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Cytotoxicity of monensin, narasin and salinomycin and their interaction with silybin in HepG2, LMH and L6 cell cultures

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ABSTRACT

The cytotoxic effect of monensin, narasin and salinomycin followed by their co-action with silybin in the cell line cultures of human hepatoma (HepG2), chicken hepatoma (LMH) or rat myoblasts (L6) have been investigated. The effective concentration of the studied ionophoric polyethers has been assessed within two biochemical endpoints: mitochondrial activity (MTT assay) and membrane integrity (LDH assay) after 24 h incubation of each compound and farther, the cytotoxicity influenced in course of their interaction with silybin was determined. The most affected endpoints were found for inhibition of mitochondrial activity of the hepatoma cell lines and their viability depended on concentration of the ionophoric polyether, as well as on the cell line tested. The rat myoblasts were more sensitive target for cellular membrane damage when compared to inhibition of mitochondrial activity. An interaction between the ionophoric polyethers and silybin resulted a considerable cytotoxicity decrease within all studied cell lines; the combination index (CI) showed differences of interaction mode and dependence on cell culture, concentration of silybin, as well as the assay used. The obtained results are of interest in respect to recent findings on applicability of salinomycin and monensin for human therapy.

1. Introduction

Monensin, narasin and salinomycin belongs to the group of carboxylic polyether ionophores, the natural antibiotic compounds produced by *Streptomyces* spp. Their mechanism of action involves formation of lipid-soluble complexes which, as the monovalent cations, are easily transported across cellular membranes by passive diffusion processes. The ionophoric polyethers are widely used as an antiprotozoal agents (coccidiostats) against coccidial parasites in poultry and other livestock to improve feeding efficiency. The drugs' residues were determined within monitoring survey of eggs and edible tissues of chicken, mainly liver and muscle. This situation occurs when the ionophoric polyethers are overdosed in feed and/or a withdrawal period for the drugs are neglected (Olejnik and Szprengier-Juszkiewicz, 2007; Dorne et al., 2013). Moreover, narrow safety margin of ionophoric polyethers is bound to fatal poisoning of husbandry and non target animals; the clinical cases and risk assessment were reported (Novilla, 1992; Oehme and Pickrell, 1999; Saller et al., 2008; Dorne et al., 2013). Also the several accidental poisoning of people affected by these drugs have been described (Kouyoumdjian et al., 2001; Story and Double, 2004). The most common clinical signs of intoxications include cardiovascular effects, necrosis of striated muscules, neuropathy and gastrointestinal disorders. The acute toxicity of the ionophoric polyethers is moderate or highly dependent on animal species (Novilla, 1992; Novilla et al., 1994; Oehme and Pickrell, 1999).

The background of considerable interspecies variation in morbidity and mortality, as well as, the ionophoric polyethers chronic impact on human health are not fully discerned. The species dependent toxicity seems to be connected with metabolism of these compounds. Dominant mechanism of ionophoric polyethers activity targets disorder of membrane transports of the ions which involves mitochondrial injure, the cell swelling and vacuolization leading to termination of muscle and liver cells (Mollenhauer et al., 1990; Novilla, 1992; Novilla et al., 1994; Kowaltowski and Vercesi, 1999; Oehme and Pickrell, 1999). Although, the extent of impairment caused by ionophoric polyethers appears to be

Abbreviations: EC_{50}, median effective concentration of drug; MTT, 3-[4.5-dimethylthiazol-2yl]-2.5-diphenyl tetrazolium bromide; LDH, lactate dehydrogenase; SD, standard deviation; SEM, standard error of the mean; ATCC, American Type Culture Collection.

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fragmentary examined in respect to basic cytotoxicity (Park et al., 2003; Radko et al., 2006, 2007). Recently, the research is focused on salinomycin specific growth inhibition of cancer stem cells and adipocytes. Hence, clinical applicability to cancer or anti-obesity therapy has been currently under intensive consideration (Gupta et al., 2009; Huczyński, 2012a, 2012b; Szkudlarek-Mikho et al., 2012).

Milk thistle (*Silybum marianum* L. Gaertn.) is a plant with well recognized cytoprotective effect due to silymarin activity and it has been widely applied in medicine. An extract from milk thistle consists of silybin, as a main flavonolignan, which represents from 50% to 70% of silymarin (Gazák et al., 2007; Loguercio and Festi, 2011). Numerous studies reported its antioxidant and chemopreventive properties which are bound to more or less defined cytoprotection pathways. Hepatoprotective and cardiomyo-protective actions of silybin have been studied in intoxicated humans and animals (Chlopciková et al., 2004; Tedesco et al., 2004; Avizeh et al., 2010; Parveen et al., 2011; Rašković et al., 2011). Data of silybin *in vitro* investigation indicated that its activity is expressed as cell membrane stabilizer and permeability regulator. In addition, promotion of ribosomal RNA synthesis, free radicals scavenging and regulation of intracellular content of glutathione have been discovered (Pradhan and Girish, 2006). Investigation on silybin interaction, e.g. with metronidazole, cisplatin or doxorubicin, have been reported (Rajnarayana et al., 2004; Tyagi et al., 2004; Rašković et al., 2011). However, there is limited information regarding ionophoric polyethers co-action with cytoprotective agents. The outreach of this process is vague when use of silybin/silymarin in medication is of interest (Kŕen et al., 2013).

Literature cites a number of works using cell line cultures in cytotoxicity/ cytoprevention studies of the xenobiotics and their interaction. The human originated HepG2 cell line was found as a sensitive model of *in vitro* basal toxicity end-points (Schoonen et al., 2005a, 2005b; Castell et al., 2006). Hepatocytes as main cells involved in metabolism of these drugs are rationale behind the good points at their anticipation of the ionophoric polyethers adoption in cancer and anti-obesity therapy (Gupta et al., 2009; Huczyński, 2012a; Szkudlarek-Mikho et al., 2012). The LMH cell line was shown to possess a liver-like enzyme pattern including the pathways involved in xenobiotics biotransformation. These model cells were used in this study, since chicken hepatotoxicity was evaluated by means of the cell line (Kolluri et al., 1999; Ourlin et al., 2000). The myoblast cell line (L6) was isolated from primary cultures of rat thigh muscles being able to maintain many morphological and metabolic characteristics of skeletal muscles (Yaffe, 1968). These cells represent a good, sensitive tool for myotoxicity testing of the ionophoric polyethers which might be relevant to *in vivo* scenario.

Although the cell lines do not display all ability of the primary cell cultures which maintain more physiological profile, nonetheless they represent some good points. The cell lines are homogeneous and the obtained results are reproducible. Moreover, they allow to avoid preliminary step using experimental animals keeping along 3R rules. Hence, adoption the model study basing on above cells lines (hepatoma and myoblasts) was assuming to allow for determination of the ionophoric polyethers cytotoxic potentials. Basing on the defined metabolic pathways of the studied cells, it is possible to find out cytoprotective ability of silybin in course of the ionophoric polyethers impact. We based on the assays determining two end-points of the cell biochemical pathways which were used in the previous studies of the drugs (Radko et al., 2006, 2007). The cell viability was examined at the MTT reduction assay as a measure of metabolism impairment of living cells. Following endpoint, the lactate dehydrogenase release (LDH) test, was served to evaluate a cell membranes stability.

The aim of the research focus on median effective concentrations (EC_{50}) estimated for monensin, narasin and salinomycin in MTT and LDH assays using above hepatoma and myoblast cell cultures. Subsequently, impact of the ionophoric polyethers in course of interaction with silybin were measured. Denomination of the interaction type and its potential for each ionophoric polyethers combined with silybin action based on mathematical method of Chou and Talalay (1984).

2. Materials and methods

2.1. Chemicals and reagents

The following were purchased from Sigma-Aldrich (St. Louis, MO, USA): monensin sodium salt (CAS:22373-78-0, purity 90–95%, FW 692.85), narasin (CAS: 55134-13-9, purity 98%, FW 765.03), salinomycin (CAS: 53003-10-4, purity 98%, FW 751.00), silybin (CAS:802918-57-6, FW 482.44), Triton X-100, dimethyl sulfoxide (DMSO), 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT), trypsin-EDTA, fetal bovine serum (FBS), antibiotic solution (penicillin and streptomycin), and L-glutamine. Dulbecco's modified Eagle media (DMEM) were purchased from GIBCO. Waymouth MB 751/1 media were purchased from ATCC. All other chemicals were obtained from commercial suppliers and were of highest available purity.

2.2. Cell cultures

All cell lines in this study were purchased from American Type Culture Collection (ATCC). Human (HepG2) and chicken (LMH) hepatoma cell lines were cultured in DMEM. The rat myoblasts (L6) cell line was cultured in Waymouth MB 751/1. The media were supplemented with foetal bovine serum, antibiotics, and L-glutamine. These cells were cultured in 75 cm^2 cell culture flasks and kept at 5% CO_2, 95% air, at 37 °C. The medium was refreshed every 2 days and cells were trypsinized when the cells reached 70–80% confluence. The cells were counted using Bürker's hemacytometer and initial cell viability was determined with the trypan blue exclusion test. The well-grown cells were seeded and 100 μl was placed into 96-well plates (NUNC) at a density of 2.5×10^5 - cells/ml (HepG2, L6) and 2×10^5 cells/ml (LMH) following the incubation for 24 h before exposure to tested drugs.

2.3. Exposure to drugs

The concentration ranges were selected on the basis of the results of the previous studies on monensin, narasin and salinomycin. Monensin was tested from 0.1 to 25 μM on HepG2 and LMH cells. Narasin was tested from 0.05 to 25 μM on HepG2 and LMH cells. Salinomycin was tested from 1 to 50 μM on HepG2 and LMH cells. However, L6 myoblasts line was exposured on the ionophoric polyethers concentrations from 10 to 250 μM. However, it should be stated that the *in vitro* exposure to these concentration levels does not mimic exactly the drug kinetics observed in *in vivo* conditions. On the other hand, the concentrations were chosen taking into consideration those results from previous cytotoxicity studies of the drugs (Radko et al., 2006, 2007). Silybin at 10, 25 and 50 μM concentrations were co-incubationed with each ionophoric polyether in the cell cultures. The clinical data on concentration in human plasma determined c-max of 1.4 μg/ml silybin which corresponds to 0.64 μM (Weyhenmeyer et al., 1992). Even when liver concentrations of silybin increase up to 10 times the plasma level, concentrations beyond 50 μg/ml will not occur with maximum plasma levels. However, silybin concentration in the portal blood can achieve levels much higher than in the systemic