BEHAVIOR OF SOME ENZYMATIC SYSTEMS TO THE ACTION OF THE CYTOSTATIC ACTIVE *EGlCP* GLUCANIC BIOPREPARATION UPON HeLa NEOPLASTIC CELLS

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Abstract: Interference of an autochthonous cytostatic active *EGlCP* glucanic biopreparation (in dose of 1.5 mg/mL) with the activity of some key enzymes, involved in the development of active transmembranary transport, of the intermediary and energetic metabolism, as well as in cellular answer to the oxidative stress, of HeLa neoplastic cells has been investigated. The study revealed: the intensification of the membranary Na^+-K^+-ATP-ase, of the cellular Mg^{2+}-ATP-ase, of the superoxide dismutase activities; the operating level attenuation of the of catalase, peroxidase, glutathion peroxidase, lactate dehydrogenase, alkaline phosphatase, acid phosphatase; the diminution of the malondialdehyde content. This functional interference with some cell enzymatic biomolecules has also induced the perturbation of the diverse membrane and metabolic processes, which was incompatible with the survival of HeLa tumoral cells

The modulations of the cellular enzymatic equipment activity can be the consequences of the glucanic components direct (with the molecules of the miscellaneous enzymes) or indirect interactions (with membrane or genetic apparatus) with some cell, subcell and molecular structures, implicated in the control and regulation of the biosynthesis and activity of the enzymatic biomolecules. The central element, which induces this enzymatic imbalance, appears to be the excess generation of the free radicals in the tumoral cells’ metabolism aggressed by glucanic constituents.

INTRODUCTION

Always the fungi represented accessible resources for obtaining some extracts used in ethnophytotherapy of many and various diseases. Simultaneous with the development of chemistry were perfomed studies for the identification of the bioactive compounds from fungal extracts as well as for the evaluation of their activity. It has been found that a major part of the biological active substances are belonging to the polysaccharides (Wasser, 2002).

Among the bioactive polysaccharidic compounds we mention the β-glucans, which are a diverse group of molecules that can vary with respect to molecular mass, solubility, viscosity, and three-dimensional configuration. They occur most commonly as cellulose in plants, the bran of cereal grains, the cell wall of baker's yeast, certain fungi, mushrooms and bacteria. Some forms of beta glucans are useful in human nutrition as texturing agents and as soluble fiber supplements, but can be problematic in the process of brewing.

Therefore, a lot of biologically active compounds are generally glycoconjugates, and particularly glucans, they having numerous and diverse positive pharmacological properties. Thus, it has been highlighted that the glucanic biomacromolecules are behaving as antibacterial, antiviral, antifungal (Gantner et al., 2005), antiparasitical (Veleby et al., 2008), antiallergic Kirmaz et al.,2005), antiarhtritic (Bauerová et al., 2009), cardiovasomotor (Keogh et al., 2003), antiinflammatory (Luhm et al., 2006), antiteratogenic (Kobayashi et al., 1993), antioxidant (Kogan et al., 2005), immunomodulating (Demir et al., 2007; Vetvicka, 2007; Rotinberg et al., 2008b; Solcan et al., 2008), even antitumor and anticancer (Ooi and Liu, 2000; Daba and Ezeronye, 2003) agents.

Also, they are free radical scavengers (Sener et al., 2006, Toklu et al., 2006), metabolic and digestive modulators, nutritional supplements (Kren & Martinkova, 2001; Tian, 2007), antigenotoxic (Tohamy, 2003), antiradiation protectors (Cramer et al., 2006), as well as potentiating agents of some antibiotics (Kernodle et al. 1998).

The therapeutical importance of the glycoconjugates has imposed to the scientific world the necessity of obtaining some efficient bioactive products (Varki, et al., 1999; Wasser, 2002).

Despite the fact that there has been continuous progress in cancer diagnosis and treatment as a result of recent discoveries in cellular and molecular oncobiology, structural and functional genomics, pharmacogenomics and toxicogenomics, proteomics and metabolomics, antineoplastic therapy - which holds pride of place - is still of little effectiveness, fact explained and by its negative impact on the normal cells of the organism under neoplasm aggression and by the development of a resistance phenomenon of the tumoral cells to the cytostatic drugs action (Bronchud, 2000, Lyden et al., 2001; Adams, 2002; Anderson et al., 2002; Abrams, DeVita, 2004, Celis and Moreira, 2010).

So, in the fight against cancer – a real scourge of contemporary times – identification of new, more effective antineoplastic agents, as well as of new ways to decrease cancer cells resistance to cytostatics represent topical and major objectives (Bronchud, 2000; Abrams, 2003; Lodish et al, 2003; DiPiro et al, 2005).

Our previous studies, on appropriate experimental models to the pharmacodynamic or cellular and molecular oncobiology researches, developed either *in vitro*, on various healthy and neoplastic cells (Mihai et al., 2008a), or *in vivo*, on rats bearing various experimental tumoral systems, have highlighted and quantified the significant antitumor property of a biopreparation of glucanic nature (*EGlCP*), extracted from submerged cultures of *Claviceps purpurea* microfungus (Gherghel et al., 2008; Mihai et al., 2008b), as well as the reactivity of some membrane and metabolic processes of cancerous cells to the action of this new potential cytostatic agent (Rotinberg et al, 2007; Rotinberg et al., 2008a).

Thus, it has imposed extending and thoroughgoing investigations necessary to preclinical pharmacological characterization of the product as new oncochemotherapeutic drug, one of very important direction being the elucidation of cellular, subcellular and molecular mechanisms of action, involved in the expression of the pharmacodynamic potential.

Consequently, a first problem investigated in the present work, complementary and explanatory for the functional membrane and metabolic modifications signaled by us in other papers, was related to the interference of the cytostatic active *EGlCP* glucanic biopreparation with the activity of some key enzymes involved in the development of active transmembranary transport, of the intermediary and energetic metabolism, as well as in cellular answer to the oxidative stress.

MATERIALS AND METHODS

The biological material used in the *in vitro* experiments was represented by mycoplasm-negative, stabilized, HeLa cellular cultures of human neoplastic origin, obtained from an uterine cervix carcinosarcoma and cultured in DMEM growing medium (Dulbeco's Modified Eagle's Medium, Biochrom AG, Germany, FG 0415), supplemented with 10.0% fetal bovine serum (Sigma, Germany, F9665), 100 µg/mL streptomycin (Biochrom AG, Germany, A 331- 26), 100 IU/mL penicillin (Biochrom AG, Germany, A 321-44) and 50 µg/mL antimycotic amphotericin B (Biochrom AG, Germany, A 2612), at a density of 2 x 10^6 cells / 300 cm^2 flask, in a humidified 5% CO_2 atmosphere at 37°C (Bissery and Chabot, 1991, Doyle and Griffiths,1998,).

The tumoral cells were incubated for a period of 144 hours, the growing medium being renewed twice in this time frame of cultures development. When the cells reached confluence in the monolayer stage, the cultures were divided into control and glucanic treated cell cultures.

The cytostatic agent used by us in the HeLa neoplastic cells treatment was a biopreparation of glucanic nature (*EGlCP*), specific extracted from submerged cultures of *Claviceps purpurea* microfungus. At the 144 hour old of tumoral cell cultures – the optimum age for achievement of an adequate cellular mass – the culture medium of treated cultures was discarded from the test flasks and replaced with a fresh medium which contain *EGlCP* bioproduct in dose of 1.5 mg/mL, the duration of treatment being of 12 hours. In case of control cultures, the culture medium was replaced with a fresh one.

The layer of tumoral cells was washed with phosphate buffered saline, precisely weighted and then subjected to the steps of obtaining the clarified cellular lysates. Adequate aliquots were used for the biochemical determination of the membranary Na^+-K^+-ATP-ase, cell $Mg2^+$-ATP-ase, lactate dehydrogenase (LDH), peroxidase (Px), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), acid (ACP) and alkaline (ALP) phosphatase activities and of malondialdehyde levels (MDA) (Artenie et al., 2008).

The estimation of the total Mg^{2+}- Na^+ - K^+ and respectively membranary Na^+-K^+-ATP-ases (tATP and mATP) activities, expressed in mg inorganic phosphate/minute/g cellular mass (mg Pi/min/gcm), was based on the amount of inorganic phosphorous released after ATP hydrolysation by ATP-ases from the cellular homogenate. Lactate dehydrogenase activity (µM/min/gcm) was determined through the measurement of NADH oxidation velocity in the case of transformation reaction of pyruvic acid in lactic acid. Peroxidase activity (peroxidase unit, UP, /min/gcm) was estimated by o-dianisidine method, which measures the intensity of the o-dianisidine oxidation product colour.

Glutathione peroxidase activity (µM GSH/ml/min/gcm) was measured on the basis of the reaction of unconsumed reduced glutathione with 2, 2'- dinitro-5, 5'- dithiodibenzoic acid (Merck), which drives to a yellow, photometrable complex.

The evaluation of superoxide dismutase activity (superoxide dismutase unit, USOD, /ml/min/gcm) is based on the enzyme capacity to inhibit the nitroblue tetrazolium reduction by the superoxide radicals generated in reaction medium through riboflavin reduction.

Catalase activity was estimated through spectrophotometric registration of the hydrogen peroxide consumed quantity, being expressed in enzymatic unit (UE/gcm).

Alkaline and acid phosphatases activities (international unit, U.I., /gcm) were determined with para-nitrophenol, which is converted in a spectrophotometrable product, p-nitrophenolat, under the action of phosphatases.

At high temperature and in acid medium, malondialdehyde – product of lipid peroxides degradation – reacts with 2-thiobarbituric acid, leading to a photometrable pink trimetinic adduct (MDA nM/ml /gcm).

Five flasks of cultures have been used for each experimental group, the results being analyzed statistically by means of Student' „t" test (Cann, 2002).

RESULTS AND DISCUSSIONS

The investigation of the consequences of the glucanic treatment upon HeLa cell cultures has conducted to a set of data – shown in figure 1 and 2 – which expresses the modulation of some cellular enzymatic activities by this chemical agent. Thus, the action of *EGlCP* bioproduct upon the activity of different enzymatic systems has materialized, comparatively with the one of the control group, through variations of its sense and amplitude.

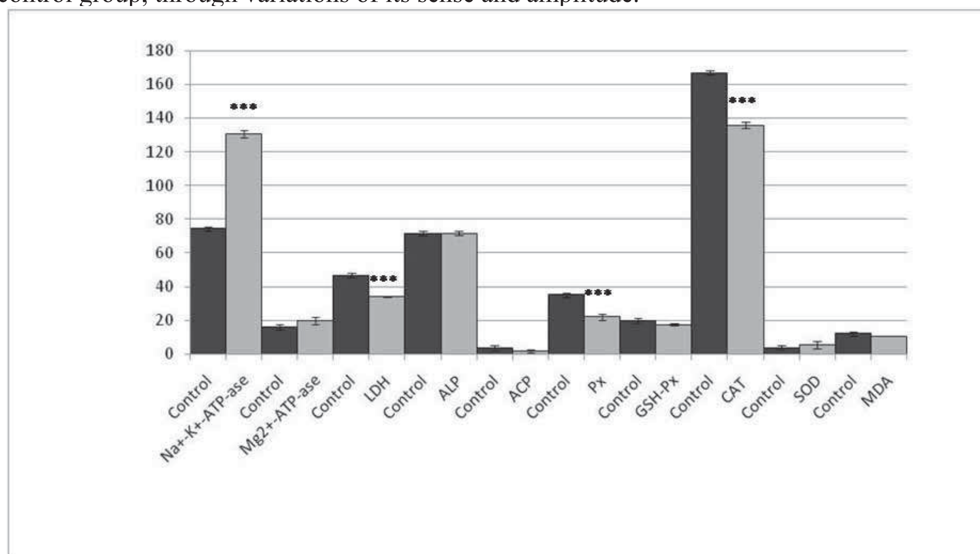

Figure 1. The impact of EGlCP bioextract (1.5 mg/mL) upon the activities of membranary Na^+-K^+-ATP-ase (mg Pi/min/g cell mass), cellular Mg2$^+$-ATP-ase (mg Pi/min/g cell mass), LDH (µM/min), ALP(U.I.) ACP(U.I.), Px(UP/g/min.), GSH-Px (µM GSH/ml/min.), SOD (USOD/ml/min), CAT (UE) enzymes and upon MDA levels (nM/ml) of HeLa neoplastic cells. Significantly different from control: ***p<0.001.

It can be seen, from Figure 1, that in the case of the control HeLa cell cultures, enzymatic activities were of: for membrane Na^+-K^+-ATP-ase, 74.49 mg Pi/min/gcm; in the case of cellular Mg^{2+}-ATP-ase, 16.15 mg Pi/min/g cm; for LDH, 46.7 µM/min/gcm; for Px, 35.28 UP/gcm/min; in the case of GSH-Px, 19.86 µM GSH/ml/min/gcm; for SOD, 3.96 USOD/ml/min/gcm; for CAT, 166.89 UE/gcm; in the case of ACP 3.69 U.I./gcm; for ALP 71.66 U.I./gcm; in the case of lipooxidation enzymes, 12.10 nM MDA/ml/gcm. We considered these quantitative estimations as reference values, necessary for the interpretation of the glucanic impact' signification upon the activity of the studied enzymes.

As compared to the control group, the interference of glucanic bioextract with enzymatic activities has determined significant functional and statistical modifications of these membranary and intracellular biomolecules. Thus, the impact of the glucanic treatment has been expressed by increases – in case of the membranary Na^+-K^+-ATP-ase (130.72 mg Pi), cellular Mg^{2+}-ATP-ase(19.74 mg Pi), SOD (5.59 USOD/ml/min) – and decreases – in case of the LDH (33.99 mM/min.), Px (22.27 UP/g/min), GSH-Px (17.61 µM GSH/ml/min), CAT (135.9 UE), ACP (1.76 UI), ALP (71.66 U.I./gcm) MDA (10.80 nM/ml) – of the studied enzymes activities.

In comparison with the control enzymatic functionality (see Figure 2), the activity of mem-

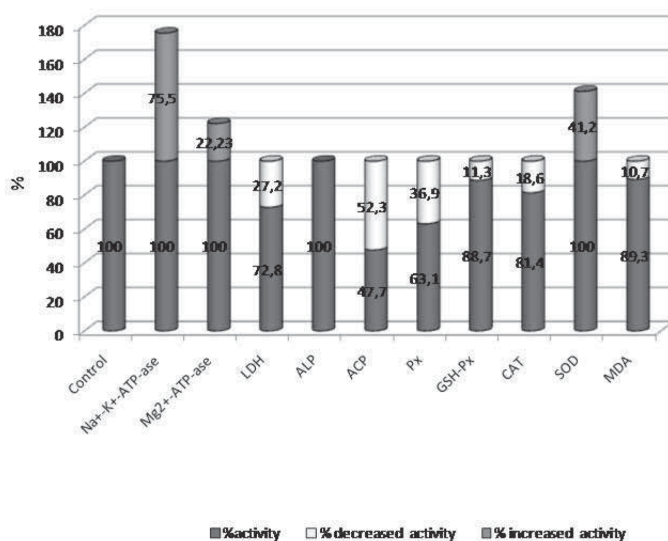

Figure 2. Modulation of the membranary Na^{+}-K^{+}-ATP-ase, cellular $Mg2^{+}$-ATP-ase, LDH, ALP, ACP, Px, GSH-Px, SOD, CAT, enzyme activities and MDA levels (nM/ml) of HeLa cell cultures caused by the EGlCP bioproduct (1.5 mg/mL).

branary Na^{+}-K^{+}-ATP-ase has been amplified with 75.5%, while the operating level of cellular Mg^{2+}-ATP-ase has been increased, just with 22.23%.

In the case of the enzymes implied in the response to oxidative stress, we assist to an attenuation of some activities with 36.9% (Px), 11.3% (GSH-Px), 18.6% (CAT), and 10.7% (MDA), respectively, or to an enhancement of SOD's activity with 41.2%.

The activity of lactate dehydrogenase was decreased with 27.2%.

It can be also observed that the operating level of the ALP was not perturbed (100%) and of ACP has registered a major regression (with 52.3%).

The *in vitro* testing on normal and tumoral cell cultures have practical importance in the selection of potential oncochemotherapeutic agents of diverse chemical nature. The cell cultures are also compatible and useful experimental models for preliminary understanding of the action mechanism implied in inducing of pharmacodynamic effect of the bioactive agent (Leiter et al., 1965; Boyd, 1989; Bissery and Chabot, 1991; Phillips et al., 1991).

The animal eukaryotic cells contain self regulation and self control mechanisms which maintain the cell homeostatic status, they being the target of the biologically active substances. The activation of molecular mechanisms of the cellular functional regulation is dependent on the transformation of the extracellular information in an action of cellular response. In this condition, the starting molecular event is logically localized at the level of the environment–cell interface, meaning in the cellular membranes. After this primary interaction between an agent and a cell membrane, there takes place the transfer and traducing of the extracellular signal. Consequently, the intracellular mechanisms of control and the activity of the enzymatic systems will be influenced. These specific modulations would stimulate and would inhibit the different metabolic processes which will exteriorize by global pharmacodynamic effect (Benga 1985; Karp, 1996; Alberts et al., 1998; Stroescu, 1998; Cruce, 1999).

The lack of balance between the structural components of the tumoral cell membranes – the decrease of the membranary fluidity, the modification of the packing degree of the membrane overmolecular structures, the different topographical location and activity of the membrane ATP-ases – is functionally expressed by perturbation of the membranary permeability. The modification of the ionic fluxes leads to the appearance of the transmineralization phenomenon.

This specific feature of the neoplastic cells consists in an abnormal distribution of the ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- etc.) correlated with other ionic ratios in extra- and intracellular compartments and with a decrease of the membrane resting potential. Among other membranary peculiarities of the tumoral cells, it is important to mention the powerful enhancement of the activity of the Na^+–K^+–depending electrogenic pump (Bannasch et al., 1998; Olbe, 1999; Miron, 2000; Owens, 2001).

Our experimental results, registered after treatment of tumoral HeLa cells with exopolysaccharidic *EGlCP* bioproduct have highlighted an increase of the inorganic phosphate, in the membrane and intracellular substratum, fact that suggests a high activity of the membrane Na^+-K^+ electrogenic pump and of the total ATP-ases. Thus, we assist to a stimulatory impact upon the activity of the cell ATP–ases system, comparatively to the one of control, which reveals diverse energetically needs for the insurance of the optimal active transmembranary fluxes of Na^+ and K^+ cations in the glucanic treated HeLa cells.

The stimulatory effect of the glucanic bioextract upon Na^+-K^+ membrane electrogenic pump can be the consequences of it direct interaction with some membrane structures (receptors or Na^+-K^+-ATP-ase biomolecules). This supposition is argued by modulation of the membranary Na^+–K^+–ATP-ase in the condition of *in vitro* treatment of the human HeLa tumoral cells with *EGlCP*, which influences consecutively the membrane permeability, transmembranary ionic fluxes, ionic equilibrium, extra- and intracellular ionic ratios. Our assumption is according to some recent bibliographical data (Akinori, 1996; Mithöfer, 2005), which suggest that the plasma membrane is the site of glucan's primary action.

We cannot exclude the intracellular penetration of the exopolysaccharidic molecules, due to increased permeability of tumoral cells for the large sizes energogenetic sources, as well as their direct or indirect interaction with the intracell enzymatic systems or intracell receptors.

A key-enzyme of the glucidic intermediary metabolism and a well-known marker of malignant cells is lactate dehydrogenase biomolecule. In the conditions of the cytostatic *EGlCP* treatment, the LDH activity was obviously perturbed, its operating level being inhibited by a probable stimulation of pyruvate or NAD^+ synthesis.

Another enzymatic system, implied in the phosphorylation and dephosphorylation cell metabolic reactions, includes the ALP and ACP phosphatases. In our experimental conditions, the HeLa cells exposure to the glucanic action has not affected the ALP functionality, while the ACP activity was significantly repressed because of some probable changes in intracellular pH due to a very increased intracell production of the free radicals.

The above explications are also supported by our experimental results upon the functional behaviour of the free radicals scavenger enzymatic system (peroxidase, glutathione peroxidase, catalase, superoxide-dismutase etc.) to the glucanic impact on HeLa cells.

Thus, it can be seen that the *EGlCP* has conditioned a nonsignificant attenuation of the Px, GSH-Px and CAT activities, consequence of the possible lack of the specificity to the currently existing substrates in cells. Surprising is the registered simultaneous increase of SOD activity, which justifies a fast elimination of superoxide radical ($O_2^{\,-}$), probably under the influence of the stress generated by the presence of the glucanic extract in the culture medium. This behaviour

leads us to the idea that SOD may be a target of glucanic action, suggesting a promising clinical and experimental way to selectively kill cancer cells (Huang et al., 2000).

The *EGlCP* treatment of the HeLa cells has been correlated with decreased MDA levels, suggesting the inhibition processes which generate the lipid peroxides.

Our experiments have highlighted that the *EGlCP* biopreparation modulates the activities of some oxidative stress enzymes (Px, GSH-Px and CAT), more in an inhibitory manner then in one stimulatory. Therefore, it seems that the glucanic extract – used for cytostatic treatment – has altered probably the unfolding of the metabolic events, generating smaller or larger intracellular amounts of specific free radicals, which have repressed or enhanced the activity of some clearing enzymes by feedback mechanism, generated by modifications of cancerous cells' intracellular medium " homeostasis ". This effect of *EGlCP* – which partially explains its cytostatic action – can be the result of an indirect or direct interaction of the glucanic compounds with the enzymatic biomolecules or with the membranary or intracell receptors.

In this moment of research, we can appreciate – in the light of our results on the behaviour of some enzymatic equipments to the glucanic action – that the oncostatic property of the *EGlCP* natural extract can be also the expression of it capacity to induce profound perturbations of the operating degree of various enzyme systems, these being not compatible with survival of tumor cells, hence the *in vitro* cytostatic action of the autochthonous fugal glucanic extract.

Modification of the tumor cells' specific homeostatic level, no longer is compensated by the specific cell control systems, leads to expression of the cytostatic effect of the glucanic extract. The central element that generates this imbalance seems to be represented by the excess generation of some specific free radicals.

CONCLUSIONS

The *in vitro* glucanic treatment of the HeLa human tumoral cells modulates the activity of some enzymatic systems, located either at membrane level or at intracellular one.

Stimulation of the Na^+-K^+-ATP-ase, of cellular Mg^{2+}-ATP-ase and SOD enzymes or inhibition of LDH, ACP, Px, GSH-Px, CAT, and those implicated in lipid peroxidation reactions have perturbated membrane and metabolic processes, justifying the cytostatic impact of the glucanic extract.

This cytostatic property of the *EGlCP* is probably due to a primary membranotrop action mechanism and/or metabolic action mechanism of the exopolysaccharide compounds, which can interact with the membrane receptors and/or intracellular ones.

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