ + 1.0 mL 7.5 mmol neocuproine + 1.0 mL 1.0 M ammonium acetate buffer + 0.1 mL of the investigated sample + 1.0 mL methanol. A blank sample was prepared in the same way, but using water instead. The reaction mixture was heated to 50°C for 20 min and the absorbance was measured at 450 nm against the blank sample. Results were expressed as mmol Trolox equivalents per gram protein, using calibration curve (absorption vs. concentration) of Trolox dissolved in methanol at different concentrations (0.1; 0.2; 0.3; 0.4; 0.5 mmol).

Statistics

The antioxidant activity of HaH was analyzed according to the above mentioned methods in triplicates. The presented values are means ($n=3$)

with the corresponding standard deviations ($\pm$ SD).

Results and Discussions

Until now there has not been a unified method that ensures full assessment of the antioxidant properties of a given compound against all radicals. The antioxidant activity of hemocyanin from marine snails *Rapana thomasiana* was tested in an experimental liposomal system (Kindekov *et al.*, 2014). The results about Fe^{2+} - induced oxidation of an aqueous emulsion system of egg liposomes showed about 30 % less antioxidant activity of Rth compared to a common antioxidant butyl hydroxytoluene (BHT). An enhanced chemiluminescence-based assay has been used to measure the antioxidant capacity of albumin and other proteins (Medina-Navarro *et al.*, 2010).

For the purpose of a more complete characterization of Hc from snails *Helix aspersa maxima*, in the present study the antioxidant activity was determined according to four complementary methods that rely on different reaction mechanisms. DPPH and ABTS utilize predominantly Hydrogen Atom Transfer reaction mechanism (HAT), while FRAP and CUPRAC methods are based on Single Electron Transfer mechanism (SET). This is the first report for evaluation of antioxidant activity of Hc through the aforementioned methods. The data are presented on Table 1.

DPPH is based on the measurement of the scavenging ability of antioxidants towards the stable radical 1,1-diphenyl-2-picrylhydrazyl. The antioxidant activity of HaH according to this method is 13.86 mM Trolox equivalents/g protein and 59.54 % inhibition of the DPPH radical.

The ABTS assay is based on the decolorization of the ABTS radical. The antioxidant activity here is higher than that measured according to DPPH - 93.94 mM Trolox equivalents/g protein and 62.31 % inhibition of the ABTS radical. This higher activity here could be explained by the structural configuration of the DPPH radical which hinders high molecular antioxidants from interacting

Table 1. Antioxidant activity of *Helix aspersa maxima* (garden snail) hemocyanin, analyzed by different methods and radical inhibition.

DPPH		ABTS		FRAP	CUPRAC
mM Trolox equivalents/g protein	Inhibition of DPPH radical, (%)	mM Trolox equivalents/g protein	Inhibition of ABTS radical, (%)	mM Trolox equivalents/g protein	mM Trolox equivalents/g protein
13,86 $\pm$ 0,18	59,54 $\pm$ 1,29	93,94 $\pm$ 2,25	62,31 $\pm$ 1,52	3,68 $\pm$ 1,00	412,65 $\pm$ 6,35

with it (Sánchez-Moreno *et al.*, 2002). Elias *et al.*, 2008, have noticed that the protein's overall antioxidant activity can be increased by disruption of its tertiary structure to increase the solvent accessibility of amino acid residues that can scavenge free radicals and chelate prooxidative metals.

The reducing power of FRAP is based on SET mechanism and cannot detect antioxidants that act by radical quenching (H transfer), especially thiols and proteins. The method is based on the reduction of Fe (III) to Fe (II) and was carried out at pH 3.6 to maintain its solubility. Originally, the method was developed to measure the reducing power in plasma and later adapted to assay other antioxidants (Prior *et al.*, 2005). But obviously these conditions are not suitable for HaH and here the lowest antioxidant values of 3.68 mM Trolox equivalents/g protein are detected.

CUPRAC is a method also based on SET mechanism like FRAP. But the method is carried out at different conditions, pH 7.0 and is based on the reduction of Cu (II) to Cu (I) (Prior *et al.*, 2005). Here, the highest values for an antioxidant activity of HaH - 412.65 mM Trolox equivalents/g protein are detected. This pH is more favourable for the redox potential, which facilitates the electron transfer and makes it suitable for hydrophilic and lipophilic antioxidants (Prior *et al.*, 2005). Nevertheless, the ability to reduce transition metal ions as Cu (II) is considered to be a potent pro-antioxidant activity of the antioxidants (Mistry *et al.*, 2003; Prior *et al.*, 2005). The physiological value of pH (7.0) is in the pH-stability region of the protein, so it is able to reveal its antioxidant potential in a better way.

The reduction of iron and copper and the formation of Cu(I) complexes is of critical importance, since when they are in "free" form they can catalyze the production of highly toxic hydroxyl radicals. When they are kept at minimum concentrations by sequestering the metals in complexes, they are unable to take part in redox reactions with activated oxygen species (Brouwer *et al.*, 1998; Levy *et al.*, 2001). In a review, Roche *et al.*, 2008, show that the antioxidant activity of serum albumin is mainly due to its ligand-binding capacity, especially the binding of free transition metals, so the protein is able to limit the damage caused by hydroxyl radicals produced from Fenton reaction between iron/copper and H₂O₂.

The high copper reduction capacity in our study suggests that hemocyanin could be the first line of defence system against copper toxicity and serve as a copper chelator by sequestering the metal

in a non-redox-active form. This mechanism of the antioxidant activity is beneficial to living organisms due to the preventive oxidative damage of the cellular membranes and is essential for cell survival.

Conclusion

In conclusion, for the first time the antioxidant activity of Hc from garden snails *Helix aspersa maxima* was determined by DPPH, ABTS, FRAP and CUPRAC methods. These methods are suitable to demonstrate the ability of this Hc to scavenge different radicals and to reduce or chelate different ions as Fe (II) and Cu (II). The highest antioxidant activity was recorded by CUPRAC method, which is evidence of the high pro-antioxidant activity of the protein and that these conditions are the most suitable for revealing its antioxidant potential. According to our investigations, Hc might perform an essential detoxification function against copper and iron as a constituent of the antioxidant defence network in the garden snail *Helix aspersa maxima*. This function would be beneficial for maintaining the metal homeostasis and protect the function of cellular structures against the damaging effects of reactive oxygen species.

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Temperature Pre-Treatment Modulates Oxidative Protection of *Aspergillus niger* Cells Stressed by Paraquat and Hydrogen Peroxide

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Abstract

The protective effect of pretreatment with low oxidative stress factors is little known in relation to the growth and development of filamentous fungi. The acquisition of new knowledge can be particularly useful for industrially important fungal strains. In the present study, the adaptive response of the filamentous fungus *Aspergillus niger* 26 to oxidative stress has been examined. The findings indicate that pretreatment with a sub-lethal temperature leads to the development of resistance to lethal concentrations of paraquat (PQ), H₂O₂ and extremely high temperatures. Fungal cultures subjected to 35°C followed by exposure to high doses of stress agents showed a higher amount of biomass accumulation compared to the single treated cells. The pre-exposure strategy exerted a protective effect with respect to the amount of oxidatively damaged proteins in *A. niger* cells, which was accompanied by a corresponding increase in the intracellular protein content and induction of antioxidant enzymes compared to the non-adapted cultures. At the same time, the results demonstrated different responses in the temperature-adapted cells. Pretreatment makes cells more resistant to both PQ and temperature than to H₂O₂.

Key words: fungi, oxidative stress, adaptive response, biomarkers, antioxidant enzymes

Резюме

Ефектът на предварителното третиране с ниски дози от фактори, индуциращи оксидативен стрес върху растежа и развитието на култури от филаментозни гъби е слабо изучен феномен. Получаването на нови знания в тази област може да бъде много полезно при използването на индустриално важни щамове. В настоящото изследване проучихме адаптивния отговор на щам *Aspergillus niger* 26 към оксидативния стрес. Получените резултати показват, че пре-третирането със сублетална температура води до проявата на резистентност към летални концентрации паракват (ПК), H₂O₂ и екстремно висока температура. При третиране с температура 35°C и последващо въздействие с високи дози от използваните стрес фактори се наблюдава натрупване на по-високо количество биомаса в сравнение с еднократното третиране култури. Стратегията на пре-третирането води до протективен ефект по отношение количеството на оксидативно увредените белтъци в клетките на *A. niger*, което кореспондира с повишено количество вътреклетъчен белтък и индукция на антиоксидантната ензимна защита в сравнение с не-адаптираните култури. Освен това, резултатите демонстрират разлики в отговора на клетките, адаптираните към температурата. Пре-третирането повишава в по-голяма степен резистентността към ПК и температурата, отколкото към H₂O₂.

Introduction

In filamentous fungi, as eukaryotic organisms, oxidative stress induced directly or indirectly through various abiotic factors such as heat, cold, herbicide applications (e.g., paraquat), drought, UV radiation, etc. leads to enhanced production of reactive oxygen species (ROS) (Lushchak, 2011).

These ROS include the superoxide anion radical ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H₂O₂), the hydroxyl radical (OH $\cdot$) and can cause peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately lead to cell death (Fridovich, 1998). They are produced by metabolic pathways localized in different cellular compartments.

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Fungi possess a complex antioxidative defence system containing non-enzymatic and enzymatic components to scavenge ROS. The enzymatic components comprise of several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPX), and glutathione reductase (GR) (Bai *et al.*, 2003). These enzymes operate in different subcellular compartments and respond in concert when cells are exposed to oxidative stress. When the level of ROS exceeds the defence mechanisms, the cells are said to be in a state of “oxidative stress”. SOD catalyze $\bullet\text{O}_2^-$ dismutation to H_2O_2 and molecular oxygen (Fridovich, 1998). SODs are metallo-proteins and are classified into Fe-, Mn-, CuZn- and Ni-containing SOD on the basis of the metals in their active sites. It is generally accepted that fungal cells contain Mn-SOD in the mitochondria and CuZn-SOD in the cytoplasm (Ito-kuwa *et al.*, 1999; Angelova *et al.*, 2005). CATs are ubiquitous enzymes, which protect aerobic organisms from the toxic effects of H_2O_2 by catalyzing the conversion to molecular O_2 and H_2O . CATs from filamentous fungi have several characteristics that distinguish them from their mammalian counterparts (Bussink and Oliver, 2001).

Although the oxidative stress responses in fungi is insufficiently studied, it has been shown that fungal cells respond to different oxidative stressors in distinct ways (Bai *et al.*, 2003). For example, herbicides as paraquat (PQ), menadion (MD) and their derivatives are strong inductors of ROS generation, mainly $\bullet\text{O}_2^-$. On the other hand, H_2O_2 is a reactive oxygen species and universally cytotoxic at high concentrations, mainly due to strong oxidant, hydroxyl radical. To our knowledge, there are few reports regarding the effects of antioxidant enzymes over expression on fungal sensitivity to PQ, MD and H_2O_2 . Li *et al.* (2008) showed that *A. niger* B1-D adapts to exposure to H_2O_2 by reducing growth and inducing a number of antioxidant enzyme activities, of which the induction of catalase is the most pronounced. Similar results have been published for *Penicillium chrysogenum* cells treated by H_2O_2 . Conversely, after addition of MD the same strain demonstrated enhanced activity of Mn- and Cu/Zn-SOD, but CAT did not included in the cell response (Emri *et al.*, 1997). Our previous study confirmed the distinct antioxidant response to PQ and H_2O_2 in 18 fungal species (Angelova *et al.*, 2005).

At the same time, the pre-treatment with low concentrations of PQ, MD and H_2O_2 significantly

increased survival of the lethal doses of each oxidant, indicating the existence of an adaptive response to oxidative stress (Lee *et al.*, 1995; Izawa *et al.*, 1995; Li *et al.*, 2008). *Candida albicans* is able to acquire adaptive oxidative tolerance by pretreatment with non-stressing concentration of H_2O_2 before exposure to a drastic oxidative challenge (González-Pàrraga *et al.*, 2003). While MD caused protection against cell killing of *Saccharomyces cerevisiae* by subsequent higher concentrations of MD or H_2O_2 , the pretreatment with H_2O_2 did not protect cells against the enhanced dose of MD (Jamieson, 1992).

Temperature pre-treatment also affected the survival of fungal cultures against induced oxidative stress. Yeast cells treated with a temperature of 40°C showed less accumulation of ROS than nontreated cells in response to heat shock and H_2O_2 (Liu *et al.*, 2011). The authors suggest that the overall improvement in stress tolerance is associated with the induction of TPS1 gene expression and trehalose accumulation. Heat-shock enhanced the trehalose content in *S. cerevisiae* and reduced the damage caused by ROS (Benaroudj *et al.*, 2001). It is important to clarify the role of the major enzymes of antioxidant defence, SOD and CAT, in the induced adaptive response. Whereas this problem is widely studied in plants, little is known about the effect of temperature pre-treatment on the modulation of antioxidant enzyme activity of fungi. The knowledge can be useful for industrially important fungal strains.

Our previous investigation demonstrated the effect of enhanced temperatures on the morphology, the level of oxidative stress biomarkers, and activity of antioxidant enzyme defence in the fungal strain *Aspergillus niger* 26 (Abrashev *et al.*, 2014). The aim of the present study was to investigate if pre-treatment with sub-lethal temperature causes an adaptive response to oxidative stress induced by heat shock, PQ and H_2O_2 as exogenous sources. For this purpose, growth, intracellular protein content, carbonylated protein level, and the antioxidant enzyme activity (SOD, CAT) variations were evaluated.

Materials and methods

Materials

Nitro blue tetrazolium (NBT), paraquat (PQ), 2,4-dinitrophenylhydrazine (DNPH), were obtained from Sigma-Aldrich (Deisenhofen, Germany). All other chemicals used in this study were of the highest analytical degree.

Fungal strain, culture conditions and temperature pre-treatment

The fungal strain, *A. niger* 26 from the Mycological Collection at the Stephan Angeloff Institute of Microbiology, Sofia, was used throughout and maintained at 4°C on beer agar, pH 6.3. All experiments under submerged conditions were carried out in the medium AN-3 (Abrashev *et al.*, 2005).

Cultivation was carried out in 3 L bioreactors, ABR-09, developed and constructed by the former Central Laboratory for Bioinstrumentation and Automatisation (CLBA) of the Bulgarian Academy of Sciences. The bioreactor was equipped with automatic temperature, pH and dissolved oxygen (DO) monitoring equipment and a control system. For the inoculum, 80 ml of medium AN-3 was inoculated with 10^9 spores in 500 ml Erlenmeyer flasks. The cultivation was performed on a shaker (220 rpm) at 30°C for 24 h. For bioreactor cultures, 200 ml of the seed culture was brought into the 3 l bioreactor, containing 1800 ml of the medium AN-3. The cultures were grown for 18 h at a temperature of 30°C with a stirrer speed of 600 rpm air flow, 1.0 v.v. m. At that time, a single administration of stress factors, PQ (1, 3 or 5 mM), H_2O_2 (5, 10 or 30 mM) or temperature (35, 40 or 50°C), was performed and cultivation was carried out for 12 h. Experiments with PQ and H_2O_2 were continued at the initial temperature. Experiments without the stress agents were also performed under the same conditions, as controls.

For adaptive stress response assays, 12-hour bioreactor cultures of *A. niger* 26 were transferred from 30 to 35°C for 6 h. The pre-treated fungal cells were then immediately challenged with 5 mM PQ, 30 mM H_2O_2 (incubation at 30°C) or 50°C for 12 h. The control variants were grown at an optimal temperature without stress agents during the whole period. Results were evaluated from repeated experiments using three parallel runs.

Cell-free extract preparation and enzyme activity determination

The cell-free extract was prepared as described earlier (Abrashev *et al.*, 2005). Briefly, mycelium biomass was harvested by filtration, washed in distilled H_2O and then in cold 50 mM potassium buffer (pH 7.8), and resuspended in the same buffer. The cell suspension was disrupted by homogenizer model ULTRA Turrax T25 IKA WERK. The temperature during treatment was maintained at 4-6°C by chilling in an ice-salt bath and by filtration through a Whatman filter, No 4 (Clifton, USA). Cell-free extracts were centrifuged at 13 000 x g for

20 min at 4°C.

SOD activity was measured by the nitro-blue tetrazolium (NBT) reduction method of Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount of enzyme protein required for inhibition of the reduction of NBT by 50% (A_{560}) and was expressed as units per mg protein. Catalase activity was determined by monitoring the decomposition of 18 mM H_2O_2 at 240 nm (Beers and Sizer, 1952). One unit of activity is that which decomposes 1 μ mol of H_2O_2 min⁻¹ mg protein⁻¹ at 25°C and pH 7.0. Specific activity is given as U (mg protein)⁻¹. Protein was estimated by the Lowry procedure (Lowry, 1951) using crystalline bovine albumin as standard.

Measurement of protein carbonyl content

Protein oxidative damage was measured spectrophotometrically as protein carbonyl content using DNPH binding assay (Levine *et al.* 1990), slightly modified by Adachi and Ishii (2000). The cell-free extracts were incubated with DNPH for 1 h at 37°C, proteins were precipitated in 10% cold TCA, washed with ethanol: ethylacetate (1:1), to remove excess of DNPH, and finally dissolved in 6 M guanidine chloride, pH 2. Optical density was measured at 380 nm, and the carbonyl content was calculated using a molar extinction coefficient of 21 mM⁻¹ cm⁻¹, as nanomoles of DNPH incorporated (protein carbonyls) per mg of protein.

Determination of dry weight

The dry weight determination was performed on samples of mycelia harvested throughout the culture period. The culture fluid was filtered through a Whatman (Clifton, USA) No 4 filter. The separated mycelia were washed twice with distilled water and dried to a constant weight at 105°C.

Statistical evaluation of the results

The results obtained in this investigation were evaluated from at least three repeated experiments using three or five parallel runs. The statistical comparison between controls and treated cultures was determined by Student's *t*-test for MIE (mean interval estimation) and by one-way analysis of variance (ANOVA) followed by Dunnett's post-test, with a significance level of 0.05.

Results

Cell response to single administration of stress factors

Fungal growth

The ability of *A. niger* cultures to produce biomass under conditions of oxidative stress indu-

ced by PQ, H₂O₂ or temperature is presented in Fig. 1. The value of stress agents was chosen based on preliminary experiments where the range was found to be wide enough to give clear contrast between control and stressed cultures.

As expected, the dry weight content decreased with an increase temperature. The results showed that the moderate temperature stress (35°C) decrease the biomass of the fungal culture by 11% compared to the control variant. However the acute stress at 50°C reduced biomass accumulation by 42% compared to the control. Similarly to the trend of tolerance to high temperatures, the growth of *A. niger* 26 decreased with exposure to increased concentrations of PQ and H₂O₂. About 20, 41 and 68% lower levels of biomass were achieved after exposure to 1, 3 or 5 mM PQ, respectively. The treatment with 5, 10 and 30 mM H₂O₂ led to approximately the same results. Thus, both agents were the more powerful stress factors that affected *A. niger* development.

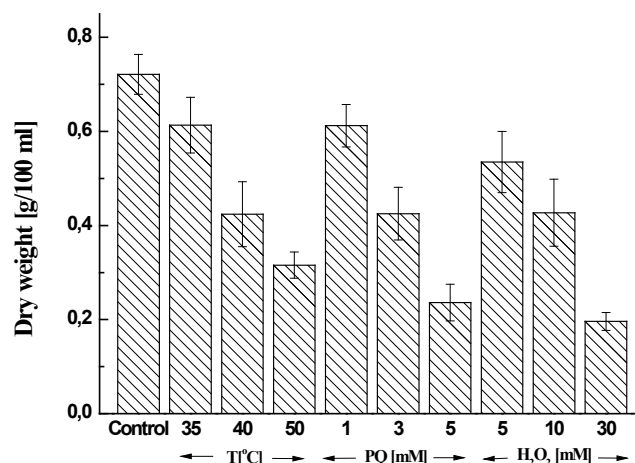


Fig. 1. Biomass production by *A. niger* 26 in response to a single administration of the stress factors, PQ, H₂O₂ or temperature. The effect of treatment was significant ($p \leq 0.05$)

Table 1. Effect of single administration of stress factors on the intracellular protein, carbonylated protein content, and specific activity of SOD and CAT

Variants	Intracellular protein [mg/g d.w.]	Carbonylated protein [nM/mg protein]	SOD [U/mg protein]	CAT [U/mg protein]
Control	40.82	1.82	32,8	11.8
Temperature [°C]				
35	33.56	3.56	58.5	13.5
40	23.12	6.02	82.2	16.2
50	16.42	8.12	109.3	18.7
PQ [mM]				
1	29.92	3.98	64.6	14.1
3	21.51	7.11	95,1	15.1
5	11.87	13.87	121,7	18.5
H ₂ O ₂ [mM]				
5	26.11	5.12	33.9	18.7
10	12.82	8.82	35.8	22.3
30	8.06	15.06	37.6	29.6

Changes in protein content and antioxidant enzyme activities

The agents used (temperature, PQ and H₂O₂) are known as inducers of oxidative stress. Their effect on the stress biomarkers, such as intracellular protein content, oxidatively damaged proteins and antioxidant enzyme activities is demonstrated in Table 1.

When cells of the tested fungal strain were

exposed to the above-mentioned factors, the intracellular protein level decreased significantly as compared with the control cultivation. After 12 h, there was a trend for a dose-dependent decline in protein content. The most dramatic reduction was related to the H₂O₂ exposure (from 40 to 80% compared to the control). PQ affected protein level to a similar extent, while increasing the temperature from 35 to 50°C resulted in a lower reduction (from 18 to 60% compared to the control).

The above-mentioned reduction in the intracellular protein content coincided with a remarkable enhancement in the oxidatively damaged proteins, measured by the protein carbonyl content. At the end of the stress treatment with 35 and 40°C, carbonyls increased about 2- and 3.3-fold in comparison with the control. Exposure at 50°C caused about 4.5-fold higher increase in carbonyls than in the control. Carbonyls in total protein of the PQ and H₂O₂-exposed fungal cells showed the same trend of increase but to a greater extent.

As expected, under oxidative stress conditions induced by $\cdot\text{O}_2^-$ and H₂O₂ generating agents, the antioxidant enzyme response of *A. niger* cultures varied greatly. The activity of SOD and CAT increased by PQ and temperature treatment, but the level of induction varied for both enzymes: about 2 - 3.7-fold for SOD compared to 35 -58% for CAT. In contrast, peroxide stress caused a 2-3-fold increase in catalase activity compared to the control, while SOD only showed a modest increase with H₂O₂.

Adaptive response of *A. niger* 26 to temperature pretreatment

To test the adaptive response induced by pre-treatment with moderate heat stress (HS) biomass production, protein content and antioxidant enzyme activities of pretreated cultures subsequently exposed to 5 mM PQ, 30 mM H₂O₂ and temperature 50°C were compared with non-pretreated cells challenged with the same agents.

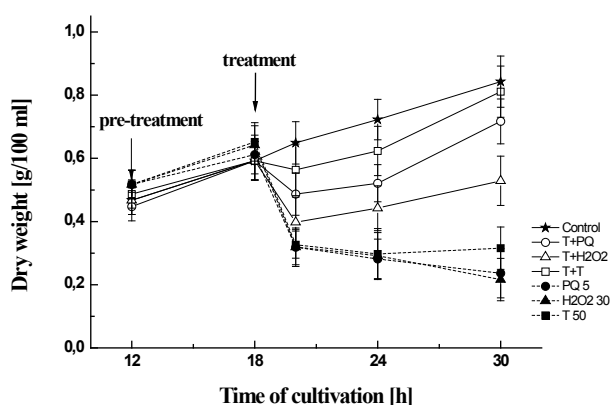


Fig. 2. Time course of biomass production for two different experiments: cultures without pretreatment (dash lines; closed symbols) and cultures pretreated with 35°C (solid lines; open symbols), when 5mM PQ (○, ●), 30 mM H₂O₂ (△, ▲) and temperature 50°C (□, ■) was added. The control (*) without treatment.

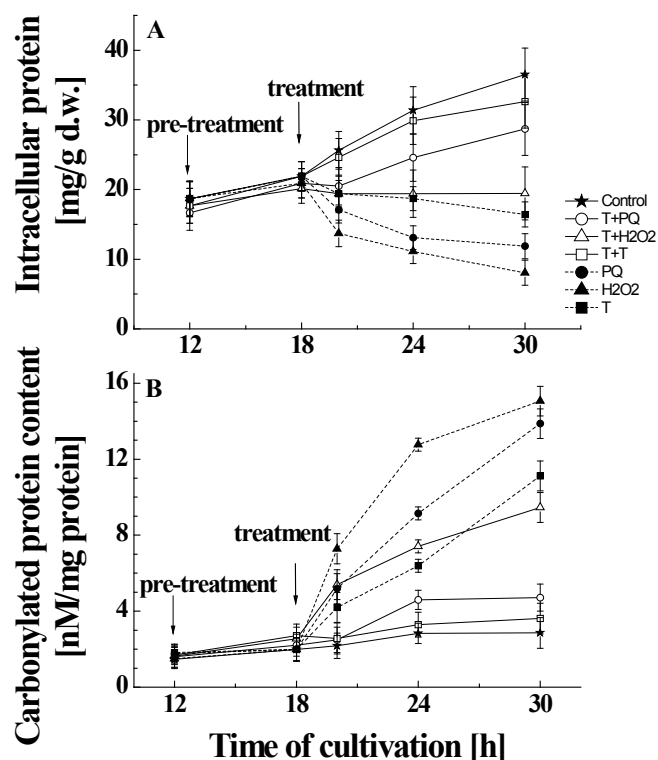


Fig. 3. Adaptive response on intracellular protein (A) and carbonylated protein content (B) cultures without pretreatment (dash lines; closed symbols) and cultures pretreated with 35°C (solid lines; open symbols), when 5mM PQ (○, ●), 30 mM H₂O₂ (△, ▲) and temperature 50°C (□, ■) was added. The control (*) without treatment.

Fungal growth

Experiments to evaluate changes between pretreated and non-pretreated cells of *A. niger* 26 in relation to the growth versus time were performed and the results are shown in Fig. 2.

Direct exposure to high concentrations of PQ, H₂O₂ and temperature lead to reduced biomass content, these doses are lethal to this culture. Sharp changes occurred during the first 2 hours of the treatment and continued thereafter until the end of cultivation. However, pretreatment with 35°C for 6 h prior to exposure to lethal doses removed the deleterious effects to a great extent. This effect was more prominent in the temperature and PQ-treated cells than in the variants with H₂O₂. When pretreated cells were subjected to oxidative stress agents, the dry weight increased 3- and 2.6-fold respectively, compared to the non-pretreated cells.

Protein changes

The effect of the adaptive response on the level of intracellular protein and carbonylated pro-

tein content was examined. As seen in Fig. 3A, the intracellular protein concentration in the pretreated bioreactor cultures of *A. niger* was different from that of non-pretreated cells.

Although the evaluated values were lower than those of the control, a significant increase in protein level was measured in the cultures adapted to HS compared to the non-pretreated. Heat-pretreated PQ-, H_2O_2 - or temperature-stressed fungal cells showed 2-, 2.5-fold higher protein level compared with those with direct exposure. As expected, the level of carbonyl groups in both pretreated and non-pretreated variants increased remarkably. But the previous adaptation resulted in 3- or 1.6-fold lower carbonyl protein level after PQ and temperature treatment and H_2O_2 , respectively. when compared with the cells subjected to stress agents without heat pretreatment. The marked effect was observed immediately after treatment and this trend continued until the end of cultivation.

Antioxidant enzyme activities

To compare the effect of heat-pretreatment on the induction of antioxidant defence of *A. niger* 26, the SOD and CAT activities were analysed. The results are illustrated in Fig. 4.

Oxidative stress caused by direct treatment with PQ and temperature immediately induced SOD and CAT to a large extent (Fig. 4A). The pretreated cultures showed a further remarkable increase in both enzyme activities. For example, 12 h after exposure to the lethal dose of PQ and temperature, 25 and 40% higher SOD activity, respectively, was measured compared to the non-pretreated cultures. At the same time, the estimated values were about 4.5-fold higher when compared with the control cells. In contrast, the effect of peroxide stress turned out to be insignificant in the variants with non-pretreated or pretreated cultures.

On the other hand, H_2O_2 addition to *A. niger* cells resulted in a 2- and 4-fold increase in CAT activity for non-pretreated and pretreated cultures, respectively, compared to the control. Enhanced activity was also found in the heat-pretreated and non-pretreated variants exposed to lethal dose of PQ and temperature.

Discussion

Adaptive stress response plays a major role in microbial cells, in particular those used in different industrial applications. Filamentous fungi involved in real biotechnological processes are subjected to multiple stresses often occurring simultaneously

(Bai *et al.*, 2003). They have evolved the ability to survive and produce valuable compounds using a system of stress response mechanisms. In spite of the great interest to clarify these mechanisms, the number of studies on adaptive stress response in biotechnologically important fungi is limited. The strain *A. niger* 26 has been selected as a promising candidate for industrial production of pectinolytic enzymes, mainly polymethylgalacturonase (PMG) (Angelova *et al.* 1998, 2000; Pashova *et al.* 1999). Moreover, our previous investigations showed that this strain is a good producer of the first antioxidant enzyme SOD. Short-term HS treatment of the spores (Abrashev *et al.*, 2005) and mycelia in mild-exponential growth phase (Abrashev *et al.*, 2008) markedly enhanced SOD activity.

In the present study, HS pretreatment (35°C for 6 h) of *A. niger* 26 cells was shown to be effective in improving fungal cell tolerance to stress agents such as PQ, H_2O_2 and high temperature. Of the agents used, H_2O_2 generated extracellular oxidative stress, whereas PQ, a redox-cycling agent, served as the source of intracellular oxidative stress

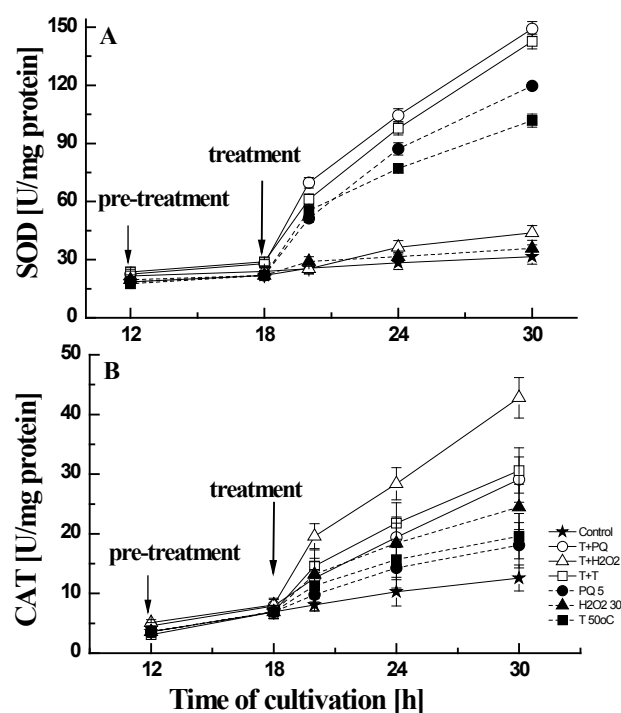


Fig. 4. Effect of heat pretreatment on activity of SOD (A) and CAT (B) in cultures without pretreatment (dash lines; closed symbols) and cultures pretreated with 35°C (solid lines; open symbols), when 5mM PQ (○, ●), 30 mM H_2O_2 (△, ▲) and temperature 50°C (□, ■) was added. The control (*) without treatment.

generating a flux of $\bullet\text{O}_2^-$ in fungal cells (Angelova *et al.*, 2005; Li *et al.*, 2008; Ponts *et al.*, 2009; da Silva Dantas *et al.*, 2015). A similar increase in $\bullet\text{O}_2^-$ and H_2O_2 levels after heat shock treatment has been demonstrated in different aerobic cells including fungal cells (Bai *et al.*, 2003; Abrashev *et al.*, 2008). The major findings in this study are that: (1) the pretreatment with mild HS induces an adaptive response, which protects cells from the lethal effects of the subsequent challenge with higher concentrations of these oxidants; (2) the mechanism of enhanced fungal resistance includes suppression of the oxidative stress; (3) the adaptive responses to superoxide and peroxide stress agents are distinct, although there is a significant overlap between many of the responses.

It was shown here that a combination of mild heat stress and subsequent exposure to lethal dose of PQ, H_2O_2 or temperature cause a significant improvement of fungal growth compared to the single treatment. Despite the evaluated decline in biomass as a result of an increase in cell autolysis in the first 2 hours of exposure, the pre-treated cultures quickly overcome the harmful effect. Probably, a preliminary exposure to 35°C might induce in cells the necessary adaptation to keep their growth. Several examples of this have been reported previously. Transient exposure to a sub-lethal HS induce tolerance to a more extreme stress in *Metschnikowia fructicola* (Liu *et al.*, 2011). Combinatorial H_2O_2 plus nitrosative stresses or cationic (NaCl) plus nitrosative stresses appear to exert adaptive effects upon the growth of *Candida albicans* cells (Kaloriti *et al.*, 2012). Li *et al.* (2008) found that the pretreatment of *A. niger* B1-D with H_2O_2 at a non-lethal concentration confers greatly enhanced resistance to killing by H_2O_2 at lethal concentrations in the early exponential phase of growth. In addition to improved biomass production of *A. niger* 26, HS pretreatment also provided increased tolerance to oxidative stress. Such inducible adaptive responses have been observed at every level of DNA damage repair to the induction of antioxidant enzymes such as SOD, CAT, peroxidase as well as small molecules, such as glutathione, atocopherol and ascorbate that scavenge reactive oxygen species before they cause damage (see Patra *et al.*, 1997).

The results of this study indicated reduced level of carbonyl groups, which can be used as a marker of oxidatively damaged proteins. As has been reported previously, when cells are exposed to severe stresses, the accelerated generation of intracellular ROS correlated well with enhanced con-

tent of oxidatively damaged protein (Davies and Goldberg, 1987). In contrast, the pretreatment with a sub-lethal dose of oxidative stress factors significantly declined ROS level in different fungal cells such as *Candida oleophila* (Reverter-Branchat *et al.*, 2004), *A. niger* B1-D (Li *et al.*, 2008), etc. This situation led to a decrease in the content of carbon-ylated proteins in stress-adapted cells compared with non-adapted cells. Protein carbonylation is an irreversible oxidative process leading to a loss of function of the modified proteins. These oxidized proteins are selectively recognized and degraded by proteolytic enzymes (Nystrom, 2005), followed by *de novo* protein synthesis or reparation (Crawford and Davies, 1994).

Furthermore, the lower level of oxidative damage may be due to the stimulation of antioxidant systems in stress-adapted cells. High activity levels of SOD and CAT were induced immediately after exposure to the stress agents in both non-adapted and adapted cells. The enhanced activity levels found for SOD and CAT in the experiments without pretreatment suggested that the cells were strongly stressed. It is noteworthy that moderate exposure to temperature promoted additional activation of the antioxidant enzymes compared to the non-adapted cultures. Thus, the increase in high-temperature tolerance caused by low-temperature pretreatment was a result of increasing ROS scavenging enzyme activities. Moreover, the enhanced antioxidant enzyme activity was implicated in the cross-tolerance of *A. niger* cells to PQ and H_2O_2 stress induced by HS treatment at 35°C . A strong correlation between HS pretreatment and antioxidant defence activity has been reported for plants (Mei and Song, 2011; Mansoor and Naqvi, 2013; Zhao *et al.*, 2014), but results about fungi could be very seldom found. Increased antioxidant enzyme activity in fungi has been demonstrated after PQ, MD, H_2O_2 , air pressure etc. (Lee *et al.*, 1995; Pinheiro *et al.*, 2002; Bai *et al.*, 2003; Li *et al.*, 2008). Similar results have also been reported for *Candida oleophila*, *Methanosarcina barkeri*, *Fusarium decemcellulare* (see Liu *et al.*, 2012). It was reported that the enzymatic detoxification of ROS in adapted cells is dependent on the upregulation of several antioxidant genes at transcriptional level including peroxisomal catalase, cytochrome c peroxidase, peroxiredoxin TSA1, thioredoxin reductase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase (Liu *et al.*, 2012). Spiró *et al.* (2012) reported evidence that a post-transcriptional element participates in the regulation of heat stress

adaptation under oxidative conditions.

It is noteworthy that the stress agents used in the present study elicited a different response in HS-adapted cells of *A. niger* 26. Our results provided clear-cut evidence that HS pretreatment makes cells more resistant to both PQ and temperature than to H₂O₂. Although the adaptive response to H₂O₂ challenge also included improved growth, reduced oxidatively damaged proteins content and enhanced SOD activity compared to non-pretreated cells, the evaluated levels were significantly lower than those in adapted cells treated with PQ and temperature. In contrast, the variants with H₂O₂ demonstrated extremely high specific CAT activity. The levels of resistance observed in HS-adapted cells exposed to PQ were similar to those found in variants with temperature treated cultures, suggesting that the same mechanism of resistance may be operative. The response to H₂O₂ appeared to be distinct from that induced by PQ, on the basis of cross-protection experiments. Similarly to our results, the adaptive HS response in yeasts was able to confer protection against stress caused by H₂O₂, superoxide anion and linoleic acid hydroperoxide (Jamieson, 1992; Evans *et al.*, 1998). Adaptation to heat increased the resistance of *Listeria monocytogenes* to H₂O₂ (Lou and Yousef, 1997). Furthermore, *Saccharomyces cerevisiae* possesses at least two distinct adaptive stress responses to oxidants: one induced by H₂O₂ and the other by exposure to compounds such as menadione, which produce a flux of superoxide anions in the cells (Jamieson, 1992; 1998). According to Hasanuzzaman *et al.* (2013), high temperature induced expression of inducible genes responsible for synthesis of heat-shock proteins (HSPs), which protect intracellular proteins against denaturation and preserve their stability and function through protein folding; thus it acts as a chaperone. The results reported by Troschinski *et al.* (2014) reveal that besides the well-documented HSPs stress response, antioxidant defence plays a crucial role in snails' (*Xeropicta derbentina*) competence to survive extreme temperatures.

Conclusion

HS-pretreatment improved growth and intracellular protein synthesis in the fungal cultures of *A. niger* 26 exposed to PQ, H₂O₂ and an extremely high temperature. The pretreatment strategy reduced the harmful effect of oxidative stress on carbonylated proteins and increased the activities of the antioxidant enzymes SOD and CAT. Taken together, the results demonstrated that the HS-pre-

liminary exposure induced a coordinated response that declined the oxidative stress degree in *A. niger* cells when this pre-exposure was followed by severe stress.

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Purification of Arabinomannan Synthesized by *Cryptococcus laurentii* AL₁₀₀

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Abstract

The Antarctic yeast strain *Cryptococcus laurentii* AL₁₀₀ was selected as an active arabinomannan producer (Pavlova *et al.*, 2011). The synthesized biopolymer was subjected to purification by two methods. In the application of molecular-sieve purification with Sephadex G75, two separate carbohydrate fractions were detected, while the use of gel-filtration system on Sepharose DEAE CL-6B showed three distinct carbohydrate fractions. The protein content of the fractions was also established.

Key words: exopolysaccharide, yeasts, purification, *Cryptococcus laurentii*, carbohydrate

Резюме

Cryptococcus laurentii AL₁₀₀ беше селектиран като активен продуцент на екзополisahарида арабиноманан (Pavlova *et al.*, 2011). Синтезираният биополимер беше подложен на пречистване чрез използването на два метода. При приложението на молекулно-ситово пречистване със Sephadex G75 бяха установени две отделни въглехидратни фракции. Използването на гел-филтрираща система със Sepharose DEAE CL-6B показа три отделни въглехидратни фракции. Определено беше и протеиновото съдържание във фракциите.

Introduction

Polysaccharides are high molecular compounds composed of a large number of monosaccharide residues joined to each other by glycosidic linkages. The composition of microbial polysaccharides is various, which determines the diversity of their physical and chemical properties (Sutherland, 1990; Nishinari, 2006). They are composed of long chains with molecular weight of 2.0×10^6 Da. Most often, the composition of the EPS includes glucose, galactose and mannose (Petersen *et al.*, 1990; Pavlova and Grigorova, 1999; Pavlova *et al.*, 2004; Radchenkova *et al.*, 2013). Taking into account the diversity of sugar components, there is a wide range of molecular structures. The study of the EPS structure is crucial for the understanding of their physicochemical and biological properties, as well as for the use of EPS-producing organisms for industrial or medical purposes (Kumar *et al.*, 2007; Freitas *et al.*, 2011). Therefore, their application in various industries, such as pharmaceuticals, requires

that these biomolecules should be purified.

Polysaccharides were purified by means of chromatographic techniques, taking into account the charge, solubility, and molecular weight of the polysaccharide molecule. Chromatographic separation based on particle size was applied for the separation of large molecules from macromolecular complexes in a solution.

Materials and methods

Biosynthesis of exopolysaccharide

The selected producer of arabinomannan *Cryptococcus laurentii* AL₁₀₀ was used for extracellular polysaccharide biosynthesis (Pavlova *et al.*, 2011). The biopolymer substance was obtained in bioreactor cultivation conditions. The nutrient medium contained (g/L): sucrose - 40; (NH₄)-SO₄ - 2.5; KH₂PO₄ - 1.0; MgSO₄·7H₂O - 0.5; NaCl - 0.1; CaCl₂·2H₂O - 0.1 and yeast extract - 1.0. A Sartorius bioreactor with a working capacity of 5L was used, equipped with a turbine stirrer, oxygen (Hamilton, Bonaduz AG, Switzerland) and pH (Hamilton, Bonaduz AG, Switzerland) electrodes.

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The temperature, agitation speed, air flow rate, foam and levels in the vessel were monitored by a software program (BioPAT ® MFCS/DA 3.0). Cultivation was performed at 22°C, with mechanical stirring -400 rpm and pneumatic -1.25 L/L/min, for 96h. The inoculum was prepared in 500 mL flasks during periodic deep cultivation on a rotary shaker with 220 rpm mechanical stirring, at 22°C for 48 h. *Isolation and purification of the EPS from the cultural liquid*

After completion of the fermentation, the biomass was separated from the cultural liquid by centrifugation at 6000×g for 30 min. The cultural liquid was concentrated at 60° C in a vacuum evaporator. The cooled concentrate was precipitated with two volumes of cold 95% ethanol and the samples stayed for 24 h at 4°C. The exopolysaccharide was separated from the ethanol solution by centrifugation at 5000×g for 10 min.

Two methods were used for the biopolymer purification. The first method was implemented through a preparative chromatography column with Sephadex G75, with NaCl as an eluent. For the second molecular-sieve purification a DAEC Sepharose CL-6B column was used, where the sample was filtered through a 2 µm pore size membrane before being let in. The system used for fractionation was ÄKTAprime plus. Elution was carried out with distilled water in 4 ml stream, and with a linear NaCl gradient at a concentration of 0 to 1.0 M. The fractions obtained were analyzed for carbohydrate (by the colorimetric method of Dubois *et al.* (1956), using glucose as a standard) and protein (using the colorimetric method of Bradford (1976)) content. The solutions were subjected to dialysis with a membrane for 24 hours.

Results

After the analysis of the exopolysaccharide molecule was carried out, its chemical composition was determined. It showed 79.1% carbohydrate, -11.7% protein (Pavlova *et al.*, 2011). The heterogeneous chemical composition determined the need for the removal of the ingredients that were different from the core molecule. According to the figures outlined above, the synthesized biopolymer is a proteoglycan, as it has a protein component.

The method of automatic preparative gel chromatography with Sephadex G75 was used for the purification of arabinomannan. The data in Figure. 1 report two fractions containing carbohydrates with different molecular weight.

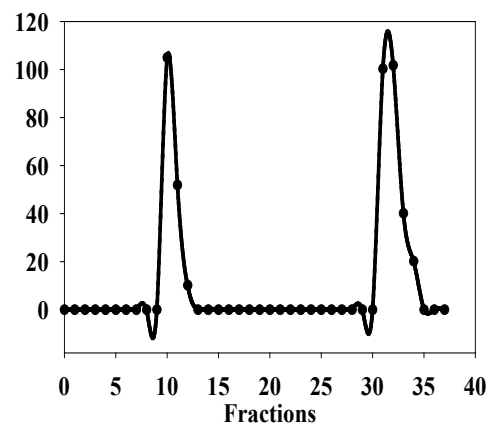


Fig. 1. Carbohydrate amounts in the fraction after purification by Sephadex G75

The first peak was obtained at 10, 11 and 12 fraction and it was a high molecular one, while the second peak appeared between 30 and 35 fractions and was determined as one of low molecular fraction. 25% of all carbohydrates were determined in the first peak, and 54% of them - in the second one. The total yield from both peaks amounted to 79.3% of the initial carbohydrates. There was about 3% of protein present in both fractions, where a carbohydrate component was established.

Purification was done using DEAE- Sepharose CL-6B, which allowed simultaneous performance of gel-chromatography and ion-exchange chromatography.

The initial color of the dissolved polysaccharide was pale pink, while after the filtration through a filter the solution became colorless, which showed that the pigment and part of the polymer had not pass through the pores of the filter.

After purification of the polymer fraction, the elution profile showed the presence of 3 different polysaccharides: the electrically neutral EPS 1 and EPS 2 (Fig 2, A), as well as the negatively charged EPS 3 (Fig.2, B). The first one was from 9 to 19 fractions, as the carbohydrates amount varied from 11.61 µg/mL to 26.32 µg/mL. The second peak was reported from 27 to 34 fractions with carbohydrate content at the highest point of 48.51 µg/mL. Elution with NaCl showed another peak with more carbohydrate content. It extends from 30 to 47 fractions and the highest content of carbohydrate - 88.77 µg/mL was in the sample 39 (Fig 2, B). The data were obtained after a 24-hour dialysis. This peak proves that part of the EPS came out after elution with NaCl through the column. Since sepharose was molecular-sieve and anion- exchangeable, the hanging parts of the polysaccharide molecule were negatively charged.

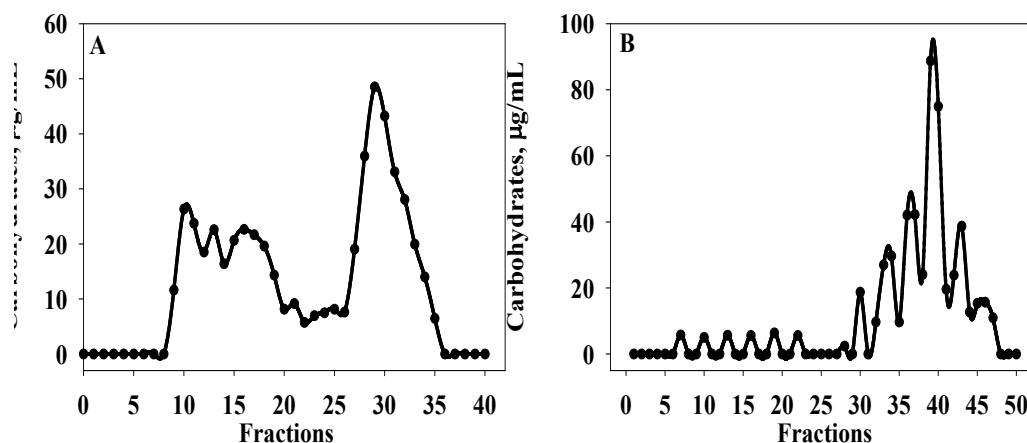


Fig. 2. Carbohydrate amounts in the fractions after purification by Sepharose DEAE CL-6B with eluent A) water and B) NaCl

The first peak contained approximately 19% of the carbohydrate, its content in the second one was 20%, and 40% was recovered in the peak after the elution with NaCl. The protein content analyses showed that during the first peak the protein content was 11.11 µg/mL, at the second peak it was 18.05 µg/mL, and at the NaCl elution peak - 45.83 µg/mL.

Discussion

Since all fractions obtained after purification with Sephadex G75 and Sepharose DEAE CL-6B contained protein (in small amounts), they could probably be determined as proteoglycans.

A more commonly used method for purification of polysaccharides is by Sepharose. Purification of exopolysaccharides by *Bifidobacterium animalis* RH was carried out in a similar manner, by anion-exchange chromatography, wherein the two peaks with a carbohydrate content were detected (Xu *et al.*, 2011). The purification of an acidic polysaccharide from *Bifidobacterium longum* BB-79 was accomplished by ion-exchange chromatography and gel-filtration (Roberts *et al.*, 1995; Andaloussi *et al.*, 1995). Microbial glucan, synthesised from *Geobacillus tepidamans* V264, isolated from a Bulgarian hot spring in Velingrad, was purified by gel filtration on Sepharose DEAE CL-6B (Kambourova *et al.*, 2009).

The results of the purification by Sepharose DEAE CL-6B of glucomannan, synthesized from *Sporobolomyces salmonicolor* AL₁, showed the presence of two fractions - the basic one with 90% carbohydrate content and low presence of protein, uronic and nucleic acids after elution with water, and the second one - after NaCl elution with 49%

carbohydrate content (Poli *et al.*, 2010).

Through the two types of arabinomannan purification, the presence of two, and in the second case - three carbohydrate fractions based on molecular weight and charge was detected. Purification by Sepharose DEAE CL-6B proved that the EPS studied contained groups with negative charge. There is a presence of proteins in both methods used.

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Spread and Manifestations of Pear Rust (*Gymnosporangium sabine*) on Different Pear Cultivars

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Abstract

The paper presents an investigation aimed at identifying the zones with regular incidence of pear rust. Such zones are appropriate for testing of cultivar susceptibility to the pathogen and fungicidal efficacy. The investigation data indicate that the disease is common on the monitored pear trees grown in the skirts of the north slopes of mountain Vitosha. Further away from the mountain, in Sofia Valley, the rate of pear rust attack is lower or the infection is missing.

Key words: pear rust, *Gymnosporangium sabinae*, *Rostellia cancellata*

Резюме

Статията представя изследване, насочено към установяването на зони, където болестта крушова ръжда се явява често. Такива зони са подходящи за изпитване на сортовата устойчивост, а така също и на фунгицидната ефикасност срещу патогена. Данните от изследването показват, че в подножието на северните склонове на Витоша болестта се явява масово по наблюдаваните дървета. В по-отдалечени от планината райони на Софийското поле нападението отслабва или няма инфекция.

Introduction

The pear is a fruit species for whose growth there are appropriate conditions in Bulgaria. Pear gardens can provide yields whose quality and quantity are sufficient for the needs of the domestic market and for export (Iliev *et al.*, Kitanov, 1986). The data for harvested land, however, indicate that the pear gives way not only to the apple but also to the rest of basic seed and stone fruit species. One of the reasons is the spread of diseases which cause pear trees to wither or perish (Aldwinckle, 1997; Borovinova, 2003). Thus the pear has become unattractive to owners of large orchards.

In the small non-market farms and orchards, however, the pear trees are not rare. Traditionally various cultivars are grown. The variety provides fresh fruits from the middle of the summer till the beginning of the winter. A part of the yield is intended for processing - drying, cooking of compote, distilling alcohol, etc. These possibilities are undoubtedly an advantage, which can be utilized mostly by small scale farmers.

In the period between August 2014 and August 2015, a research was done to establish zones with pear trees suffering from pear rust (Fig. 1). The investigation was part of a bigger research



Fig. 1. Symptoms of pear rust on leaf lamina

project on economically important diseases on fruit species in Bulgaria. It was aimed at testing the cultivar resistance and to the fungicidal efficacy against pathogens.

Materials and methods

An investigation was carried out in the regions on the territory of Sofia, Capital municipality and in Kostinbrod municipality (Table 1).

Small orchards, country house gardens and plots among residential and public buildings with planted pear trees were visited. Subject of the supervision were trees which had not been treated with fungicides.

The spread of the disease was traced out on the leaves of the common and standard Bulgarian cultivar Popska (synonyms Pastornice, Curé,

Table 1. Spread and manifestations of pear rust in some parts of Sofia' field.

Municipality / district / quarter	Location latitude, longitude	Cultivar	Rate of attack
Capital / Pancharevo	42°35'32.71" N; 23°24'48.38" E	Popska	1
		summer pear	3
Capital / Vitosha, close to Vrana residency	42°37'59.57" N; 23°26'19.83" E	Popska	2014 - 3
			2015 - 3
Vitosha Dragalevtsi quarter	42°37'22.07" N; 23°19'07.71" E	Popska	6
		Passe Crassane	6
Vitosha Boyana quarter	42°38'50.66" N; 23°15'53.09" E	Popska	2
Vitosha Boyana quarter	42°39'29.05" N; 23°16'02.26" E	Passe Crassane	5
Studentski University of forestry	42°39'08.83 N; 23°21'32.65" E	unknown small fruit variety	1
Slatina Institut of microbiology	42°40'33.05" N; 23°22'06.66" E	unknown summer cultivar	1
Slatina, close to Romanian embassy	42°41'13.32" N; 23°21'10.60" E	Popska, summer unknown	0
Ovcha kupel New Bulgarian university	42°40'09.14" N; 23°15'34.25" E	Popska	1 - 2
Novi Iskar Kurilo quarter	42°49'43.01" N; 23°21'14.30" E	Passe Crassane	0
		unknown summer	0
Municipality Kostinbrod Distict № 1	42°49'06.87" N; 23°13'57.85" E	Popska	0
District № 2	42°48'11.98" N; 23°11'03.02" E	unknown wild small fruit variety	1*

**Fig. 2.** Pear cultivar Popska (synonyms: Pastornice, Curé, Pastorenbirne, Belle de Berry, Bon - papa)

Pastorenbirne, Belle de Berry, Bon - papa) (Fig. 2) as well as cultivar Passe Crassane (synonym Edel Crassane) and some pear trees with unspecified cultivar affiliation.

When the disease is present the rate of attack on the leaves of the trees is determined on a scale (0 - 5) proposed by Lazarov *et al.* (1979) and Yoncheva *et al.* (1979) as follows: 0 - healthy leaves; 1 - single small spots; 2 - up to 5% of leaves with spots; 3 - up to 10% of leaves with spots; 4 - up to 25% of leaves with spots; 5 - up to 50% with spots. For the specifying of the present research to the scale was added rate 6 - above 50% of leaves are spotted.

The injuries on the leaf lamina were determined by means of HP Scanjet 4850 desktop scanner. Canon Powershot SX20 IS and Panasonic DMC TZ1 digital cameras were used. The background was a white sheet of paper. Measuring the area of digital image was done by Adobe Photoshop. In our

case the software product was used to list the pixels on which the image was located (Kostadinov and Moteva, 2014).

Results and discussion

The disease was found in five Sofia districts: Vitosha, Pancharevo, Studentski, Slatina, Ovcha kupel" and in Kostinbrod municipality (Table 1). The trees in Dragalevtsi and Boyana were the most severely affected, where almost all leaves

manifested symptoms characteristic of the disease. Besides, some of the leaves manifested two and more spots on the lamina.

In Slatina and Ovcha kupel, symptoms were rarely observed or were almost missing. Only one spot was recorded in Kostinbrod municipality. No symptoms were observed on the trees in district Novi Iskar.

The damage recorded at the end of September 2014 on the leaves of Popska tree grown in location

Table 2. Manifestation of pear rust on the leaves of different pear cultivars grown in different locations

Number of samples	Gauged leaf area cm ²	Part of the injury cm ²	Number spots on the leaves	Average area of one spot cm ²
Cultivar Popska (30.09.2014) location: avenue „Tsarigradsko shose” (close to Vrana residency)				
14	42.63	1.38	20	0.9
Ratio 3.24%				
Cultivar Popska (04.08.2015) location: avenue „Tsarigradsko shose” (close to Vrana residency)				
9	22.60	0.06	18	0.39
Ratio 2.65%				
Cultivar Popska (06.08.2015) Location: district Vitosha, quarter Dragalevtsi				
7	31.10	0.78	19	0.303
Ratio 2.51%				
Cultivar Passe Crassane (06.08.2015) Location: district Vitosha, quarter Dragalevtsi				
9	40.29	3.09	53	0.53
Ratio 7.62%				
Cultivar Passe Crassane (?) (06.08.2015) Location: district Vitosha, quarter Boyana				
10	34.83	1.32	23	0.537
Ratio 3.85%				
Unknown summer pear (06.08.2015 г.) Location: district Vitosha, quarter Dragalevtsi				
10	17.45	0.74	14	0.495
Ratio 4.24%				
Unknown small fruit pear (06.08.2015) Location: district Studentski, University of forestry				
3	5.05	0.08	3	0.074
Ratio 1.58%				
Unknown summer pear (04.08.2015) Location: district Slatina, Institute of microbiology				
9	19.84	0.64	9	0.614
Ratio 3.23%				
Unknown summer pear (04.08.2015) Location: district Pancharevo				
10	31.41	0.67	13	0.519
Ratio 2.13%				

42°37'59.57" N; 23°26'19.83" E (close to Vrana residency - Table 2) was more severe than the damage observed in the beginning of August 2015. This is probably due to the longer period for development of the lesions. At the same time (4 - 6 August) there was not significant difference from the tree grown in Dragalevtsi. This may be a result of similar levels of resistance to the pathogen.

The most severe manifestation was recorded on the leaves of cultivar Passe Crasane grown in Dragalevtsi and on the tree resembling the mentioned cultivar grown in Boyana.

The comparison between the injuries on the leaves of summer pears shows the highest level in Dragalevtsi quarter. However, further investigations have to be implemented for clarifying the cultivar affiliation of every one pear tree.

The small fruit pear trees observed in Sofia (Studentski) and Kostinbrod municipality seem to be resistant to *Gymnosporangium sabinae* Dickson (syn. *G. fiscum*), *Basidiomycetes* (*Rostellia cancellata* Rebentish, *Uredinales*.)

Generally the results outline the districts near by mountain Vitosha as suitable for investigations for resistance of cultivars to pear rust. There is a constant infectious background due to the presence of *Juniperus* species which are natural host plants of *G. sabine* (Aldwinckle, 1997; Borovinova, 2003; Shishkova, 1960; Tsanova *et al.*, 1979)¹.

The instructions for fungicide control against pear rust (*G. sabine*) are the same with those recommended against apple scab *Venturia inaequalis* Cooke (Winter) (anamorph *Spylocea pomi* Fr. et Fr., syn. *Fusicladium dendriticum* Wallr. (Fuckel) and pear scab *V. pirina* Bret. (syn. *Endostigma pirina* Sydow, *Ascomycetes*) (anamorph *Fusicladium pirinum* Libert (Fucke) (Aldwinckle, 1997; Borovinova, 2003). However, the suitability of such treatment is questionable because systemic fungicides permitted in Bulgaria for control of *V. inaequalis* have not been tested for efficacy against *G. sabine*.

Conclusion

In the Sofia Valley pear rust is spread in districts of municipalities of the Capital and the town of Kostinbrod. The level of attack by the pathogen is higher in locations near the northern slopes of the mountain Vitosha than the level recorded in other locations not so close to the mountain. This

is due to the presence of *Juniperus* species which are the main natural host plants of the *G. sabine*. These circumstances may facilitate the research to determine the resistance of pear cultivars and the efficacy of different fungicides against *G. sabine*.

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¹See also Administration of Park Vitosha
<http://park-vitosha.org>



Maastricht, The Netherlands, 07-11 June 2015

This year is remarkable for the Federation of European Microbiological Societies' (FEMS) history - 40 years have passed since its foundation. The Federation is a huge family which unites 52 European microbiological societies. President of the FEMS is Prof. Jean-Claude Piffaretti from Switzerland and its Vice-President is Prof. Bauke Odega from the Netherlands. The 40th Anniversary was celebrated during the VIth FEMS congress in Maastricht (NL) which was held on 7-11 June 2015. More information about the Congress is available on: <http://fems-microbiology.kenes.com/>. The Congress was an immense scientific event which was attended by approximately 2000 microbiologists gathered from 78 countries across Europe, North and South America, Africa, Asia and Australia.

Prof. Jean-Claude Piffaretti (FEMS President): The FEMS congresses are great opportunities to gather European scientists from all over Europe working on the many different fields of microbiology. Quality of science is the main driving force of the programme, which makes these events attended also by numerous scientists outside Europe, including South America, USA and Asia. A particular attention is given to young scientists through the awarding of a great number of grants. Young scientists have thus the opportunity to develop networking and interact with well-established researchers.

The delegates presented their research through intensive program of plenary lectures, symposia, workshops, poster discussions, poster presentations and special events in 58 research topics: Anaerobic physiology, Antibiotic resistance and environment, Antimicrobial resistance, Bacterial cell biology, Bacterial nanomachines, Bacterial pathogenicity, Bacterial spores, Biofilms, Biotechnology and industrial microbiology, Cell-cell communication / Quorum sensing, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), Computational microbiology, Emerging diseases, Environmental microbiology, Extreme environments, Food biotechnology, Food microbiology, Fungal bacterial interactions, Fungal development, Fungal pathogenicity in humans, Fungal pathogenicity in plants, General virology and bacteriophages, Global regulatory networks, High-throughput approaches, Host manipulation and bacterial survival, Imaging, Insect-microbes interactions, Intracellular survival, Metagenomics in humans / animals, Metagenomics in the environment, Metals and microbes, Microbes and alternative energy sources, Microbial cell biology and structure, Microbial communities, Microbial genomics, evolution, phylogeny,



Award ceremony for best presented poster of young scientists (left to right: Prof. Vaso Taleski, Ellio Rossi, Clement Masson, Tessa Quax, Maria di Martino, Ahmad Khodr, Arun Sharma and Prof. Jean-Claude Piffaretti)

Microbial persistence and chronic infections, Microbial population genetics, Microbial proteomics, Microbiology education, Microbiology of aquatic ecosystems, Mobile genetic elements, Mycobacteria, New antimicrobial mechanisms, New antimicrobials for resistant organisms, New approaches for typing, New diagnostic approaches, Plant/microbes interactions, Pollutant degradation, Secondary metabolites, metabolomics, Secretion, Signalling, Small regulatory RNAs, Toxin / antitoxin: activities and functions, Vector-borne pathogens, Veterinary microbiology, Viral infections and host, Virome, Free subjects.

It's a tradition for FEMS to support young microbiologists to participate in the FEMS congresses. Prof. Vaso Taleski, Macedonia (Grants Committee's Chairperson): During the last four FEMS congresses over 660 young microbiologists were supported with approximately 400 000 Euro lump sum, both from European and Non-European countries. This year, 157 out of 182 applicants FEMS grants to participate the VIth FEMS Congress. At the end of the Congress five European (Belgium, France, Germany, 2 x Italy) and one non-European (India) early career scientists were additionally awarded for best presented posters with a 500 EURO FEMS extra grant and 250 \$ voucher from Elsevier.

At FEMS-2015 Congress Closing ceremony with the Andre Lwoff Award were honored two eminent microbiologists - Prof. Fernando Baquero (Spain), for his research on virulence and antibiotic resistance and Prof. Rudolf Thauer (Germany) - for his work on anaerobic bacteria.

Four Bulgarian participants attended the congress and have presented their research on environmental, medical and veterinary microbiology. They belong to New Bulgarian University, MVR Hospital, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, and Medical University of Pleven.

The next, VIIth Congress of European microbiologists will be held in Valencia, Spain, July 9-13, 2017.

Galina Satchanska

New Bulgarian University Biolaboratory Head



The consecutive 9th Balkan Congress of Microbiology, Microbiologia Balkanica'15, was carried out in Thessaloniki at October 22-24, 2015. The number of registered participants was 231, 53 of which young scientists. The participants were from the following countries: Albania - 17, FYROM - 38, Greece - 80, Hungary - 1, Montenegro - 2, Romania - 13, Serbia - 34, Turkey - 19. Bulgaria was presented by 28 scientists, which gave 4 plenary lectures, 12 oral reports and 15 posters. Prof. M. Murdjeva's lecture was on microbiological and immunological aspects of autism, Prof. A. S. Galabov - on a novel and original approach preventing the drug-resistance for treatment of enterovirus infections, Prof. H. Najdenski on the role of migrating birds as carriers of zoonotic pathogens, and Prof. S. Groudev - on the achievements and perspectives of biohydrometallurgy. Three Bulgarian scientists co-chaired the Sessions "Immunology-Vaccines" (M. Murdjeva), "Food Microbiology-Parasitology-Mycology" (G. Sachanska) and "Environmental Microbiology" (H. Najdenski). Quite impressive was the direction of medical microbiology, in which could be emphasized the plenary lecture of Prof. Athanasios Tsakris "Phenotypic Strategies for Detection and Differentiation of Carbapenemases" with several newest and original methodological approaches. Among the participants was Prof. Jean-Claude Piffaretti (Massagno, Switzerland), the President of FEMS, with a lecture "Antibiotic Resistance: a Perspective on the Future". The Congress was very well organized and provided a very favorable atmosphere for fruitful, extensive, and critical discussions.



Before the Congress Opening Ceremony the traditional meeting of the Board of the Balkan Society for Microbiology (BSM) was performed. The BSM Board consists of three representatives from each South-East European Country's society (Bulgaria, Greece, Serbia, FYROM, Romania, Turkey, Albania, Bosnia and Herzegovina, and Montenegro). Bosnia and Herzegovina and Montenegro representatives did not attend the meeting. The BSM President (Prof. A. S. Galabov) presented a short report on BSM activities within the period 2013-2015. The discussion carried out pointed to the future development of the Society and new perspective for scientific cooperation in BSM. Following the By-Laws of BSM Prof. A. Tsakris (Athens), the 9th Congress organizer, was proved as the BSM President for the next two years (2015-17). Prof. Cigdem Kayacan (Istanbul), President of the Turkish Microbiological Society, proposed Istanbul as the host of the 10th BSM Congress in October 2017. The BSM Board accepted this proposal. Thus, the BSM officers for 2015-17 were announced: A. Tsakris (Athens) - President, C. Kayacan (Istanbul) - President-Elect, A. S. Galabov (Sofia) - Past-President, H. Najdenski (Sofia) - General Secretary.



70 Years Anniversary of Department of Microbiology and Immunology, Medical University of Plovdiv

The Department of Microbiology at the Medical Faculty in Plovdiv was established in 1945 by Prof. Elisey Yanev. Since then it has developed as a strong training and research structure at the Medical University of Plovdiv, generating and disseminating knowledge. The academic progress is due to the enthusiasm of Department's leaders and the academic staff - highly motivated and hard-working people, dedicated to microbiology and immunology.

Today the Department of Microbiology and Immunology trains nearly 1000 students annually from four faculties - Medical, Dental Medical, Pharmaceutical and Public Health, and the Medial College. The traditions in education are interwoven with stateliness and innovation, immersion and originality in research. The quality in education is the main priority attempting to establish the Department as a symbol of knowledge and attractive university place where traditions, maturity, youth and enthusiasm meet to share dreams and ideas.

The ceremony was held on November 20 this year. A day before the celebration started at the Auditorium Building with the inspiring lecture of Prof. Beatrice Riteau, a famous virologist and immunologist from Aix-Marseille Université in France. She shared her experience on the good and the bad side of cytokines during influenza virus infections and involvement of hemostasis giving suggestions for new strategies in treatment of influenza. Dr. Riteau was introduced to students and teachers at the Medical University of Plovdiv by Prof. Dr Angel S. Galabov, virologist, regular member of the Bulgarian Academy of Sciences.

The impressive history of the Department and the new tendencies in research and training were presented by Prof. Marianna Murdjeva, Head of the Department. An exhibition of paintings, art photography and lace was demonstrated by talented microbiologists and immunologists from the Department. A small museum collection containing old microscopes, scales, tubes and loops was opened. Students, teachers and guests from other academic organizations in Sofia and Stara Zagora attended the festivity.

The research of the Department is focused on studying phenotypic and genetic mechanisms of resistance to antimicrobial agents of clinically important bacteria - *Staphylococcus*, *S. pneumoniae*, *Acinetobacter*, *Pseudomonas*; microbiological and epidemiological characteristics of vaginal infections in women at reproductive age; viral markers of occult hepatitis; immunological aspects of chronic stress.

Marianna Murdjeva

Vice-Rector of Medical University of Plovdiv and
Head of Department of Microbiology and Immunology

Forth Congress of Virology (Days of Virology in Bulgaria) with International Participation

**Big Hall of Bulgarian Academy of Sciences
1 “15^h November” Str., Sofia, Bulgaria**

May 19-20, 2016

The Organizing Committee informs that the Forth Congress of Virology (Days of Virology in Bulgaria) with International Participation will be held on May 19-20, 2016 in the Big Hall of the Bulgarian Academy of Sciences, 1 “15^h November” Str., Sofia, Bulgaria.

The Congress will be attended by virologists, specialists of the medical, veterinary and plant virology from all over the country. The scientific program contains plenary lectures given by leading scientists from Europe (Balkan region included), USA and Japan, large poster session and organized discussions on present milestones in virology.

The registration details will be given in the website: www.virologycongress.bg

The Congress reports in English will be published in *Acta Microbiologica Bulgarica*, volume 32, issues 2 and 3 (2016). The reports have to be prepared according to the ‘Guide of Authors’ of the Journal.

Acta Microbiologica Bulgarica

GUIDE FOR AUTHORS

The journal *Acta Microbiologica Bulgarica* is organ of the Bulgarian Society for Microbiology (Union of Scientists in Bulgaria). The journal is continuation of the edited till 1993 journal of the same name, cited in Index Medicus and Medline. The new edition is published two times per year.

Types of articles

The journal publishes editorials, original research works, research reports, reviews, short communications, letters to the editor, historical notes, etc from all areas of microbiology. The manuscripts should not represent research results which the authors have already published or submitted in other books or journals. The papers submitted for publication in *Acta Microbiologica Bulgarica* are peer-reviewed by two experts from the respective scientific field who remain anonymous to the authors.

Article structure

The papers are published in English, accompanied by a bilingual summary (English and Bulgarian). The papers should be typed with double-spacing (28-30 lines), on a white paper in A4 format, with margins of 3 cm.

The length of the original research paper, including the annexes (tables, figures, etc.) should not exceed a signature or printer's sheet (30,000 signs, that is, 16 pages with 30 lines each) in Times New Roman 12. The length of shorter reports should not exceed seven pages. The submitted manuscript must contain the name/s and surname/s of the author/s, the name and address of the institution and or organisation where it was prepared. The name and address of the corresponding author should be noted. The abstract should not exceed 250 words and should represent briefly the goals, methods, main results (with numerical data) and basic conclusions of the research. The most essential six key words must be added to the abstract. The manuscript contains the following sections: introduction, materials and methods, results, discussion, acknowledgements, references. The introduction must be concise with a clearly defined goal and with previous knowledge of the problem. The materials and methods ought to contain sufficient data to enable the reader to repeat the investigation without seeking additional information. The results should be presented briefly and clearly, and the discussion should explain the results. The measuring units and other technical data should be given according to the SI-system. Illustrations (tables and figures) are submitted separately and their places in the text should be clearly indicated. Tables should be given in a separate sheet, numbered and above-entitled. The figures must be accompanied by legends in a separate sheet.

Reference style

The references used are cited in the manuscript as follows:

- In the case of single author - the author's surname and the year of publication (Petrov, 2012);
- In the case of two authors - the authors surnames and the year of publication (Petrov and Vassileva, 2013);
- In the case of more than two authors - the first author's surname, *et al.*, and the year of publication (Christova *et al.*, 2014).

The references included in the section "References" of the manuscript should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters "a", "b", "c", etc. place after the year of publication.

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Reference to a book:

John, R., W. Villiam, G. Wilmington (2011). New approach for purification of enteroviral proteins, in: Peterson, K., A. Smith (Eds.), *Methods in Proteinology*. Elsevier, London, pp. 281-304.

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<https://library.caltech.edu/reference/abbreviations/>

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