IN VITRO SELECTION OF SOME POTENTIAL CYTOSTATIC AGENTS FROM NEW FUNGAL EXOPOLYGLUCIDIC EXTRACTS

MIHAI COSMIN[1*], ROTINBERG PINCU[1], GHERGHEL DANIELA[1], CAPRARU GABRIELA[1], TRUTA ELENA[1], SURDU STEFANIA[1], OLTEANU ZENOVIA[2], ROTINBERG HELLEN[3]

Keywords: glucanic extracts, HEp-2p tumoral cells, proteinsynthesis, culture development, cytostatic effect
Abstract: The *in vitro* action of some new autochthonous glucanic biopreparations, specifically extracted from diverse submerged strains of *Claviceps purpurea*, upon the proteinsynthesis of Hep-2p cancerous cells as well as upon the development of the corresponding cellular cultures was investigated. The significant perturbation of proteic biogenesis, the modification of the protein dynamics, the inhibition of the cell cultures development and the existence of a dose-response relationship argue the behaviour of the low intensity and frequency electromagnetic field as *in vitro* active cytostatic agent. This primary characterization of some glucanic extracts as cytostatic agent justifies the study of their effect upon cell proliferation and viability in order to enlarge the reasoning basis for the introduction of those exopolysaccharidic agents in the *in vivo* antitumoral screening program on different experimental tumoral systems.

INTRODUCTION

At present, the human antineoplasic chemotherapy still holds a priority, but its relatively low efficiency imposes the broadening of the investigations both for finding new antitumor substances as well as for discovering new therapeutic ways of action on the malignant process [Stroescu, 1998; Miron, 2000; Goodman&Gilman, 2001; Owens, 2001; Weinstein, 2001; Adams, 2002; Brown&Strathdee, 2002; Wong, 2002; DeVita, 2004].

These major objectives justify the great financial support the all over the world for the identification of new agents with antitumor action.

Identification of some new substances with cytostatic action, preferentially towards the malignant cell and less upon the host's normal cells, represents, at present, a major concern in the general effort to struggle against the cancerous disease (Grunberger et al., 1988; Bradley, 2001; Lyden, 2001; Adams, 2002; Anderson&Chiplin, 2002; Habeck, 2002).

The introduction of a new drug in the category of pharmacological cancerostatic agents is the result of a long, complex and careful process of investigation. The chemotherapeutic screening programs meant for the identification of new antitumor preparations require a step-like investigation of the drugs with possible antitumor activity, "in vitro" and "in vivo", at various levels of organization characterized by different reactive potencies [Borenfreund et al., 1990; Bissery&Chabot, 1991; Bannasch et al., 1998; Bindseil et al., 2001; Donger et al., 2002].

The multitude of biosynthesis, semisynthesis and synthesis substances with supposed antitumor action imposed in the last 20 years the introduction and intensive utilization of an "in vitro" test on neoplasic cell cultures of human origin as part of the screening programms meant for a preliminary selection of the potential cytostatic and/or cytotoxic drugs [Dold, 1988; Boyd, 1989; Hrushesky, 1990; Skehan et al., 1990].

A lot of biologically active compounds are generally glycoconjugates, and particularly glucans, having numerous and diverse pharmacological properties. Thus, it has been highlighted that the glucanic biomacromolecules are behaving as antibacterian, antiviral, antifungic, antiparasitical, antioxidant and even antineoplastic agents. Also, they are free radical scavengers, metabolic and digestive modulators, as well as nutritional supplements (Kren&Martinkova, 2001; Tian, 2007).

The antitumoral effect, signaled in the case of some glucanic products, has been explained by their immunostimulatory action and not by their direct interaction with the cancerous cells. In this context, we have proposed to investigate the tumoral cells reactivity to the action of some autochthonous fungal biopreparations of glucanic nature as substratum of their cytostatic and/or cytotoxic action.

This paper presents the results of a first step of the research, which has tested the *in vitro* cytostatic action of some new glucanic biopreparations of fungal origin, isolated at Biological Research Institute Iaşi, carried out on HEp-2p neoplastic cell cultures and evaluated according to their effect on cell proteinsynthesis and cell culture development.

MATERIAL AND METHODS

In vitro testing of the cytostatic action on HEp-2p cell cultures included a series of hydrosoluble exopolysaccharidic extracts, coded GE14.1, GE 28.2, GE 31.1, GE34.1, GE35.2, GE36.1, GE37.1, GE37.2.

The glucanic biopreparations of fungal origin were performed from diverse submerged strains of *Claviceps purpurea*: Cl.p.-14.1, Cl.p.-28.2, Cl.p.-31.1, Cl.p.-34.1, Cl.p.-35.2, Cl.p.-36.1, Cl.p.-37.1, Cl.p.-37.2. First, we selected,

from the microbiological collection of the Biological Research Institute (Iassy, Romania), the above mentioned strains of *C. purpurea* phytopathogen fungus with increased bioproductive outturn and we adapted them to the submerged cultivation conditions.

From the cultivation medium supernatants of six days old strains, eight total glucanic biopreparations were separated and partially purified, by ethanolic precipitation, centrifugation, sediment resuspendation in distilled water, dialization, ethanolic reprecipitation and final centrifugation.

The evidencing and evaluation of the *in vitro* cytostatic action were based on the comparative analysis of the total protein concentrations, of the proteinsynthesis dynamics and of the cell cultures development degrees during the evolution of control and treated cell cultures.

Human laryngeal carcinoma (HEp-2p) cells, were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, 100 IU/ml penicillin and 50 µg/ml amphotericin B, at a density of $5x10^5$ cells in 75 cm^2 flasks, in a humidified 5% CO_2 atmosphere at 37°C.

When the cells reached confluence they were detached from the flask with 0.25% trypsin + 0.02% EDTA in the normal medium and then centrifuged at 1800 rpm for 2 minutes. The cells were seeded at a density of $1x10^5$ cells / ml in the experimental tubes containing 2 ml of DMEM medium. The medium of the 24 hours cell cultures was changed either with a normal one (control cultures) or with one containing the fugal extracts (treated cultures), in a dose of 1.5 mg/ml [Leiter et al., 1965; Doyle&Griffiths, 1998; Seethala&Prabhavathi, 2001]. After 24 and 48 hours of *in vitro* treatment, the total protein amount (Lowry method modified by Oyama) [Oyama, 1956], protein dynamics and the cell cultures development degrees were evaluated. The cytostatic property of the studied biopreparations was appreciated on the basis of the American prescreening program, which imposed a minimum inhibitory impact of 50% for the *in vitro* selection of the potential antitumoral agents [Leiter et al., 1965; Dold, 1988; Boyd, 1989; Philips et al., 1990].

For each culture type and time interval five culture tubes were used and the results were evaluated statistically by Student's "t" test [Snedecor, 1968].

RESULTS AND DISCUSSIONS

The experimental data obtained in the investigation of the glucanic biopreparations' action, in a dose of 1.5 mg/ml, on proteinsynthesis process of HEp-2p cells as compared to controls are shown in Table I.

Table I The total protein contents (µg/culture) of the HEp-2p cellular cultures submitted to the action of exopolysaccharidic extracts, in a dose of 1.5 mg/ml, comparatively with control cultures. Figures in brackets indicate the number of experimental cultures for each type.

Experimental group	HEp-2p cell culture				
	24 hours	48 hours (24 h drug incubation)		72 hours (48 h drug incubation)	
	X ± ES	X ± ES	p	X ± ES	p
Control	301.0 ± 23.0 (5)	373.4±8.4 (5)	–	420.4 ± 10.9 (5)	-
GE14.1	301.0 ± 23.0 (5)	185.7 ± 6.5 (5)	<0.002	99.4 ± 9.1 (5)	<0.01
GE28.2	301.0 ± 23.0 (5)	200.1 ± 25.3 (5)	NS	247.6 ± 11.3 (5)	NS
GE31.1	301.0 ± 23.0 (5)	311.2 ± 34.2 (5)	NS	240.1 ± 20.5 (5)	NS
GE34.1	301.0 ± 23.0 (5)	310.5 ± 12.1 (5)	NS	264.5 ± 10.1 (5)	NS
GE35.2	301.0 ± 23.0 (5)	188.8 ± 8.6 (5)	<0.002	109.5 ± 8.4 (5)	<0,01
GE36.1	301.0 ± 23.0 (5)	368.4 ± 9.8 (5)	NS	236.2 ± 10.1(5)	NS
GE37.1	301.0 ± 23.0 (5)	156.0 ± 7.0 (5)	<0.002	59.2 ± 6.5 (5)	<0.001
GE37.2	301.0 ± 23.0 (5)	165.5 ± 9.4 (5)	<0.05	83.3 ± 9.8 (5)	<0,.002

In the case of control HEp-2p neoplastic cell cultures it can be observed a progressive augmentation of the total protein content from 24 hours age up to 72 hours age, which assures the normal development of this culture. The cultures incubated with the glucanic extracts, as compared to the control values, were characterized by a progressive decrease of the protein concentrations, which sometimes attended the statistical significance both at 48 hours and at 72 hours after an incubation with polyglucidic extracts for 24 and 48 hours. Therefore,

we assist at an inhibition of the protein biosynthesis induced by some bioactive extracts. The amplitude of the proteinsynthesis potential is correlated to the used glucanic extract. Thus, the exopolysaccharidic bioproducts GE14.1, GE35.2, GE37.1 and GE37.2 have caused a very profound diminution of the protein concentrations (99,4 µg, 109.5µg, 59.2µg and 83.3µg) while the impact of the others extracts (GE28.2, GE31.1, GE34.1, GE36.1) upon the protein biogenesis of the HEp-2p cultures was much more attenuated (247.6 µg, 240.1µg, 264.5 µg and 236.2 µg).

From Figure 1 we can follow the consequences of the *in vitro* treatments with GE14.1, GE35.2, GE37.1 and GE37.2 glucanic extracts, the most active bioproducts from the tested gamut, upon the proteinsynthesis dynamics of the HEp-2p cells.

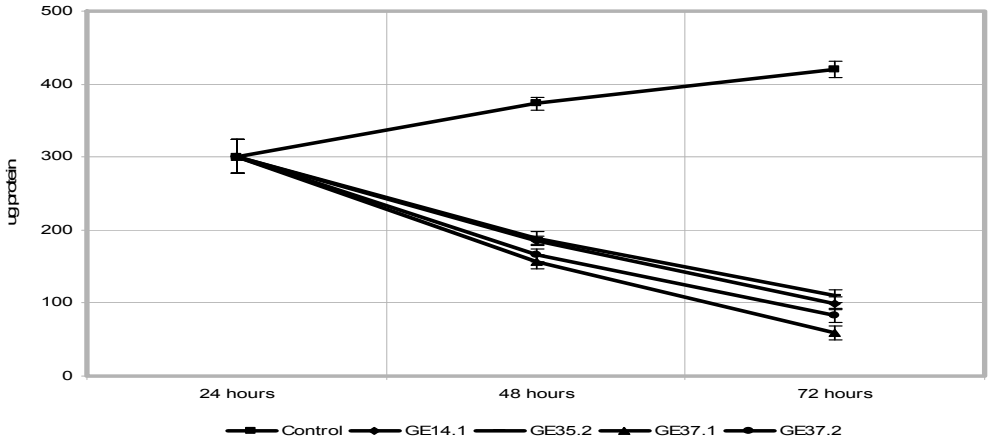

Fig. 1 The dynamics of the cell proteinsynthesis process, illustrated by the mean protein concentrations during the evolution of the HEp-2p cellular cultures, incubated or not with some glucanic extracts.

The successive graphical transposition of the total proteins value, obtained at different time intervals of cell cultures evolution, traces the proteinsynthesis dynamics, which in the case of the untreated tumoral cellular cultures, are characterized by an ascendant route with progressive increased amplitude. These peculiarities of the untreated cell cultures dynamics are the expression of an inherent proteinsynthesis and cell proliferation enhancement, which assures the normal development of the control cultures, considered by us as reference percentage value (100%).

Contrary, in the case of the treated tumoral cell cultures, it can been seen, during the evolution, that the protein dynamics is characterized by a descendent route and by a decrease amplitude, the most active biopreparatins being cronologically: GE37.1, GE37.2, GE14.1 and GE35.2.

The comparative analysis of the total protein values, estimated at the different ages of the treated cultures, with the control ones, has highlighted the interference of the glucanic treatment with the cultures development process, as it can be seen from Figure 2.

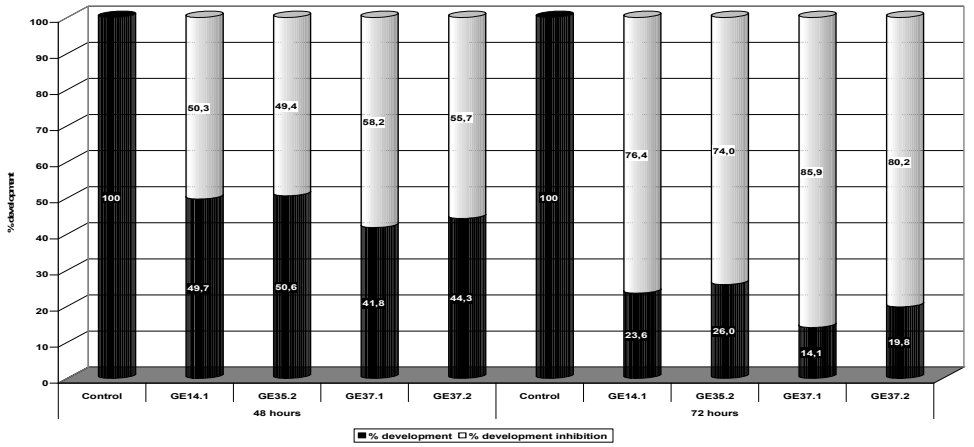

Fig. 2. The development degree of Hep-2p cancerous cell cultures, during their evolution, in the conditions of their incubation in the presence of the glucanic extracts.

The cultures treatment with the active glucanic biopreparations has been correlated to different degrees of development, the following levels were registered:

- in the case of 24 and 48 hours GE14.1 treated culture: 49.7% and 23.6%;
- for the 24 and 48 hours GE35.2 treated culture: 50.6% and 26.0%;
- in the case of 24 and 48 hours GE37.1 treated culture: 41.8% and 14.1%;
- for the 24 and 48 hours GE37.2 treated culture: 44.3% and 19.8%;

As compared to the 100% reference development value of the control cultures, an inhibitory impact of the glucanic exposure upon the HEp-2p cultures development can be evidenced and assessed, its intensity being chronologically:

- in the case of 24 and 48 hours GE14.1 treated culture: 50.3% and 76.4%;
- for the 24 and 48 hours GE35.2 treated culture: 49.4% and 74.0%;
- in the case of 24 and 48 hours GE37.1 treated culture: 58.2% and 85.9%;
- for the 24 and 48 hours GE37.2 treated culture: 55.7% and 80.2%;

Conferring the attribute of antitumoral agent to a new substance is the result of a complex, multidirectional and multistage process of investigation, on various and adequate experimental models, represented by biological testing systems of different organizational levels and characterized by specific reactive capacities (Jungstand et al., 1971; Cook, 2002; Crouch&Slater, 2001; Hrushesky, 1990; Philips et al., 1990).

The methodology and experimental protocol applied in our study correspond to the chemotherapeutical prescreening program on cancer cells cultures elaborated by the National Institute for Cancer Chemotherapy of USA, for the selection of some possible active cancerostatic agents. For a first step of investigation, the reference program imposes the induction of a minimum inhibitory impact upon the cell culture development by the tested product of at least 50%, for it to be considered a potential cytostatic or cytotoxic agent [Leiter et al., 1965; Riddell et al., 1986; Dold, 1988; Boyd, 1989; Philips et al., 1990].

Our *in vitro* preliminary testing of the effect of some new fungal exopolysaccharidic preparations upon the HEp-2p tumoral cells has highlighted a more or less pronounced negative impact induced by these bioactive products. This consequence of some of the tested

glucanic extracts can be due to an inhibitory effect upon cell proteinsynthesis process.

According to the alteration degree of the cells protein biosynthesis, we can classify the eight polyglucidic extracts in two categories:

− the former group, including the preparations GE28.2, GE31.1, GE34.1 and GE36.1, that was characterized by the induction of a moderate decrease of the protein concentrations during the evolution of the cultures, which haven't surpassed the minimum 50% level and

− the second group, that includes the GE14.1, GE35.2, GE37.1 and GE37.2 glucanic extracts, which has conditioned a very strong reduction of the cellular protein stocks (over 50%).

The negative impact of the four glucanic products upon the proteinsynthesis is observed during the entire evolution of the treated HEp-2p cultures, this conditioning the modification of the protein dynamics route and amplitude as well as the positive correlation between that two evaluation indices.

The inhibitory impact of this glucanic treatment upon the protein biosynthesis of HEp-2p neoplastic cells, which was accentuated during the evolution of the treated cell cultures, reacts on their development process. Thus, in comparison with 100% reference development of the control culture, the incubation of the cancerous cells with GE14.1, GE35.2, GE37.1 and respectively GE37.2 exoglucanic biopreparations has induced a profound perturbation of the cultures development, the inhibitory effect touching finally levels of 76.4%, 74.0% 85.9% and respectively of 80.2%, values which are superior to the minimum reference level imposed by the American prescreening program (50%).

Therefore, we consider that the last exopolysaccharidic products can be characterized as potential cytostatic agents, this property being the consequence of their proteinsynthesis inhibitory effect. We can, also, perform a hierarchy of the glucanic biopreparation from the point of view of their cytostatic effectiveness: on the first position is the glucanic extract codified GE37.1; on the second position is the GE37.2 and on the last place are the GE14.1 and GE35.2 extracts.

The signaled differences between the above mentioned glucanic biopreparations, in relation to their cytostatic potential, could be determined, on one hand, by a qualitative and/or quantitative compositional heterogeneity of those extracts, and on the other hand, by the different outturn of *Claviceps purpurea* strains in the glucanic bioproduction.

Generally speaking, the cytostatic property of a chemical, physical or biological agent represents the expression of its interaction with different cell structures and processes. For the time being, this property is supported, in our case, by the inhibitory impact of the bioactive agents upon the proteinsynthesis during the cell cultures evolution, with an immediate consequence upon the culture development. But, the culture development inhibitory impact can be determined by the drug interaction with other cellular processes.

Therefore, for an objective appreciation of the *in vitro* antitumoral property mechanism of the glucanic extracts, we proposed to study the effect of the active glucanic bioproducts upon cell mitosis and cellular viability, for identification of its cytostatic and cytotoxic components.

CONCLUSIONS

The *in vitro* testing of eight glucanic biopreparations, specifically extracted from diverse submerged strains of microfungus *Claviceps purpurea*, on HEp-2p neoplastic cells cultures, has highlighted the strong interaction of some of these biological extracts

with the cell proteinsynthesis process.

The attenuation of the proteinsynthesis intensity, the modification of the protein dynamics and the inhibition of the HEp-2p cell cultures development, during the evolution of the biological material, outline a significant cytostatic property of GE14.1, GE35.2, GE37.1 and GE37.2 glucanic extracts.

Further investigations are necessary, on cellular models adequate to *in vitro* prescreening, to establish the effect of the selected glucanic bioproducts upon the cell mitosis and viability.

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Acknowledgments: The authors wishes to thank Miss Rotinberg Myriam for linguistic revision.

1. Biological Research Institute, Str. Lascar Catargi, nr. 47, 700505, Iaşi, Romania, *cosmin.mihai.2005@gmail.com
2. Faculty of Biology, "Al. I. Cuza" University, Bd. Carol I, 20 A, 700505, Iaşi, Romania
3. „Gr. T. Popa" University of Medicine and Pharmacy, University Street, Iaşi, Romania