

THE BEHAVIOUR OF THE MEMBRANARY $\text{Na}^+ - \text{K}^+ - \text{ATP-ase}$ OF NEOPLASTIC CELLS TO THE IN VITRO CYTOSTATIC TREATMENT WITH BIOSYNTHESIS OR SEMISYNTHESIS FUROSTANOLIC-GLYCOSIDES

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Key words: furostanolic glycoside cytostatics; neoplastic cell cultures; $\text{Na}^+ - \text{K}^+ - \text{ATP-ase}$ activity; membranary ionic transport; mechanism of action.

Abstract: The in vitro short lasting antitumoral treatment of the HEp-2p and HeLa human tumoral cells with some active cytostatic furostanolic-glycoside biopreparations has induced an inhibitory impact upon $\text{Na}^+ - \text{K}^+$ -depending electrogenic pump. These furostanolic glycoside agents interferes, probably, direct or indirect with the membrane $\text{Na}^+ - \text{K}^+$ -ase activity, either by their binding of the enzyme macromolecule or by perturbing of the energetical metabolism which leads to a decreased level of the ATP biosynthesis.

INTRODUCTION

A lot of biologically active compounds are glycosides. Sometimes, the glycosidic residue is crucial for their activity, in other cases glycosylation only improves pharmacokinetic parameters. Recent developments in molecular glycobiology brought better understanding to the aglycone versus glycoside activities, and made possible to develop new, more active or more effective glycodrugs based on these findings (Kren and Martinkova, 2001).

Our previous preclinical studies – performed on experimental models adequate to the in vitro investigation on neoplastic cell cultures – were relevant for the appreciation of some original biopreparations of furostanolic glycoside type, extracted from *Digitalis purpurea* leaves, as potential cytostatic drugs with possible biomedical significance (Gherghel et al., 2004).

The pharmacological characterization of a new biosynthesis, semisynthesis or synthesis product as chemotherapeutic agent is assured not only by in vitro and in vivo highlighting, confirmation and quantification of the pharmacodynamic effect, but also by data regarding the action mechanism at cellular, subcellular and molecular level implied in the overall expression of the specific pharmacological property (Calabresi and Parks, 1985; Goodman and Gilman, 1985; Chiricuță, 1988; Boyd, 1989; Phillips et al., 1990; Bissery and Chabot, 1991; DeVita, 1991; Stroescu, 1998; Lyden et al., 2001; Sheetala and Prabhavathi, 2001; Weinstein, 2001; Habeck, 2002; Wong, 2002).

Consequently, in the light of the above affirmations, the extending and thoroughgoing of the research have been required in order to establish the probably action mechanism involved in inducing of the in vitro antitumoral effect. Thus, in a first step, we have decided to investigate the reactivity of the cancerous cell's membrane phenomena in the conditions in the in vitro treatment with some glycosidic cytostatics.

The purpose of the present paper, which includes results of a preliminary research, is the analysis of the interference of B1 and E5 cytostatic agents of furostanolic glycoside nature with $\text{Na}^+ - \text{K}^+ - \text{ATP-ase}$ activity from the membrane level of the HEp-2p and HeLa tumoral cells.

MATERIALS AND METHODS

The active cytostatic compounds of furostanolic glycoside nature which were used in the in vitro experiments have been the following:

- B1, a biosynthesis product, extracted and specifically purified from leaves of *Digitalis purpurea*;
- E5, which is a semisynthesis product, derived from the former by partial oxidation until to obtaining of some aldehydic groups.

The biological material used in the in vitro investigations was represented by the control and treated HEp-2p and HeLa cellular cultures of human neoplastic origin (laringeal carcinoma and cervix carcinosarcoma, respectively).

The test tubes have been inoculated with 1×10^5 tumoral cells in Eagles' MEM growing medium supplemented with 10% calf serum, they being incubated at 37°C for a period of 72 hours.

After 72 hours of cultures development, when the monolayer stage was attained, the initial medium was replaced with a medium containing one from the two furostanolic glycoside biopreparations in a dose of 5 mg/ml. The cultures were incubated again at 36.5–37°C for 180 minutes in the presence of the drugs.

After this short in vitro antitumoral treatment, the medium was discarded from the test tubes. The layer of tumoral cells was washed with PBS and then subjected to the biochemical determination steps of membrane enzyme implied in the transmembranary active transport of Na^+ and K^+ ions.

The activity of these cations –depending electrogenic pump was assessed by the spectrophotometrical quantitative analysis of inorganic phosphate ($\text{mg P}_i/\text{g}$ of cell protein) from the cell homogenate, after Ca^{2+} -ATP-ase blocking with EDTA and ATP hydrolysis by Na^+ - K^+ -ATP-ase action (Artenie and Tănase, 1981).

Five tubes of cultures have been employed for each culture type, the results being analyzed statistically by means of Student' „t” test (Snedecor, 1968).

RESULTS AND DISCUSSIONS

In a first test we have investigated the effect of B1 and E5 active cytostatic furostanolic-glycoside biopreparations upon the membranary Na^+ - K^+ -ATP-ase activity from the HEP-2p tumoral cells which has been expressed by quantitative and percentage values.

Table 1. The behaviour of the Na^+ - K^+ -ATP-ase ($\text{mg P}_i/\text{g}$ protein) from the membranes of the HEP-2p neoplastic cells submitted to the short treatment with B1 and E5 cytostatic agents, in a dose of 5 mg/ml. Figures in brackets indicate the number of experimental cultures for each type.

Culture types	$\bar{X} \pm \text{ES}$	p
Control	56.90 \pm 3.95 (5)	
B1	21.45 \pm 1.80 (5)	<0.001
E5	26.10 \pm 2.05 (5)	<0.001

It can be seen, in Table 1, that the in vitro short antitumoral treatment of the 72 hours old HEP-2p cellular cultures has induced a statistically significant decrease of the inorganic phosphate contents from the membranes of the treated neoplastic cells, comparatively with the control level.

Another in vitro experiment has been performed in the same conditions for the appreciation of the membrane Na^+ - K^+ -ATP-ase reactivity to the cytostatic impact of the bioactive agents of glycosidic nature upon HeLa cell cultures.

Table 2. The membrane Na^+ - K^+ -ATP-ase activity ($\text{mg P}_i/\text{g}$ protein) in the HeLa tumoral cells treated with 5 mg/ml of B1 and E5, comparatively with the control cultures. Figures in brackets indicate the number of experimental cultures for each type.

Culture types	$\bar{X} \pm \text{ES}$	p
Control	55.77 \pm 4.05 (5)	–
B1	23.40 \pm 1.90 (5)	<0.001
E5	33.15 \pm 2.47 (5)	<0.002

From Table 2, it can be observed that the inorganic phosphate concentrations have again presented an important diminution as compared to the control value.

The reduced contents of the inorganic phosphate, released by enzymatic hydrolysis of the ATP, have revealed lower degrees of the activity of the Na^+ - K^+ -depending membrane electrogenic pump in the case of HEP-2p and HeLa tumoral cells submitted to the in vitro short cytostatic treatment.

Thus, it can be highlighted, from the following figures and in comparison with 100% control values, that the membranary Na^+ - K^+ -ATP-ase activity reaches intensities of 37.7% (B1) and 45.9% (E5), respectively, in the case of the HEP-2p treated neoplastic cells, as well as of 41.9% (B1) and respectively 59.4% (E5) in the case of the HeLa treated cancerous cultures.

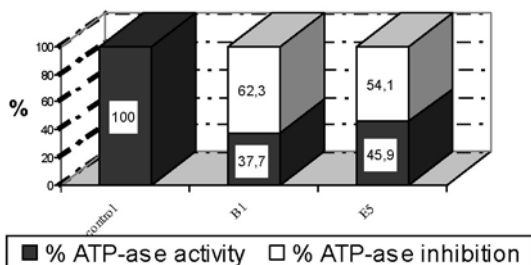


Fig. 1. Modulation of the $\text{Na}^+\text{-K}^+$ -dependent electrogenic pump's activity (%) from the HEp-2p tumoral cells submitted to the furostanolic glycoside treatment, in comparison with the control cultures.

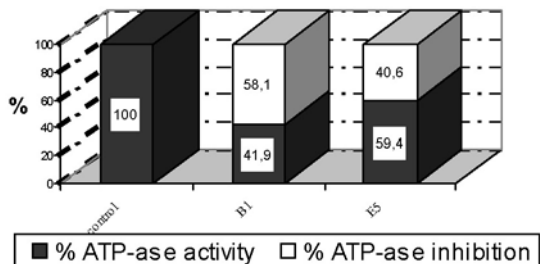


Fig. 2. Variations (%) of the membranary $\text{Na}^+\text{-K}^+$ -ATP-ase activity at HeLa cancerous cells treated with the glycosidic cytostatic agents, comparatively with the control one.

The various *in vitro* testing systems with tumoral cell cultures have practical importance both in the selection of potential oncochemotherapeutic agents of diverse chemical nature and in the understanding of the action mechanism responsible for therapeutic impact. The tumoral cell cultures are compatible and useful experimental models for preliminary appreciation of the mechanism implied in inducing a pharmacodynamic effect of an active biological agent (Leiter et al., 1965; Bissery and Boyd, 1989; Chabot, 1991; Phillips et al., 1991; Lyden, 2001; Habeck, 2002; Wong, 2002).

The animal eukaryotic cells contain self regulation and self control mechanisms which maintain the cell homeostatic state, they being the target of the biologically active substances. Generally speaking, 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 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 poise 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 are 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 (Benga, 1979; Chaubal and Firket, 1979; Binggeli and Cameron, 1980; Bianchi et al., 1986; Rusu et al., 1988; Bannasch et al., 1998; Cruce, 1999; Olbe, 1999; Miron, 2000; Owens, 2001; Wong, 2002).

This informational context justifies our objective of investigating of the effect of the B1 and E5 cytostatic agents of biosynthesis and semisynthesis upon the neoplastic cell membrane phenomena and to appreciate our experimental results in order to explain the possible mechanism of inducing their in vitro antitumoral impact.

Thus, in the present study we have followed the effect of the furostanolic-glycoside cytostatics upon the membranary Na^+ - K^+ -depending ATP-ase activity of the HEp-2p and HeLa tumoral cells.

The experimental results, registered after short lasting treatment of HEp-2p and respectively HeLa cells with the B1 or E5 antitumoral agents, have highlighted inferior amplitudes of the membrane Na^+ - K^+ -ATP-ase activity, comparatively with the one of control. Therefore, the furostanolic-glycoside biopreparations have induced an inhibitory impact upon the membrane electrogenic pump of Na^+ - K^+ . The intensity of the depressing effect was assessed at 62.3% (HEp-2p) and 58.1% (HeLa) for the B1, as well as at 54.1% (HEp-2p) and 40.6% (HeLa) in the case of the E5.

The decreased levels of the ATP enzymatic hydrolysis don't assure the energetical needs for the active transmembranary fluxes of Na^+ and K^+ cations, which are also perturbed.

Consequently, new extra- and intracellular ionic ratios will be established. These will modify both optimal conditions for the diverse intracellular enzymatic systems' activity and the unfolding of the metabolic events. Finally, these disturbances induced by the glycosidic compounds could represent the molecular substratum of their cytostatic activity.

The inhibitory effect upon membranary Na^+ - K^+ -ATP-ase activity can be the consequence of a direct binding of the furostanolic-glycoside substances with the electrogenic pump molecule. This appreciation is a plausible hypothesis if we remind that ouabain – a membranotropic modulator – blocks the active transmembranary transport of the Na^+ and K^+ cations by coupling with the macromolecule of the membrane enzymes (Baker et al., 1980; Churchill and Churchill, 1980; Dawsen and Smith, 1986; Tran and Farley, 1986), therefore, with the same cellular substratum.

Certainly, we can't exclude the idea that the inhibitory impact of B1 and E5 -glycosidic agents upon the activity of the Na^+ - K^+ -ATP-ase could be the secondary result of another possible interaction with the intracellular receptors. This kind of interactions could be followed by alteration of the energetical metabolism, with negative consequences both on level of ATP biosynthesis and on membranary Na^+ - K^+ -depending ATP-ase pump activity.

In this moment, the analysis of the experimental results allows us to suggest that the main mechanism probably involved in the expression of the cytostatic effect seems to be one of membranary type, the furostanolic glycoside agents would interact with one from the specific membrane receptors of the tumoral cells.

In future studies we will investigate other aspects of the in vitro interference of the glycoside antitumoral drugs with the membrane and metabolic processes of the neoplastic cells in order to elucidate the mechanism responsible for their pharmacodynamic property.

CONCLUSIONS

The short lasting in vitro antitumoral treatment of the HEP-2p and HeLa human cancerous cells with the active cytostatic biopreparations of furostanolic glycoside nature have induced an inhibitory impact upon $\text{Na}^+\text{-K}^+$ -depending electrogenic pump.

The decreased ATP enzymatic hydrolysis has negative consequences upon the active membrenary transport of Na^+ and K^+ , as well as on cell metabolic processes.

The similar sense and different amplitude of the membrane reactivity of the HEP-2p and HeLa cultures highlights the various sensibilities of the tumoral cells to the B1 and E5 action.

In this preliminary step of the research, the results indicate that the most probably mechanism involved in the expression of the antitumoral impact of the biosynthetic and semisynthetic glycoside compounds seems to be one of membrenary type.

It can be possible that B1 and E5 agents to interacts with the membrane $\text{Na}^+\text{-K}^+\text{-ATP}$ -ase, their binding of the enzyme macromolecule partially blocking the electrogenic pump of Na^+ and K^+ and perturbing – by the consequences upon the active membrenary transport of ions – the metabolic events of the tumoral cells.

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