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Original article

Cytoprotective effect of silybin against lasalocid-induced toxicity in HepG2 cells

L. Radko[1], W. Cybulski[1], W. Rzeski[2]

[1] Department of Pharmacology and Toxicology National Veterinary Research Institute, Partyzantow 57, 24-100 Pulawy, Poland
[2] Department of Virology and Immunology, Institute of Microbiology and Biotechnology, Maria Curie-Sklodowska University, Akademicka 19, 20-033 Lublin, Poland
[2] Department of Toxicology, Institute of Agricultural Medicine, Jaczewskiego 2, 20-950 Lublin, Poland

Abstract

Lasalocid is an ionophore coccidiostatic agent frequently used in poultry. Its extensive use causes the formation of residues in edible tissues and eggs which may pose a risk to consumers. Silybin is the main compound extracted from the herb milk thistle *Silybum marianum* and its hepatoprotective effect has been reported in literature.

The aim of the study was to compare lasalocid and silybin cytotoxic effects followed by their combined use in HepG2 cell line. A cytoprotective effect resulting from the interaction of both pharmacologically active substances was measured.

In this study, an MTT test, coomassie brillant blue binding test, and LDH release test determined the effective concentration (EC_{50}) of the compounds. The isobolograms and combination index were used to assess the nature of interaction.

The lowest EC_{50}-value for lasalocid was established via the MTT test. This study revealed a lack of silybin cytotoxic effect on the cells. Co-actions of the two drugs led to a significant decrease of lasalocid cytotoxicity. The isobolograms and combination index showed a remarkable antagonistic effect in the course of silybin and lasalocid interaction.

The results indicate that silybin revealed a cytoprotective effect when incubated with lasalocid since its cytotoxic impact on HepG2 cells has been significantly diminished.

Key words: silybin, lasalocid, cytotoxicity, protecion, HepG2 cells

Introduction

Lasalocid belongs to ionophoric antibiotics, a group of chemotherapeutic agents used in veterinary practice for protection against gastrointestinal parasites (coccidiosis, caused by *Eimeria* sp.). These antibiotics are characterized by a narrow margin of safety, therefore they are not used in medicine because of their high toxicity to humans. Accidental cases of ionophore poisoning of people have been reported (Safran et al. 1993, Kouyoumdjian et al. 2001). In addition, concern of public health is involved due to lasalocid residues in excessive permissible concentrations revealed by monitoring of eggs and edible

Correspondence to: W. Cybulski, e-mail: wojciech.cybulski@piwet.pulawy.pl, tel.: +48 81 889 32 95

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tissues (Mortier et al. 2005, Olejnik and Szprengier-Juszkiewicz 2007, Wong and Roxburgh 2010). *In vitro* studies on liver cells reported that lasalocid disturbed the ions membrane transport, induced a process of mitochondrial injury and decrease of cell viability (Grinde 1983). Humans acutely poisoned by ionophore antibiotics manifested an increase in liver enzymes activity and myoglobinuria (Safran et al. 1993, Kouyoumdjian et al. 2001).

Cytoprotective agents are regarded as suitable, biologically active substances preventing pathogenesis of chemically-induced injury of human or animal's cells by restoring physiological and biochemical cell function (Gazak et al. 2007, Avizeh et al. 2009). Naturally occurring flavonoids have received increased attention in the past few years because of their chemopreventive properties. Silybin is one of the flavonolignans, tested in clinical trials, used for treating different types of liver disease. Silybin represents 50% to 70% of silymarin, which is an active extract from fruits and seeds of milk thistle (*Silybum marianum* L. Gaertn). Studies on animals and humans indicated that silymarin has got a broad spectrum of hepatoprotective effects in cases of the liver injured by acetaminophen, carbon tetrachloride, ethanol, iron overload, amanita mushroom poison and radiation. Chemopreventive efficacy has been demonstrated for different cell cultures (Wellington and Jarvis 2001, Ramadan et al. 2002, Fraschini et al. 2002, Kren and Walterova 2005). Data of silybin study indicate that its activity is expressed as cell membrane stabilizers and permeability regulators. In addition, promotion of ribosomal RNA synthesis, antioxidative action, free radicals scavenger properties and regulation of intracellular content of glutathione have been discovered.

Results of our previous study on rat FaO cells line indicated considerable cytoprotective effect of silybin when incubated with lasalocid. Hence, diminishing of the ionophore cytotoxicity developed in course with silybin interaction has been evidenced (Radko et al. 2011). The rationale for next step of the study was a choice of human hepatoma cell lines. The literature on HepG2 cell line proved it to be a good model of cytotoxicity assessment impacted by xenobiotics, as well as to estimate an interaction nature. (Ponsoda et al. 1995, Thabrew et al. 1997, Pook et al. 2006, Clothier et al. 2008, Chen et al. 2009).

The aim the presented study was focused on assessment of silybin and lasalocid effective concentrations followed by their combined co-action testing in the cultures of human liver-derived cell line, HepG2. Impact on cell viability was evaluated by three different endpoints at cellular level: reduction of metabolism activity of living cells (MTT), total cellular protein level (CBB) and lactate dehydrogenase release (LDH) tests. Isobolography and combination index (CI) revealed the nature of lasalocid and silybin interaction and its scale.

Materials and Methods

Chemicals

The following were purchased from Sigma-Aldrich (St. Louis, MO, USA): silybin ≥ 98%, lasalocid sodium salt > 97%, dimethyl sulfoxide (DMSO), 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT), coomassie brilliant blue R-250 dye, trypsin-EDTA, fetal bovine serum (FBS), antibiotic solution (penicillin and streptomycin), and L-glutamine. Minimal Essential Medium Eagle (MEME) was purchased from GIBCO. All other chemicals (acetic acid, hydrochloric acid, ethanol, potassium acetate) was obtained from commercial suppliers and were of highest available purity.

Cell cultures

The human hepatoma cell line HepG2, obtained from American Type Culture Collection (Manassas, VA, USA) was incubated in MEME culture. The culturing was performed according to cell line protocol obtained from supplier. Briefly, the medium was 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 Burker'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 and incubated for 24 h.

Exposure to drugs

The concentration ranges (1-250 μM) of lasalocid and the same range of silybin were selected on the basis of the results of the preliminary studies. The stock solution of lasalocid was dissolved in DMSO, while silybin was dissolved in ethanol. The final concentration of DMSO and ethanol was 0.1% in the medium. The same final concentrations of the solvents were used in the corresponding control. The substances background effect was measured after the

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incubations run without presence of cells, (a blank assay) in order to correct OD absorbance reading against tested samples. Each drug concentration was tested in six replications for 24 h. The study was repeated as three independent experiments.

MTT assay

The metabolic activity of living cells was assessed by the activity of dehydrogenases (Mosmann 1983). The MTT (3-[4.5-dimethylthiazol-2yl]-2.5-diphenyl tetrazolium bromide) was dissolved in a sterile phosphate buffer saline solution at the concentration of 5 mg/ml and sterilized by filtration through a 0.22 μm filter and protected from light by covering into aluminium foil. After incubation of the cells with the substances, 10 μl of the MTT solution was added to every well of 96-well plates and incubated for 3 h at 37°C in humidified atmosphere of 5% CO_2. Formazan crystals were solubilized overnight in an SDS buffer (10% SDS in 0.01 N HCl) and the product was quantified in the spectrophotometer by measuring absorbance at 570 nm wavelength using E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

Coomassie brilliant blue (CBB) assay

Total cellular protein was measured by the dye uptake being incorporated into the cells of the culture, which reflected the degree of cytotoxicity effects caused by the tested substances (Bradford 1976). The procedure was based on the INVITTOX Protocol No. 3b.

LDH assay

The lactate dehydrogenase release was determined by using the commercially available Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Poland). The assay is a mean to measure membrane integrity as a function of the amount of cytoplasmic LDH released into the medium (Sasaki et al. 1992).

Drug interaction analysis

The nature of the interaction between lasalocid and silybin was analysed with the help of isobolography based on the estimation of cytotoxic median effect (EC_{50}) (Chou and Talalay 1983).

The HepG2 cells were simultaneously incubated for 24 h with lasalocid at a median effect concentration (EC_{50}) combined with silybin range concentrations, 1-250 μM. Isoboles were defined by the effects of pair of the studied drugs. Lasalocid paired with silybin formed the basis for the additivity line. Synergism or antagonism was depicted by the linear cell-kill effects obtained by the toxic drug in combination with the interacting drug in different concentrations (Gessner 1995). The combination index (CI) mathematically compiled a two-drug pharmacological interaction and denominated its nature (Chou and Talalay, 1983, 1984). CI values were generated over a range 0.05 to 0.95 (5%-95%) of fractional cell-kill levels. CIs of less than 1 indicated synergy, CIs equal to 1 indicated additivity, and CIs exceeding 1 indicated antagonism.

Statistical analysis

The results representing a mean values ± standard deviation (SD) were analysed using one way analysis of variance (ANOVA) with *post hoc* Dunnett test to determine significance relative to unexposed control. The EC_{50} values, concentrations necessary for 50% of inhibition of viability of the cells for each substance were calculated according to Hill's equation. These values were expressed as mean + standard error of the mean (SEM) from three independent experiments. Statistical comparisons among EC_{50} results were performed by analysis of variance (ANOVA) followed by Tukey test. Differences were considered as statistically significant at $P \leq 0.05$.

Results

Effects of lasalocid on HepG2 cells

The cell metabolism, cellular protein content, and the integrity of cell membrane were significantly affected in a concentration-dependent manner after 24 h exposure of HepG2 cells in tested concentrations ranging from 1.0 to 250 μM, when compared to the control ($P<0.05$) (Fig. 1). The results of MTT, CBB and LDH assays with lasalocid EC_{50} in HepG2 cells are shown in Table 1. The mean values of effective concentrations in MTT assay were twofold lower than for other performed tests and statistically different ($P<0.05$).

Effects of silybin on HepG2 cells

Silybin at concentrations higher than 25 μM decreased the cell metabolism, cellular protein content,

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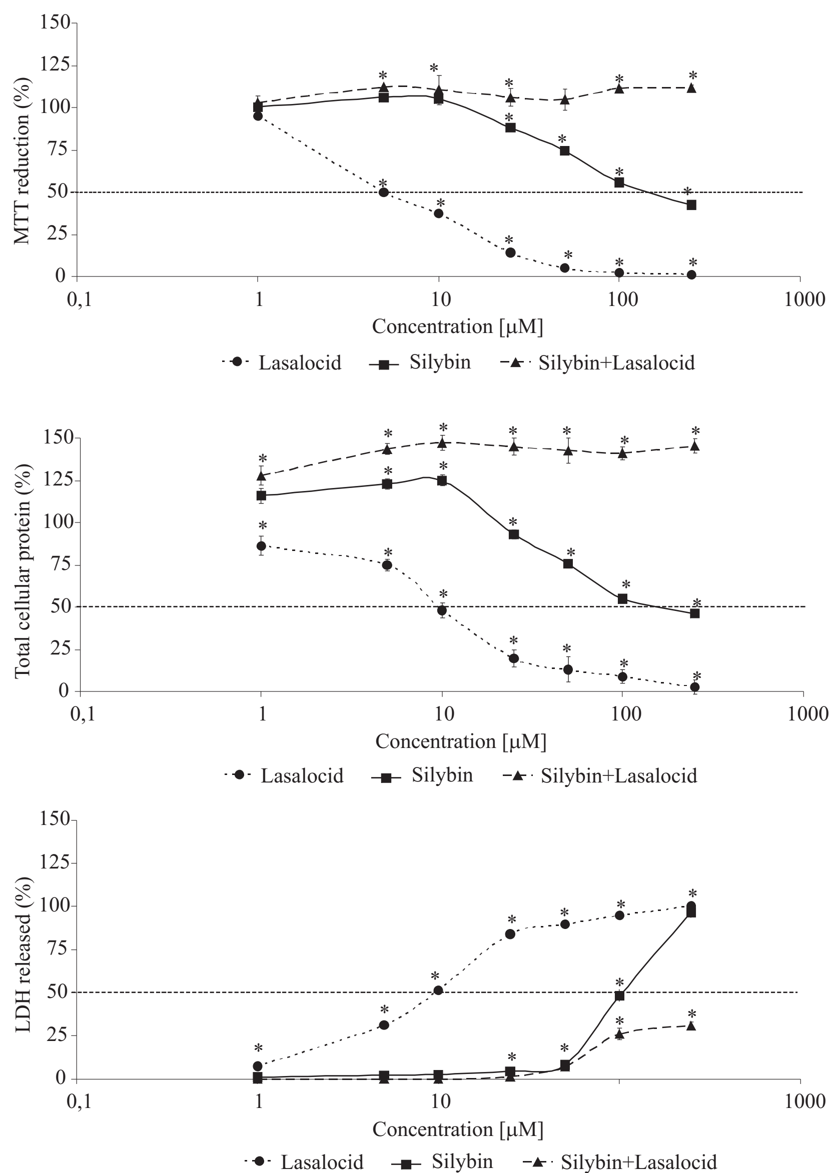

Fig. 1. The effect of lasalocid, silybin and silybin range concentrations in co-action with lasalocid (EC_{50}) on metabolism (MTT), total cellular protein and lactate dehydrogenase release (LDH) in human hepatoma cell line (HepG2). The values are expressed as percentage of control response and are means ±SD (n=3), * $P < 0.05$.

Table 1. Effective concentration, EC_{50} (µM) of lasalocid, silybin and lasalocid EC_{50} in co-action with silybin 1-250 µM range concentrations estimated by the metabolic activity (MTT), total cellular protein (CBB) and membrane integrity (LDH) assays on human hepatoma cell line (HepG2), mean ± SEM, (n=3).

Methods	Lasalocid	Silybin	Silybin with lasalocid(~ EC_{50})
		EC_{50}	
MTT	4.8 ± 1.20[a]	166 ± 4.0[a]	N/D
CBB	9.6 ± 0.50[b]	178 ± 5.0[b]	N/D
LDH	9.7 ± 0.59[b]	98 ± 5.0[c]	N/D

The different superscripts (a, b, c) within a column indicate significant differences between the methods (P<0.05), N/D – not determined.

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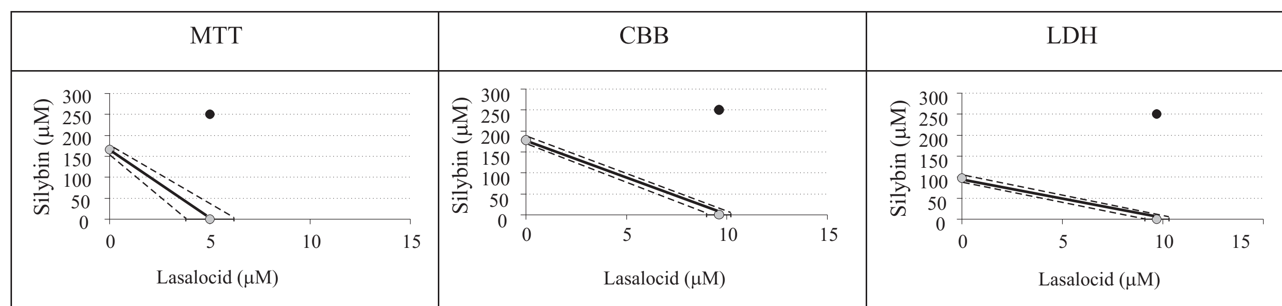

Fig. 2. Isobolograms describing the interaction of silybin with lasalocid in HepG2 cells. The isobolograms were constructed by connecting the EC_{50} values of lasalocid with the EC_{50} of silybin estimated by the metabolic activity (MTT), total cellular protein (CBB) and membrane integrity (LDH) assays. The black heavy lines indicate the theoretical line of additivity. The results below the additive line indicate synergism and those above the additive line denote antagonism.

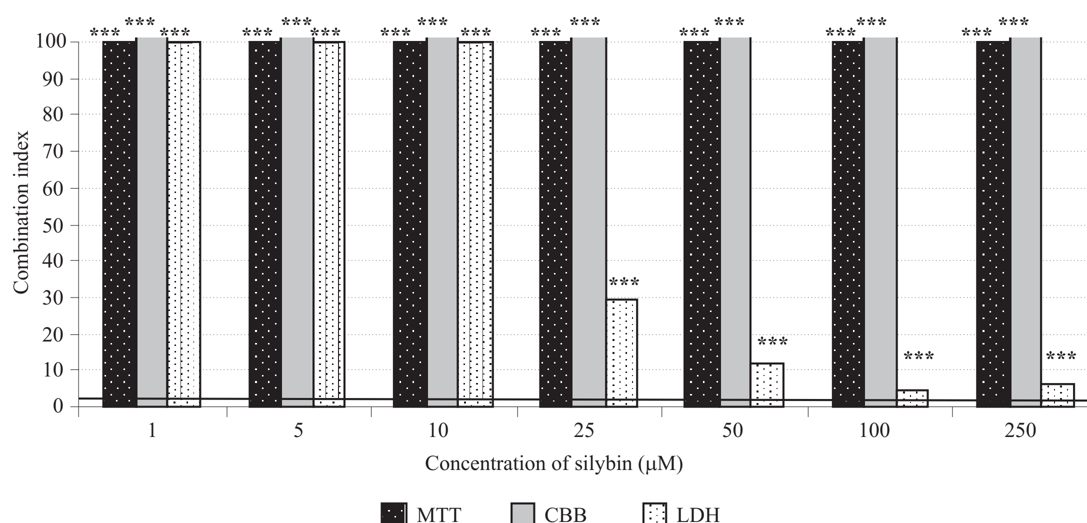

Fig. 3. Values of the combination index (CI) when lasalocid (EC_{50}) was combined with silybin concentration range in HepG2 cells culture used conversion of tetrazolium salt (MTT) as an indicator of cell metabolism, coomassie brilliant blue (CBB) assay to evaluate total cellular protein and lactate dehydrogenase release (LDH) as an indicator of membrane integrity. CI value significantly higher than 1 indicates antagonism, CI not significantly different from 1 indicates addition, and CI significantly less than 1 indicates synergism (***$P<0.001$).

and integrity of the cell membrane when compared to the control ($p<0.05$) (Fig. 1). The EC_{50} for LDH release assay was about twofold lower than EC_{50} from MTT and CBB tests (Table 1). The EC_{50} values for silybin were of one to two-orders higher than for lasalocid.

Effects silybin on cytotoxicity of lasalocid

Silybin within 1-250 μM range concentrations in co-action with lasalocid EC_{50} prevented a decrease of cellular metabolism, synthesis of total cellular protein and membrane integrity. The protective effect of silybin on the cells was not dose-dependent and reflected a strong interaction between these two substances (Fig. 1).

The nature of interaction

The nature of the interaction between silybin and lasalocid in the HepG2 cells line culture analyzed with the help of isobolography was depicted in Fig. 2. It shows an antagonistic character of the interaction in cell metabolism (MTT assay), synthesis of total cellular protein (CBB assay) and membrane integrity (LDH assay). Moreover, the data computed from the fractional cell-kill levels (Fa) confirmed that co-active effects of silybin and lasalocid represented antagonistic interaction. Antagonism was proven statistically, achieving combination index above 1 (CI >1) in all tested assays on HepG2 cells (Fig. 3).

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Discussion

In the present study, we investigated the effective concentrations and the action of silybin on HepG2 cells simultaneously treated with lasalocid. Possible protective effects of silybin were measured using three parameters: cellular metabolism, synthesis of cellular protein and integrity of membrane.

The experimental data showed that lasalocid at low concentrations induces cytotoxic effects on HepG2 cells due to inhibition of cellular metabolism (MTT assay), diminishing cells proliferation and desintegrating of cell membrane. These results are in accordance with other study on mitochondria in different rat cultures, both primary cells and cell lines (Antonio et al. 1991, Bolkent and Zierold 2002, Radko et al. 2007, 2011). The cytotoxic mechanism of lasalocid action is associated with an impaired flow of ions (monovalent and divalent) through cellular membranes (Antonio et al. 1991). It leads to Ca^{2+} overload within mitochondria. As a result, mitochondrial damage and cellular energy deficiency occurred. Lasalocid modulates calcium levels, its excess induces swelling of liver mitochondria and inhibits lysosomal protein degradation by affecting its pH (Grinde 1983, Mehrotra et al. 1993). Discussing the mechanism of lasalocid toxicity, microsomal enzymes involvement should be considered. Their catalytic activity can lead to the formation of toxic metabolites, including reactive and free radical derivatives of the antibiotic (Grijalba et al. 1998). It is assumed that changes of the enzymes activity measured by the MTT assay may result from a mechanism induced by those reactive metabolites (Kowaltowski and Vercesi 1999).

There are many plants with medicinal and detoxication properties. One of them is milk thistle (*Sylibum marianum L.*) containing silymarin. Silymarin is a safe substance as no acute toxicity was demonstrated. Silybin is the main component of silymarin and being a chemically standardized substance was applied in this study.

All *in vitro* studies indicated a lack of cytotoxicity of silybin (Antonio et al. 1991, Oh et al. 2004, Varghese et al. 2005, Dvorak et al. 2006, Pook et al. 2006, Angeli et al. 2010, Radko et al. 2011). Using cytotoxicity tests, we showed that silybin in concentrations above 25 μM led to decrease of viability and growth inhibition of HepG2 cell lines after 24 h incubation. The literature data show that at higher concentrations of silybin (> 100 μM) and exposure longer than 24 h, an apparent inhibition of cell growth with no cytotoxic effect occurred. (Varghese et al. 2005, Pook et al. 2006, Lah et al. 2007, Chen et al. 2009, Angeli et al. 2010). Comparing the silybin EC_{50} results, value over 34 times (LDH assay), 18 times (CBB assy) and 10 times (MTT) higher than the EC_{50} values for lasalocid were recorded. The EC_{50} values for silybin in the MTT and CBB were 4 times higher on HepG2 cells than the EC_{50} values for that drug on FaO cells (Radko et al. 2011). The lowest EC_{50} values for silybin in the LDH assay mean that the primary mechanism of silybin action could be membrane integrity dysfunction and subsequently a decrease of cellular metabolism and depletion of total protein content. This mechanism of silybin action may be explained by its hydrophobic character and penetration into membrane mainly to lipid bilayer structure. However, there are no changes of biophysical properties of the membrane. Such behavior of silybin in membranes is in line with its postulated biological functions. Membrane stabilization and cell protection may correspond to the observed lack of serious side effects of this drug (Wesołowska et al. 2007). Silybin at concentrations higher than 50 μM decreased LDH activity and total cellular protein content. However, an activity decrease may result from cells getting detached from the well surface in primary human hepatocytes cultures (Dvorak et al. 2003).

Silybin and lasalocid interaction analyzed according to Chou and Talalay method provided fundamental data for assessing whether cytotoxicity induced by combination of the drugs tested is greater, equal to or smaller than would have been expected for the individual substances. Our study showed a lack of cytotoxicity effects after simultaneous lasalocid and silybin action on HepG2 line. The EC_{50} values obtained from MTT, CBB and LDH assays analysed by isobolograms showed the lasalocid and silybin interaction which resulted in antagonistism leading to protection of HepG2 cells. The *in vitro* study revealed that antagonistic interaction between both drugs could be accounted for different mechanisms of toxicity. Competitive binding is probably involved in these mechanisms. If the mixed components have different affinities towards the same binding site of the target cell, some of them could not be able to induce their toxicity. This nature of interaction is observed in cases of combination of silybin and phalloidin or ethanol (Loranger et al. 1982). Results of our previous studies showed an antagonistic interaction between these drugs on FaO cell line leading to a decrease of cytotoxicity of lasalocid in presence of silybin. Range of inhibition of cell viability in the previous study with rat FaO cells was similar to human HepG2 cells. However, the sensitivity of rat hepatoma cells (Radko et al. 2011) was higher than the human hepatoma cells. Hitherto, results from human cell line assays support the finding from rat FaO cell line observation on marked cytoprotective interaction between both studied drugs.

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In general, it is difficult to clarify the mechanism underlying the effects of these two drugs used in combination. It is possible that the interactions of these drugs are due to some unknown mechanism related to complex perturbations of biochemical processes. Hepatocellular injury due to high level of lasalocid seems to be the primary event. This is rarely due to the drug action itself as toxic metabolites are usually responsible for lipid peroxidation and membrane damage. The final result is hepatocytes death related to failure and depression of mitochondrial function (Sherlock and Dooley 2002). Silybin has a regulatory action on cellular and mitochondrial membrane permeability in association with an increase in membrane stability against xenobiotic injury. It can prevent the absorption of toxin into the hepatocytes by occupying the binding sites as well as inhibiting many transport proteins at the membrane. Silybin was capable of reducing not only the cytotoxicity but the genotoxic effect induced by benzo(a)pyrene, bleomycin and aflatoxin B_1 (Angeli et al. 2010). However, silybin enhances the therapeutic potential of anticancer drugs such as doxorubicin, cisplatin, carboplatin and baicalein by synergistic effects for cell growth inhibition (Tyagi et al. 2004, Chen et al. 2009). The determination of either synergism or antagonism is probably an intricate balance between the concentration and type of anti-oxidants used, the class of chemotherapeutic agents administered as well as the category of cell involved (Pook et al. 2006).

The results of this study provided a complement to our previous evaluation of high cytotoxicity of lasalocid and protective properties of silybin revealed on FaO cell line (Radko et al. 2011). Since the interaction between silybin and lasalocid has never been discussed in literature, this mechanism needs more investigation in primary culture hepatocytes and verification in animal models.

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