CYTOTOXICITY STUDIES OF LASALOCID AND SILIBININ IN RAT HEPATOMA CELL CULTURE

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Received: August 3, 2010  Accepted: May 26, 2011

Abstract

The cytoprotective effect of silibinin in course of cytotoxicity induced by lasalocid had been measured in rat hepatoma FaO cell line. In the course of the study, MTT test (cellular metabolism), coomassie brillant blue binding test - CBB (total cellular proteins), and LDH release test (membrane integrity) were applied. In addition, changes in the cell morphology after 24 h treatment were observed by light microscopy. The effective concentrations, EC₅₀ were quantified for each compound alone, whereas the nature of their co-action was assessed by isobologram plotting. Lasalocid EC₅₀ ranged from 4 to 10 µM and microphotographs showed significant morphological changes of the cells after 24 h exposure. Silibinin EC₅₀ ranged from 40 to 42 µM for MTT and CBB assays, and 63 µM for LDH assay, and no significant morphological changes occurred. When lasalocid EC₅₀ was used in combination with silibinin in 1-250 µM concentrations, the EC₅₀ values were plotted at 36 µM and 72 µM in MTT and LDH assays, respectively. Thus co-actions of the two drugs led to significant diminishing of lasalocid cytotoxicity in respect to cellular metabolism and membrane integrity. The isobolograms showed remarkable antagonistic effect of silibinin in the course of lasalocid cytotoxic action. Although a considerable interaction was concisely relevant to hepatoma cell line FaO, the promising results incline to extend the study on other in vitro models (primary hepatocytes), as well as to investigate in vivo the cytoprotective effect of silibinin against lasalocid in target animals.

Key words: rats, hepatoma cell line, lasalocid, silibinin, cytotoxicity.

Lasalocid is a polyether carboxylic ionophore antibiotic produced by Streptomyces lasaliensis. Antiprotozoal activity enables its use in veterinary practice for the prevention of coccidiosis, prevalently in poultry. Previous reports on anti-coccidial application of lasalocid in animals showed high risk of intoxication even after slight over-dosed treatment. Toxic events may occur after uncontrolled accidental access to diet and errors in medicated feedingstuff mixing (19). Public health concerns relate to the occurrence of lasalocid residues in animal tissues and eggs, which were reported in monitoring surveys of the coccidiostats determined with the use of HPLC and LC-MS/MS methods (20). The mechanism of lasalocid activity is clearly attributed to its ionophoric properties (3). The disorder of mitochondrial injury and cell swelling, vacuolisation, and finally death, complete the events sequence (13, 16, 17, 21). In addition, generation of free oxygen radicals by lasalocid has been proposed to contribute to the mechanism of toxicity (12).

Cytoprotective agents are regarded as a suitable tool to prevent pathogenesis of chemically induced injury of human and animal organs (2, 10). Silymarin, the herbal drug of choice, is an active extract from fruits and seeds of milk thistle (Silybum marianum (L.) Gaertn (Asteraceae). The major (60%-70%) and most bioactive component of the silymarin complex is silibinin. The silymarin also includes isosilibinin, silydianin, silychristin, which can be classified as flavonolignans and related compounds (10). The data in the literature for silymarin and silibinin indicate that their hepatoprotective activity is expressed in four different ways. They act as (a) cell membrane stabilisers and permeability regulators that prevent hepatotoxic agents from entering hepatocytes; (b) as promoters of ribosomal RNA synthesis, stimulating liver regeneration; (c) as antioxidants, scavengers, and regulators of the intracellular content of glutathione; and (d) as inhibitors of the transformation of stellate hepatocytes into myofibroblasts, the process responsible for the deposition of collagen fibres leading to blocking of cirrhosis binding sites and hindering the uptake of...
toxins (9, 22, 27). In clinical studies, silibinin showed protective and curative effects on liver damage resulting from highly toxic compounds such as phallolidin and α-amanitin from *Amanita phalloides* (25). It has been used with success in toxic liver damage, hepatic cirrhosis caused by toxic drugs, or exposure to irradiation (26).

In this paper we report the effects of silibinin on lasalocid induced cytotoxicity in cultured rat hepatocyte cell line. Evaluation of the cell metabolism, synthesis of cellular protein, and integrity of cell membranes in course of 24 h incubation of the studied agent admixtures were performed. Prior, median effect concentrations (EC50) were estimated separately for lasalocid and silibinin. Then EC50 were estimated for combined lasalocid and silibinin effects by plotting the data on isobolograms, in order to find out the nature of interaction. In addition, cell morphology was evaluated under the light microscope.

**Material and Methods**

**Chemicals.** Silibinin ≥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, foetal bovine serum (FBS), antibiotic solution (penicillin and streptomycin), and L-glutamine were purchased from Sigma–Aldrich (USA). All other chemicals were purchased from commercial suppliers and were of the highest available purity.

**Cell cultures.** Rat hepatoma cell line (FaO), obtained from European Collection of Cell Cultures (Center for Applied Microbiology and Research, Salisbury, UK) (ECACC 89042701) was cultured in F-12 nutrient mixture Ham Kaign’s modification with L-glutamine (Gibco). The medium was supplemented with foetal bovine serum (10% of FBS), antibiotics and L-glutamine. These cells were cultured in 75 cm² cell culture flasks and were kept at 37°C in humidified atmosphere of 95% air and 5% CO₂. The medium was refreshed every 2 or 3 d and the cells were passaged three times before using them in the assays. The cells were counted using Bürker’s haemacytometer and initial cell viability was determined by the trypan blue exclusion test. The well-grown cells were seeded at 100 µl into 96-well plates (NUNC) at a density of 2.5×10⁵ cells/mL following the incubation for 24 h.

**Exposure to drugs.** The concentrations ranging from 1 to 250 µM for lasalocid and the same range for silibinin have been selected on the basis of previously performed preliminary studies. The stock solution of lasalocid was dissolved in DMSO, while silibinin was dissolved in ethanol. Final concentration of DMSO and ethanol in the culture medium was 0.1%. The same final concentrations of the solvents have been used in the corresponding control cultures. Each drug in seven concentrations was tested at the same time for 24 h.

**MTT assay.** This method is a good mean of measuring metabolic activity of living cells by assessing the activity of dehydrogenases (18). The MTT was dissolved in sterile phosphate buffer saline (PBS-Mg²⁺-Ca²⁺) solution at concentration of 5 mg/mL, sterilised by filtration through a 0.22 µm filter, and protected from light. After incubation of the cells with the substances, 10 µl of the MTT solution was added to each well of 96-well plates and incubated for 3 h at 37°C in humidified atmosphere of 5% CO₂. Formazan crystals were solubilised overnight in 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, USA).

**Coomassie brilliant blue (CBB) assay.** This test determines total cellular protein by measuring the uptake of the dye. The amount of dye incorporated by the cells in the culture determines the degree of cytotoxicity caused by the tested substance (5). The test medium was removed and 100 µl/well of the fixative was added to the plates and incubated for 10 min at room temperature. The fixative was removed and 100 µl of the coomassie brilliant blue R-250 stain solution was added. The plates were incubated at room temperature for 10 min. After the staining period, the stain was removed and the cells were rinsed twice with 100 µl/well of the desorbing solution (1 M potassium acetate) was added to the plates and shaked for 10 min. The absorbance was measured at the test wavelength of 600 nm by spectrophotometry in the microplate reader, the same as in previous test.

**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 of measuring membrane integrity as a function of the amount of cytoplasmic LDH released into the medium (24). After 24 h exposition to the drugs, 100 µl/well medium without cells was removed carefully and transferred into corresponding wells of an optically clear 96-well flat bottom microplate. To determine the LDH activity in these supernatants, 100 µl of freshly prepared reaction mixture was added to each well and incubated for up to 30 min at room temperature in the dark. After this time, 50 µl/well of 1 N HCl was added and the colour product was quantified spectrophotometrically at 450 nm wavelength using E-max Microplate Reader.

**Light microscopy.** To evaluate changes in cell morphology, May-Grünwald-Giemsa staining was applied. The FaO cells were plated on Lab-Tek Chamber Slide (Nunc) at a cell density of 1.2×10⁵/mL. Next day, the culture medium was replaced with medium containing the drugs in median effect concentrations (EC50) or vehicle in the control. After 24 h incubation, the medium was removed and cells were washed with PBS. Next, the specimens were incubated with May-Grünwald stain for 3 min and then in May-Grünwald stain diluted with an equal volume of distilled water for next 3 min. The dye was removed and the cells were
stained for 30 min with Giemsa stain diluted 1:20 with distilled water. After washing with distilled water and drying at room temperature, the stained FaO cells were observed under light microscope Olympus BX51 System Microscope (Olympus Optical Co., Japan) and the micrographs were prepared in analySIS® software (Soft Imaging System, Germany).

**Drug interaction analysis.** The nature of the interaction between studied drugs was analysed with the help of isobologram according to Chou and Talaly based on the estimation of cytotoxic median effect (EC₅₀) data (7, 8, 11).

The FaO cells were simultaneously incubated for 24 h with lasalocid at median effect concentration (EC₅₀) and silibinin in a range of concentrations: 1-250 µM. Isoboles were defined by effects of pair of the studied drugs. The EC₅₀ for MTT and LDH assays of lasalocid in the pair with silibinin formed basis for the additivity line. Synergism or antagonism was depicted by the linear cell-kill effects obtained by the toxic drug with combination of the interacting drug in different concentrations (11). The CI (combination index) method is a mathematical quantitative evaluation, which denominates a two-drug pharmacological interaction (7, 8). CI values were generated over a range 0.05 to 0.95 (5%-95%) of fractional cell-kill levels. CIs of <1 indicated synergy, CIs equal to 1 indicated additivity, and CIs >1 indicated antagonism.

**Statistical analysis.** The obtained results are presented as mean values ± SD (standard deviation). This data were assessed using one way analysis of variance (ANOVA) with post hoc Dunnett test to determine significance relative to unexposed control. The EC₅₀ 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 ± SEM (standard error of the mean) from three experiments. Statistical comparisons among EC₅₀ results were performed by analysis of variance (ANOVA) followed by Tukey post hoc test. Differences were considered as statistically significant at P<0.05.

**Results**

**Effect of lasalocid.** The cell metabolism, cellular protein, and integrity of cell membrane were significantly reduced in dose dependent manner after 24 h exposure of FaO cells to tested concentrations ranging from 1.0 to 250 µM when compared to the control (P<0.05) (Fig. 1).

The correlation coefficients (r²) estimated from the inhibitory concentration-response curves were above 0.9. Based on the results of MTT, CBB, and LDH assays with lasalocid in FaO cells, the EC₅₀ were determined between 4.4 and 5.5 µM (2.7-3.4 µg/mL); 5.4 and 14 µM (3.3-8.8 µg/mL), and between 6.9 and 7.9 µM (4.2-4.9 µg/mL), respectively. The mean values from these assays were not statistically different, as it is shown in Table 1. The cytotoxic effect is documented on microphotographs, which show significant morphological changes occurring after 24 h treatment of FaO cells followed by significant reduction of cell number with increased drug concentration in comparison to control. These phenomena were accompanied by marked vacuolisation of the cytoplasm, contraction of the nucleus, and decrease in cell size (Fig. 2).

**Effect of silibinin.** Silibinin at concentrations higher than 25 µM decreased the metabolism, as well as the cellular protein content and integrity of the cell membrane when compared to the control (P<0.05) (Fig. 1). The correlation coefficients (r²) between the mean concentration values and percentage of inhibition of FaO cell viability exceeded 0.9. The EC₅₀ values for silibinin were about 10 times higher than those for lasalocid. The EC₅₀ of silibinin was calculated in range of 40-41 µM (19-20 µg/mL) for MTT assay and ranged from 40 to 42 µM (19-20 µg/mL) in CBB assay. The EC₅₀ for LDH release assay was around 1.5-fold higher, 56-70 µM (27-34 µg/mL) than EC₅₀ from above tests (Table 1). The effect of silibinin is documented on microphotographs. No significant morphological changes occurred after 24 h treatment of FaO cell culture. However, a significant reduction of cell number dependent from silibinin concentration in comparison to control was observed (Fig. 2).

**Combined effect of silibinin and lasalocid.** Silibinin, ranging from 1 to 250 µM concentrations, significantly prevented the disruption of cellular metabolism and membrane integrity induced by lasalocid at median effect concentrations; EC₅₀ results are shown at Fig. 1 and Table 1. Thus silibinin co-action with lasalocid (EC₅₀ at 5 µM) in the MTT assay enabled to calculate at EC₅₀ 31-41 µM (15-20 µg/mL) range, whereas co-action with lasalocid (EC₅₀ at 7 µM) assayed by the LDH leakage test yielded EC₅₀ at 68-76 µM (33-37 µg/mL) range (Table 1). The CBB assay showed an apparent increase in synthesis of total cellular protein during incubation of silibinin with lasalocid reflected by EC₅₀ value; >250 µM (Fig. 1).

The nature of the interaction between silibinin and lasalocid in the FaO cells analysed with the help of isobolograms was depicted in Fig. 3. The figure shows remarkable antagonistic interaction in the case of both: mitochondrial metabolism (MTT assay) and membrane integrity (LDH assay).

In addition, the data (Table 1) computed from the fractional cell-kill levels (Fa) confirmed that co-active effects of silibinin and lasalocid represented antagonistic interaction. Antagonism was proved statistically, achieving combination index ranging: CI from 1.4 to 51 and from 1.6 to 66 at MTT and LDH assays, respectively (Fig. 4).
Fig. 1. The effect of lasalocid, silibinin, and silibinin range concentrations in co-action with lasalocid EC$_{50}$ on rat cell line (FaO). The values are expressed as percentage of control response and are means ±SD (n=3), * P<0.05.
Fig. 2. Morphology of FaO cells stained with May-Grünwald-Giemsa after treatment with tested compounds: A: lasalocid exposure (5 µM); B: silibinin (40 µM); C: controls cells (100x).
Fig. 3. Isobolograms describing the interaction of silibinin with lasalocid in FaO cells. The isobolograms were constructed by connecting the EC$_{50}$ values of lasalocid with the EC$_{50}$ of silibinin. The black heavy lines indicate the theoretical line of additivity. Results below the additive line indicate synergism and those above the additive line depict antagonism.

Fig. 4. Values of the combination index (CI) when lasalocid (EC$_{50}$-MTT and EC$_{50}$-LDH) was combined with silibinin concentration range in FaO cells culture. 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<0.05, ***P<0.001.

Table 1
Effective concentrations, EC$_{50}$ (µM) of lasalocid (A), silibinin (B), and lasalocid EC$_{50}$ in co-action with silibinin 1-250 µM range concentrations (C) estimated by the MTT, CBB, and LDH assays on rat hepatoma cell line (FaO), mean ± SEM (n=3)

<table>
<thead>
<tr>
<th>Methods</th>
<th>A</th>
<th>P&lt;0.05</th>
<th>B</th>
<th>P&lt;0.05</th>
<th>C</th>
<th>P&lt;0.05</th>
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<tbody>
<tr>
<td>MTT</td>
<td>4.9 ± 0.57$^a$ vs B, C</td>
<td>40 ± 1$^a$</td>
<td>36 ± 5$^b$ vs A</td>
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<tr>
<td>CBB</td>
<td>9.9 ± 4.49$^a$ vs B</td>
<td>41 ± 1$^a$</td>
<td>72 ± 4$^b$ vs A</td>
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<tr>
<td>LDH</td>
<td>7.4 ± 0.52$^a$ vs B, C</td>
<td>63 ± 7$^b$</td>
<td>N/D</td>
<td>N/D</td>
<td></td>
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The different superscripts (a or b) within a column indicate significant differences between the methods (P<0.05); The different superscripts (A, B or C) within a line indicate significant differences between the substances (P<0.05), N/D - not detected.
Discussion

For the first time, a considerable moderation of FaO cell functions under the influence of silibinin and lasalocid was demonstrated in this model study. The drugs given alone strongly affected cell metabolism, cell total protein synthesis, and in case of lasalocid integrity of the membrane. However, the data for silibinin showed an approximately one magnitude order lower effect when compared with the EC$_{50}$ values for lasalocid (Fig. 1). This indicates a safe margin for silibinin use when confronted with lasalocid in FaO cells exposed to the examined compounds.

In the presented study, lasalocid was shown to disrupt intensively the mitochondrial metabolism of FaO cells. The effects of carboxylic ionophores, including lasalocid, on mitochondria have been investigated in different cultures of both primary cells and cell lines (1, 4). High levels of divalent cations, 10-50 µM lasalocid in Ca$^{2+}$ and Mg$^{2+}$ supplemented medium, produced mitochondrial osmotic swelling, which was associated with an increase in influx of these cations. In a divalent cation-free medium, lasalocid caused depletion of membrane-bound Ca$^{2+}$ and release of endogenous Ca$^{2+}$ and Mg$^{2+}$ from mitochondria (1). In our studies, reduction of mitochondrial metabolism by lasalocid in hepatoma cells was found in the concentrations even below 5 µM. Alterations in cellular Ca$^{2+}$ concentration induced by lasalocid have been associated with disturbances in normal physiological function dynamically unregulated by Ca$^{2+}$ (1). In addition, ionophore cytotoxicity is thought to involve the influx of Na$^+$ and Ca$^{2+}$ ions with simultaneous efflux of K$^+$ ions. It leads to the excess of Ca$^{2+}$ within mitochondria and in consequence to mitochondrial damage, cellular energy deficiency, and ultimately to necrosis. Another mechanism of Ca$^{2+}$ mediated cell death occurs via apoptosis. It is thought that ionophores lead to apoptosis via calcium-activated endonucleases, which interfere with energy metabolism of the cell. Alteration of the cellular ionic gradient caused by ionophores can deplete intracellular ATP level followed by expending ATP to maintain normal Na$^+$ balance inside the cell. Failure of ion pumps results in elevation of intracellular Na and Ca concentrations, which cause cellular swelling, metabolic disruption, and necrosis. Furthermore, lasalocid can exchange different metal ions against H$^+$ in the cell membrane. This exchange leads to pH changes and to an increase in the osmotic pressure inside the cell, which finally is a reason of the cell death. Low concentrations of lasalocid (below 10 µM) caused irreversible changes in the functioning of cells as well as in their appearance. It seems that the studied hepatoma cell line proved to be more sensitive to lasalocid when compared to the primary hepatocytes (4).

The exposure of primary rat hepatocytes to ionophore antibiotics revealed less dense structure of the cytoplasm and nuclei, arise of vacuoles, swollen and rounded mitochondria, and finally disintegration of the cell structure (4). These observations are in agreement with the morphological findings in the studied FaO cells.

The experiments carried out by other authors indicated low cytotoxicity of silibinin, which has been also shown in our study. Its relatively high concentrations in the culture medium led predominately to cell metabolism disorder and inhibition of cellular proteins synthesis. In higher concentrations of silibinin, the damage of cell membrane was seen. The silymarin in concentration of 100 µg/mL significantly inhibited HepG2 cells growth (6). Another study indicated that silibinin in concentrations of 75 and 35 µg/mL reduced by 50% cell viability in Fet, Geo, and HCT116 cell lines (14). In our study, the values of EC$_{50}$ obtained at 20 and 34 µg/mL of silibinin appeared at comparable or slightly lower range as the cited references.

The mechanism by which silibinin protects from lasalocid cytotoxicity has not been univocally elucidated so far. Silibinin is a strong antioxidant and scavenger of free radicals (15, 23). In addition, inhibition of Na$^+$ and K$^+$-ATPase activity and lipid peroxidation have been reported (22, 23). Possible protective mechanisms of silymarin disposition have been proposed toward four target-sites, as it is mentioned in the introduction of this paper (9, 22, 27); whereas lasalocid toxicity is associated with the generation of free oxygen radicals, stimulation of lipid peroxidation, and loss of mitochondrial membrane potential (4, 16). Silibinin regulates the function of DNA-dependent RNA polymerase I in liver cells. It leads to an increased number of ribosomes and might counteract the decrease in protein synthesis in hepatocytes.

Our data, showing the mechanism of silibinin cytoprotection in hepatoma cell line cannot be extrapolated easily to other cell types. However, the observations of our study, pointing at the remarkable antagonistic interaction in the chosen in vitro model, confirmed cytoprotective properties of silibinin when incubated with lasalocid in FaO cell line. The obtained data constitute a substantial basis to lead further studies on primary cultures of liver cells and in vivo studies on target animals in order to establish the extend of silibinin cytoprotection in the course of xenobiotic harmful effects.

Acknowledgments: The studies were conducted in the Department of Toxicology and Environmental Protection, Faculty of Veterinary Medicine, University of Life Sciences in Lublin. This study was supported by a research grant from the State Committee for Scientific Research (KBN) No. N30801332/135.

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