Stimulatory Effects of Silibinin and Silicristin from the Milk Thistle Silybum marianum on Kidney Cells

JOHANN SONNENBICHLER, FORTUNATO SCALERA, ISOLDE SONNENBICHLER, and ROLAND WEYHENMEYER
Max Planck Institute for Biochemistry, Martinsried, Germany (J.S., F.S., I.S.); and Madaus AG, Köln, Germany (R.W.)
Accepted for publication May 25, 1999

ABSTRACT
The biochemical influence of flavonolignans from the milk thistle Silybum marianum has been tested on kidney cells of African green monkeys. Two nonmalignant cell lines were selected, with the focus of the work on the fibroblast-like Vero line. Proliferation rate, biosynthesis of protein and DNA, and the activity of the enzyme lactate dehydrogenase (as a measure of the cellular metabolic activity) were chosen as parameters for the effect of the flavonolignans. Silibinin and silicristin show remarkable stimulatory effects on these parameters, mainly in Vero cells; however, isosilibinin and silidianin proved to be inactive. In vitro experiments with kidney cells damaged by paracetamol, cisplatin, and vincristin demonstrated that administration of silibinin before or after the chemical-induced injury can lessen or avoid the nephrotoxic effects. The results warrant in vivo evaluations of the flavonolignan derivatives.

The extracts of the flowers and leaves of Silybum marianum (St. Mary’s thistle) have been used for centuries to treat liver diseases. In 1960, Janiak and Hänsel (1960) isolated the biological active principles of the extracts, and the chemical structures were elucidated by Pelter and Hänsel (1968), as well as by Wagner et al. (1968, 1971). The isolation led first to a mixture that was named silymarin, and it was with this mixture that most of the clinical studies were carried out. The main constituents are silibinin, isosilibinin, silicristin, and silidianin (Fig. 1).

Most of the clinical findings can be summarized by the term “increased liver regeneration”. Fintelmann and Albert (1980) observed that after toxic liver damage, the administration of silymarin resulted in a significantly accelerated normalization of serum glutamate-oxalacetate-transaminase and glutamate-pyruvate-transaminase values.

A double-blind study by Feher (1989) demonstrated that after administration of silymarin to patients with alcohol-induced liver disease, a distinctly more rapid normalization of the transaminases and bilirubin takes place. After treatment with silymarin, Berenquer and Carrasco (1977) observed a faster normalization of the plasma albumin values in patients with chronic inflammatory liver diseases. A multicenter study by Ferenci and colleagues (1989) in Vienna showed that the average survival rate of cirrhotic patients in the Child A stage could be markedly lengthened by silymarin.

Numerous investigations into the biochemical mechanism of action of silymarin have been performed by Sonnenbichler et al. It was confirmed that the transcription process in the livers of rats and mice in vivo (Sonnenbichler et al., 1976) is accelerated under the influence of silymarin and purified silibinin. In in vitro experiments with isolated cell nuclei and nucleoli, Machicao and Sonnenbichler (1977) demonstrated that the enzymatic activity of DNA-dependent RNA-polymerase I is stimulated by silymarin. Sonnenbichler and Zetl (1984) subsequently found that silibinin accelerates the synthesis of 28S, 18S, and 5.8S ribosomal RNAs and also promotes the formation of complete ribosomes. Thus, protein biosynthesis is indirectly intensified (Sonnenbichler and Zetl, 1986). Stimulation of RNA and protein biosynthesis was actually found to result in an increase in the rate of DNA biosynthesis in partially hepatectomized rat livers (Sonnenbichler et al., 1986). In these experiments, no significant difference was observed, irrespective of whether silibinin or the silymarin mixture was used. These findings provide biochemical confirmation of the clinical data on accelerated cell regeneration.

In view of this biochemical mechanism, silibinin was expected to exhibit a similar effect on other cell types. The effect on kidney cells was of particular interest, as these are frequently damaged by analgesics, cytostatics, and other drugs. For this reason, the extent to which silibinin influences the molecular biology of kidney cells was examined. The question as to whether silibinin is capable of antagonizing toxin-induced damage was of particular interest. To this end, nephrotoxins such as paracetamol, cisplatin, and vincristine were used to examine the effect of silibinin applied before or after

ABBREVIATIONS: TCA, trichloroacetic acid; LDH, lactate dehydrogenase; NADH, nicotine adeninedinucleotide.
the chemical-induced injury. It was discovered in earlier experiments (Andres, 1996), that of the four most important flavonolignans of the silymarin mixture (Fig. 1), only silibinin and silicristin have a stimulating effect on transcription and translation in liver cells. This was also to be tested in the case of kidney cells. Two nonmalignant cell lines from the kidneys of African green monkeys were selected for these experiments to warrant optimal reproducibility, to minimize statistical problems, and to follow ethical aspects concerning animal experiments. Proliferation rate, biosynthesis of DNA and protein, and the activity of the enzyme lactate dehydrogenase (LDH; as a measure of the cellular metabolic activity) were taken as the parameters defining the effect of the silymarin components.

Materials and Methods

Cell Cultures. All experiments were performed under sterile conditions. The adherent permanent cell lines from the kidneys of Cercopithecus aethiops (African green monkey): Vero: ATCC CCL 81 (passages 160 to 180) and BSC-1: ATCC CCL 26 (passages 176 to 182) were cultivated in 15 ml of Medium 199 (Gibco BRL, Life Technologies, Inc., Berlin, FRG) with Earle's salt, 2.2 g/l NaHCO₃, L-glutamine, T-tryptophan, and 5% fetal bovine serum (Gibco) in culture flasks (Falcon; Becton-Dickinson, Heidelberg, FRG) lying horizontally in an incubator (type B5060EK-CO₂; Heraeus, Hanau, FRG) in a moist atmosphere at 37°C with a gas mixture of 5% CO₂/95% air (5.1 × 10⁴ cells/ml, growth area 75 cm²). Seven days after the transfer, the media were renewed and the cells were transferred after an additional 7 days. To this end, after removal of the medium, the cells were washed and dispersed in 1.5 ml of 0.05% trypsin plus 0.02% EDTA for approximately 30 min at 37°C. Then 4 ml of Medium 199 and 5% fetal bovine serum were added.

For the storage in liquid nitrogen, the cells were first trypsinized and then transferred to a sterile centrifuge tube and centrifuged at room temperature for 5 min at 2000 rpm (Centrifuge Minifuge GL, type 4400; Heraeus, Hanau, FRG). Then the supernatant was pipetted and the cell pellet suspended in Medium 199 with 15% fetal bovine serum and 10% dimethyl sulfoxide (MSD Isotopes, Montreal, Canada; Dougherty, 1962).

Determination of Vitality. In all cases, the cells were counted after trypsinization and the vitality determined according to Widholm (1972) by means of staining with fluorescein diacetate (0.5% w/v) in acetone.

Determination of Proliferation Rates. In each case, the cells were transferred in 400 µl to the wells at a concentration of 7.5 × 10⁴ cells/ml. Trypsination followed after 0, 1, 3, 4, 5, 6, and 7 days and counting was performed in a Fuchs-Rosenthal chamber (Brand GmbH, Wertheim, FRG) to determine the proliferation rates. The cells were prepared, and 24 h later the test substances were added in different concentrations to determine their effects on the cells.

Measurement of DNA Biosynthesis. The cells were transferred to wells at a concentration of 7.5 × 10⁴ cells/ml. After 24 h, the flavonolignans were added at concentrations of 20 and 100 µM, followed immediately by 0.5 µCi/ml of [methyl-14C]thymidine (specific activity 51 mCi/mmol). Trypsination was performed after 0, 24, 48, and 72 h, whereupon 2 × 100-µl samples were taken, to which 250 µl of ice-cold 0.066 M thymidine solution were pipetted. After 10 min, 5 ml of cold 10% trichloracetic acid (TCA) and 0.02 M thymidine were added. After the samples had been allowed to stand on ice for at least 30 min, they were filtered through nitrocellulose filters (pore size, 0.2 µm; diameter, 25 mm; Sartorius, Göttingen, FRG) that had been preincubated in 10% TCA with 0.02 M thymidine. Then each sample was washed with 20 ml of 10% and 30 ml of 5% TCA solution (both with 0.02 M thymidine), and the filters were transferred to scintillation vessels and dried for 30 min, and the radioactivity was determined.

Measurement of Protein Biosynthesis. Immediately after dissemination (7.5 × 10⁴ cells/ml in well), the flavonolignans were added to the cells at the appropriate concentrations (0, 10, 20, 60 µM). The cells were incubated for 72 h, and then 0.125 µCi/ml of sample [2-14C]leucine (specific activity, 310 mCi/mmol) were added. Trypsination was performed after 0, 4, 8, 12, and 28 h, whereupon...
Effects of Silibinin on Kidney Cells

In the present study, two adherent permanent cell lines from the kidneys of the African green monkey (C. aethiops) were selected: fibroblast-like Vero and epitheloid BSC-1 cells.

The growth curves in culture flasks show the typical sigmoid course with a resting phase of approx. 2 days followed by an exponential increase in cell count for up to 8 days in the case of Vero cells and with a resting phase of approx. 1 day and an exponential increase in cell count for up to 7 days for BSC-1 cells. The cells double approx. every 24 h before the stationary phase with a cell count of $6.3 \times 10^6$/ml. The cultures exhibit confluent growth in contrast to malignant cells. Both cell lines show similar behavior in the wells of the culture clusters. The logarithmic phase has a duration of approx. 5 days, after which the growth curves reach a plateau with a cell count of $1.8 \times 10^6$/well. Culture clusters were used exclusively for testing the substances because they allow for eight samples per experiment in multiple parallel batches.

**Effect of Silibinin on the Proliferation of Vero Cells.**

Silibinin hemisuccinate (10 $\mu$M) was added to the cells and the proliferation rate monitored for a period of 5 days, with constant measurement of the vitality. In comparison to the controls, an increase in growth of 14% is observed 3 days after addition of silibinin (Table 1). After 5 days, this stimulation is no longer evident. Then the treatment was continued as described above.

Paracetamol was dissolved in ethanol, cisplatin and vincristine in twice-distilled water. The suitable toxicant concentrations were determined in preliminary experiments on proliferation measurements without the addition of silibinin.

**Results**

Paracetamol was dissolved in ethanol, cisplatin and vincristine in twice-distilled water. The suitable toxicant concentrations were determined in preliminary experiments on proliferation measurements without the addition of silibinin.

2  x  100-$\mu$l samples each were taken to which 50 $\mu$l of cold 0.012 M L-leucine solution were added. After an additional 10 min, the proteins were precipitated with 5 ml of ice-cold 10% TCA (with 0.02 M L-leucine). Cleavage of the amino acid-carrying RNA was induced by heating the samples to 85°C for 35 min in a drying cabinet, cooling, and then filtering through nitrocellulose filters that had been incubated in 10% TCA with 0.02 M L-leucine solution before filtration. Additional processing is as described above.

**Determination of LDH Activity (Stevens et al., 1986).**

The test substances were added to the cells 24 h after transfer ($7.5 \times 10^4$ cells/ml in well). With gentle shaking, the cells were suspended for 5 min with 0.1% Triton X-100 after an additional 0, 24, 48, and 72 h, then 400 $\mu$l of Medium 199 was added, and the cells were centrifuged for 6 min (Eppendorf centrifuge) before being stored on ice. After the addition of 1 ml of 0.1 mM nicotine adeninedinucleotide (NADH) + 1 mM pyruvate in 0.2 M tris(hydroxymethyl)-aminomethane-HCl (pH 7.3), the LDH activity in 10 $\mu$l samples was measured by determining the decrease in extinction at 340 nm for 20 min and calculating $\Delta$E/min. The molar extinction coefficient $\epsilon$ of NADH at a layer thickness of 1 cm at 340 nm is 6.22 cm$^2$/mu mol.

$$\text{activity (units/ml) = } \frac{\Delta E/\text{min}}{6.22} \times F$$

**Tests.** For the purpose of determining the growth curves and the protein and DNA synthesis and for measuring the LDH activities, 7.5  x 10$^4$ cells/ml were cultivated in wells (growth area 2 cm$^2$) of cell cluster (Costar, Bodenheim, FRG) each in 400 $\mu$l medium. Removal of the medium was followed in each case by washing with 100 $\mu$l of 0.15 M NaCl, adding 100 $\mu$l of trypsin cocktail, heating to 37°C for 20 min, and then adding 100 $\mu$l of Medium 199 + 5% fetal bovine serum.

**Statistics.** Each value of the tables and figures was determined at least 8-fold. To characterize the experimental significance, the S.D.s and the statistical probabilities were calculated using Student’s t distribution (Meyer, 1975).

**Application of Test Substances.** To improve solubility, the water-soluble silibinin hemisuccinate was used in the majority of experiments. In the experiments comparing silibinin with sildianin, and isosilibinin, unesterified silibinin and the other flavonolignans were added after dissolution in ethanol owing to the greater solubility. Consequently, the final concentration of ethanol in the cell cultures was 1%. The controls were performed without ethanol and with 1% ethanol.

**Damage by Toxicants and Administration of Silibinin.** For subsequent administration after the chemical induced injury, 7.5  x 10$^4$ cells/ml were incubated in the wells. The toxicants were added to the cells 23 h after dissemination as shown in Fig. 2. When the cells were exposed to the toxicants for a period of 60 min, they were washed 3 times each with 100 $\mu$l of Medium 199. This was followed by the addition of 400 $\mu$l of Medium 199 with 5% bovine serum, followed immediately by silibinin hemisuccinate in various concentrations (generally 20 and 40 $\mu$M) and radioactive precursor, where necessary. Then the treatment was continued as described above.

For preadministration of silibinin before the chemical-induced injury, 7.5  x 10$^4$ cells/ml were incubated. Twenty-four hours later, silibinin hemisuccinate was added (Fig. 3) and when an additional 24 h had elapsed, the toxicant and, where appropriate, the radioactive precursor were added. Then the treatment was continued as described above.

Fig. 2. Treatment of the cells with administration of the flavonolignans after the chemical-induced injury.

Fig. 3. Treatment of the cells with administration of the flavonolignans before the chemical-induced injury.
100 μM, with the growth rate decreasing to 92% of that of the controls (Table 1).

**Effect of Silibinin on DNA Biosynthesis in Vero Cells.** The DNA biosynthesis was measured over a period of 72 h. Forty-four hours after dissemination, varying quantities of silibinin hemisuccinate were added to the Vero cells followed shortly by [14C]thymidine. Seventy-two hours after addition of 20 μM silibinin hemisuccinate, the DNA biosynthesis is found to increase by 19%, and the addition of 100 μM silibinin hemisuccinate again results in an 8% inhibition of precursor incorporation in newly synthesized DNA, comparable to the inhibition observed in the proliferation measurements.

**Effect of Silibinin on Protein Biosynthesis in Vero Cells.** The protein biosynthesis was determined at intervals by measuring the nonhydrolyzable radioactivity in the TCA precipitates of the high-molecular cell constituents after incorporation of radioactive leucine. The protein biosynthesis in cells treated with 20 μM silibinin hemisuccinate is stimulated by 16% after 24 h and by 21% with 40 μM (Table 1). Increasing the concentration to 60 μM silibinin hemisuccinate does not affect protein biosynthesis. Evidently, this is the threshold concentration for the above-mentioned inhibition. At 100 μM, the incorporation drops to 90% of that of the controls.

To establish whether the concentrations decrease as a result of silibinin degradation, 20 μM silibinin hemisuccinate were added 1 day after transfer, and this addition was repeated for 2 days. The cells were counted on day 4 after transfer, i.e., after the addition of a total of 60 μM silibinin hemisuccinate. The outcome was that the repeated addition of silibinin hemisuccinate results in an increase in protein biosynthesis initially, but that this effect decreases once more on further addition of silibinin to such an extent that growth corresponds to that of the untreated controls after the addition of a total of 60 μM. This confirms our assumption that silibinin (in contrast to the liver) is not metabolized in kidney cells.

**Effect of Silibinin on BSC-1 Cells.** BSC-1 cells failed to exhibit a stimulation by 10 μM silibinin hemisuccinate, yet a growth increase of up to 11% was observed 4 days after the addition of 20 μM silibinin hemisuccinate and 19% with 40 μM (Table 1). With regard to protein biosynthesis, too, stimulation is at first less pronounced and only approximates the Vero cell values at concentrations of 40 μM silibinin hemisuccinate (Table 1). Again, the inhibition threshold occurs at concentrations >60 μM. For this reason, we decided to focus our further work on the more active Vero line.

**Effects of Additional Flavonolignans from the Milk Thistle on Vero Kidney Cells.** In our department, the four flavonolignans were tested in transcription and translation systems with liver cells (Andres, 1996). These investigations indicated that it could be pertinent to test these substances on kidney cells. For reasons of solubility, the flavonolignans silidianin, silicristin, isosilibinin (not available as hemisuccinate), and pure silibinin were dissolved in ethanol for these experiments and added to Vero cells at a concentration of 20 μM after dissemination. The final ethanol concentration in the test mixture was 1%, a concentration at which ethanol has no detectable effect on the parameters measured in the cells.

**Effects on Proliferation.** Flavonolignans (20 μM) were added to Vero cells 24 h after transfer. The cells were counted 72 h later and their vitality determined. The measured values are listed in Table 2. The data obtained show a 16% increase in growth rate after addition of silibinin. A 14% stimulation of proliferation was also observed in the case of those cells treated with silicristin. On the other hand, the flavonolignans silidianin and isosilibinin show slight to moderate inhibitory effects compared with the controls (6 and 10%, respectively).

**Protein Biosynthesis under the Influence of Silibinin, Silicristin, Silidianin, and Isosilibinin.** Twenty-four hours after addition of the flavonolignans, [2-C14]leucine was added to the culture clusters. Figure 4 shows the leucine incorporation into proteins of Vero cells under the influence of flavonolignans as a function of time.

**Table 2**

Influence of the main flavonolignans from *Silybum marianum* in concentrations of each 20 μM on the proliferation of Vero kidney cells

<table>
<thead>
<tr>
<th>Final Cell Concentration</th>
<th>Living Cells</th>
<th>Growth Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95</td>
<td>%</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td>96</td>
<td>100 ± 1.5</td>
</tr>
<tr>
<td>Silibinin</td>
<td>96</td>
<td>99 ± 2.6</td>
</tr>
<tr>
<td>Silidianin</td>
<td>96</td>
<td>116 ± 2.5**</td>
</tr>
<tr>
<td>Silicristin</td>
<td>96</td>
<td>94 ± 3.0*</td>
</tr>
<tr>
<td>Isosilibinin</td>
<td>96</td>
<td>114 ± 2.7**</td>
</tr>
</tbody>
</table>

Included S.D. (n = 8) and statistical significances in comparison with the untreated control.

*p < .01; **p < .001.

---

**Table 1**

Comparison of growth rates and protein biosynthesis of Vero and BSC-1 cells in the presence of different silibinin concentrations

<table>
<thead>
<tr>
<th>Silibinin</th>
<th>Vero Cells</th>
<th>BSC-1 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>Growth Rate</td>
<td>Protein Synthesis</td>
</tr>
<tr>
<td>10</td>
<td>114 ± 2.9***</td>
<td>114 ± 2.8***</td>
</tr>
<tr>
<td>20</td>
<td>116 ± 2.5***</td>
<td>116 ± 2.3***</td>
</tr>
<tr>
<td>40</td>
<td>123 ± 2.0***</td>
<td>121 ± 2.8***</td>
</tr>
<tr>
<td>60</td>
<td>101 ± 2.6 (N.S.)</td>
<td>102 ± 3.0 (N.S.)</td>
</tr>
<tr>
<td>100</td>
<td>92 ± 2.8***</td>
<td>90 ± 3.1***</td>
</tr>
</tbody>
</table>

Included S.D. (n = 8) and statistical significances in comparison with the untreated controls (100%).

*p < .1; **p < .01; ***p < .001, N.S., not significant.
calculated in comparison with the corresponding control (silicristin (\(\text{L}\)), silidianin (\(\text{H}\)), and isosilibinin (\(\text{O}\))) for all \(p < .001\).

late translation, whereas silidianin (−6%) and isosilibinin (−10%) exhibit an inhibitory effect.

Effects of Flavonolignans on the LDH Activity of Vero Kidney Cells. To determine the effect of flavonolignans on the LDH activity of cells, silibinin, silidianin, silicristin, and isosilibinin were added to the cells at a concentration of 20 \(\mu\)M 24 h after transfer. The LDH activity was determined over a period of 72 h. Figure 5 shows the change in activity with and without flavonolignans as a function of time.

Compared with the controls, silibinin increases the LDH activity after 72 h by approximately 18% and silicristin by approximately 14%. Silidianin and isosilibinin again show inhibition of LDH activity in Vero kidney cells (−9 and −12%, respectively).

Effect of Silibinin on Vero Kidney Cells Damaged by Various Toxicants. In preceding experiments, the concentration dependence of the toxicity of paracetamol, cisplatin, and vincristine was examined to achieve suitable inhibitions of cell activities in the following examinations. The cell activities in the absence of toxicants were taken as control values. Because those cells treated with toxicants in the experiments on the postadministration of silibinin (after the chemical-induced injury) were exposed to the toxicants for only 1 h (Fig. 2), whereas they were exposed constantly in the case of previous administration of silibinin (Fig. 3), paracetamol and vincristine must be present in different concentrations in the experiments. The permanent presence of toxicants in the wells of the culture clusters implies that the toxicant concentration may not be as high as when the duration of exposure is only 1 h. This does not apply to experiments with cisplatin, in which the same cisplatin concentrations could be used irrespective of whether administration of silibinin was before or after the chemical injury, because in addition, cisplatin exerts its effect on kidney cells instantly and fully (blocking of DNA biosynthesis).

Paracetamol and Administration of Silibinin after the Chemical-Induced Injury. To examine the effect of silibinin after damage by paracetamol, the proliferation rates, protein biosynthesis, and LDH activity were measured again. It can be seen from the data in Table 3 that 6.62 mM paracetamol inhibits the proliferation rate by 18%. The addition of 20 \(\mu\)M silibinin hemisuccinate reduces this inhibition to 3% and results in growth similar to that of untreated controls. Silibinin hemisuccinate on its own increases the growth rate as described above.

Increasing the paracetamol concentration to 13.23 mM inhibits the proliferation rate by 27%. Compared with undamaged kidney cells, this inhibition can be reduced to 12% on addition of 20 \(\mu\)M silibinin hemisuccinate and to 8% on addition of 40 \(\mu\)M.

Protein biosynthesis is inhibited by 11% 72 h after the addition of 6.62 mM paracetamol. The addition of 20 \(\mu\)M silibinin hemisuccinate not only compensated completely for this inhibition, but actually increased the rate of protein biosynthesis somewhat compared with the controls which had not been treated with toxicants.

Paracetamol (13.23 mM) reduces protein biosynthesis by 25%, compared with the rate of synthesis of the controls. This inhibition is lowered to 9% by the addition of 20 \(\mu\)M silibinin hemisuccinate and to 6% by the addition of 40 \(\mu\)M.

Addition of 6.62 mM paracetamol inhibits the LDH activity by 10%. Addition of 20 \(\mu\)M silibinin hemisuccinate not only causes the LDH activity to rise to the normal value, but actually increases it by 6%. After damage by a paracetamol concentration of 13.23 mM, the LDH activity is found to be inhibited by 20%. This inhibition is reduced to 4% by 20 \(\mu\)M silibinin hemisuccinate and is neutralized fully by 40 \(\mu\)M.

Paracetamol and Administration of Silibinin before the Chemical-Induced Injury. The following experiments examined the extent to which the previous administration of silibinin is capable of lowering the inhibition of proliferation resulting from paracetamol in Vero kidney cells. In this series of experiments, lower paracetamol concentrations were selected because paracetamol was permanently present in the culture clusters.

It is clear from Table 4 that in the presence of 330 \(\mu\)M paracetamol, the proliferation rate decreases by 33% versus that of the control cells. This inhibition can be lowered to 18%
Effect of silibinin addition 72 h after the chemical-induced injury on the reduced proliferation, protein synthesis, and LDH activity of Vero kidney cells that were intoxicated before with paracetamol

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Growth Rate</th>
<th>Protein Synthesis</th>
<th>LDH Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 1.7</td>
<td>100 ± 2.0</td>
<td>100 ± 1.9</td>
</tr>
<tr>
<td>Silibinin</td>
<td>20</td>
<td>116 ± 2.5*</td>
<td>116 ± 2.3*</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>6 620</td>
<td>82 ± 3.0*</td>
<td>89 ± 3.0*</td>
</tr>
<tr>
<td>Paracetamol + silibinin</td>
<td>6 620</td>
<td>97 ± 3.0**</td>
<td>103 ± 3.0**</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>13 230</td>
<td>73 ± 3.0*</td>
<td>75 ± 3.0*</td>
</tr>
<tr>
<td>Paracetamol + silibinin</td>
<td>13 230</td>
<td>88 ± 3.0**</td>
<td>91 ± 3.0*</td>
</tr>
<tr>
<td>Paracetamol + silibinin</td>
<td>13 230</td>
<td>92 ± 3.0**</td>
<td>94 ± 3.1**</td>
</tr>
</tbody>
</table>

Included S.D. (n = 8) and statistical significances in comparison with the untreated controls or for second comparison with the experiments with intoxication by different paracetamol concentrations.


df All corresponding values are marked by superscript letters.

Effect of silibinin (added 24 h before the chemical-induced injury) on the proliferation of Vero kidney cells in the presence of 330 µM paracetamol

<table>
<thead>
<tr>
<th>Paracetamol</th>
<th>Silibinin Hemisuccinate</th>
<th>Final Cell Concentration</th>
<th>Living Cells</th>
<th>Growth Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>µM</td>
<td>3.62 × 10^5</td>
<td>3.42 × 10^5</td>
<td>3.28 × 10^5</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>3.62 × 10^5</td>
<td>3.42 × 10^5</td>
<td>3.28 × 10^5</td>
</tr>
<tr>
<td>330</td>
<td>0</td>
<td>3.46 × 10^5</td>
<td>3.28 × 10^5</td>
<td>2.90 × 10^5</td>
</tr>
<tr>
<td>330</td>
<td>20</td>
<td>3.86 × 10^5</td>
<td>3.12 × 10^5</td>
<td>3.16 × 10^5</td>
</tr>
</tbody>
</table>

Included S.D. (n = 8) and statistical significances in comparison with the experiments with intoxication by paracetamol.

*p < .001.
* Corresponding values.

by administering 20 µM silibinin hemisuccinate 24 h earlier or to 11% with 40 µM silibinin hemisuccinate.

Cisplatin and Administration of Silibinin after the Chemical-Induced Injury. After cisplatin damage induced with 8.4 µM, measurements showed a decrease in growth of approx. 34%, which was reduced to 19% on addition of 20 µM silibinin hemisuccinate and to 13% on addition of 40 µM (not listed in Table 5).

Cisplatin (33.3 µM) reduced growth by 85%, and 20 and 40 µM silibinin hemisuccinate lowered this growth inhibition slightly to 82 and 81%, respectively. In other words, the effect of silibinin on proliferation is only slight in these cases (Table 5).

The data show that protein biosynthesis is inhibited by 28% 72 h after the addition of 33.3 µM cisplatin, a concentration that has caused a significantly greater inhibition of growth. Administration of 20 and 40 µM silibinin hemisuccinate lowers this inhibition to 13 and 8%, respectively.

A cisplatin concentration of 33.3 µM inhibits the LDH activity by 22%. After administration of 20 µM silibinin hemisuccinate, this inhibition is reduced to 5%. With 40 µM, the activity of the untreated control cells is attained once again. One noteworthy phenomenon in these experiments with cisplatin was that the volume of the cells exceeded the normal value by a factor of approximately 5 or 6.

Cisplatin and Administration of Silibinin before the Chemical-Induced Injury. Despite the inevitable differences in experimental procedure with the permanent presence of toxicants, it was possible to work with cisplatin concentrations similar to those of the experiments with intoxication by paracetamol.

It is evident from Table 6 that proliferation is inhibited by...
50% after the addition of 16.7 μM cisplatin to Vero cells. The addition of 20 and 40 μM silibinin hemisuccinate 24 h before the toxicant can lower the growth inhibition to 40 and 37%, respectively.

**Vincristine and Administration of Silibinin after the Chemical-Induced Injury.** To determine the effect with the flavonolignan silibinin after damage by vincristine, the toxicant can lower the growth inhibition to 40 and 37%, respectively. The 30% inhibition with 60.8 μM vincristine is lessened with silibinin to 37 or 35%.

At a concentration of 36.5 μM, vincristine inhibits protein biosynthesis in Vero cells by 16%. The addition of 20 and 40 μM silibinin hemisuccinate reduced this inhibition to 9 and 2%, respectively. The 30% inhibition with 60.8 μM vincristine is decreased by the flavonolignan to 25 and 22%, respectively.

At a concentration of 36.5 μM, vincristine inhibits the LDH activity by 12%. This effect is neutralized by adding 20 and 40 μM silibinin hemisuccinate, respectively. The LDH activity is inhibited by 24% after the addition of 60.8 μM vincristine, which is reduced to 18 and 14% by the application of 20 and 40 μM silibinin hemisuccinate, respectively, after the chemical-induced injury.

**Vincristine and Administration of Silibinin before the Chemical-Induced Injury.** Table 7 shows the data on the proliferation of kidney cells damaged by vincristine under the constant influence of 20 and 40 μM silibinin hemisuccinate. At a concentration of 0.3 μM vincristine inhibits growth by 60%. The administration of 20 and 40 μM silibinin hemisuccinate results in a reduction in growth inhibition to 56 and 51%, respectively.

**Discussion**

Because of its greater solubility, silibinin in the form of the hemisuccinate was used in the majority of experiments. This hemisuccinate is hydrolyzed rapidly and fully by the cells’ esterases (Koch and Tscherny, 1983). Equivalent quantities of succinate fail to show any effect on the experimental data. Pure silibinin was used in the comparison of silibinin with silecristin, silidianin, and isosilibinin. The most notable finding is that silibinin increases the proliferation rate by up to 23% in Vero kidney cells, compared with the control cells. The concentration at which the efficacy is greatest is 40 μM silibinin hemisuccinate. Stimulation of comparable intensity was detected in the parallel measurements of DNA and protein biosynthesis with radioactive precursors. This stimulation decreases at higher concentrations, reaches the values of the controls at 60 μM, and changes into inhibition at concentrations in excess of 100 μM (Table 1). In the corresponding experiments with cells of the BSC-1 line, the results were essentially the same but the stimulatory effects were less pronounced (Table 1). Therefore, we focused our studies on Vero cells.

Sonnenbichler and Zetl (1986) found silibinin to have an

---

**Table 7**

Effect of silibinin 72 h after addition on the reduced proliferation, protein synthesis, and LDH activity of Vero kidney cells that were intoxicated before with vincristine

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Growth Rate</th>
<th>Protein Biosynthesis</th>
<th>LDH Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>100 ± 1.8</td>
<td>100 ± 2.1</td>
</tr>
<tr>
<td>Vincristine</td>
<td>36.5</td>
<td>80 ± 3.0*</td>
<td>84 ± 2.9**</td>
</tr>
<tr>
<td>Vincristine + silibinin</td>
<td>36.5</td>
<td>85 ± 3.0**</td>
<td>91 ± 3.0***</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vincristine + silibinin</td>
<td>36.5</td>
<td>89 ± 2.8***</td>
<td>98 ± 2.6***</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>60.8</td>
<td>60 ± 2.9f</td>
<td>70 ± 3.0*</td>
</tr>
<tr>
<td>Vincristine + silibinin</td>
<td>60.8</td>
<td>63 ± 3.0*(N.S.)</td>
<td>75 ± 3.1**</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vincristine + silibinin</td>
<td>60.8</td>
<td>65 ± 3.0***</td>
<td>78 ± 3.0***</td>
</tr>
</tbody>
</table>

Included S.D. (n = 8) and statistical significances in comparison with the experiments with intoxication by different vincristine concentrations.

*p < .05; **p < .01; ***p < .001; N.S., not significant.

*Corresponding values.
The effect of silibinin added 24 h before the chemical-induced injury on the proliferation of Vero kidney cells in the presence of 0.3 μM vincristine was measured. At concentrations in excess of 100 μM, similar inhibitions were observed in liver cells in vitro (Sonnenbichler et al., 1981). This substantiates the assumption that the same stimulation mechanism applies to kidney cells, as has been described in the case of liver cells in great detail (Machicao and Sonnenbichler, 1977). For liver it was shown that the DNA-dependent RNA-polymerase I is stimulated by interaction of silibinin to an enzyme effector binding site (Sonnenbichler and Zetl, 1996).

Repeated addition of stimulating silibinin hemisuccinate concentrations, staggered with respect to time, results in a reduction of the stimulatory effect. This confirms that the flavonolignan is not metabolized, but is accumulated by the kidney cells and thereby approaches the “inhibition range” of higher silibinin concentrations.

For the purpose of comparing the main components of the milk thistle extract, silibinin, isosilibinin, silicristin, and silidianin were also determined on Vero kidney cells with respect to the proliferation rates, the protein biosynthesis, and the LDH activities according to Stevens et al. (1986). LDH catalyzes the final stage of glycolysis, the reduction of pyruvate to lactic acid by NADH, one of the most important metabolic steps within cells. The enzyme plays an important role in the cytosol and reflects the vitality of the cells. Increases in protein synthesis also have an indirect effect on the LDH activity. It is noteworthy that the stimulatory effect of silibinin and silicristin on the LDH activities (Fig. 5) with increases of 18 and 14%, respectively, closely resembles the increases in proliferation of 16 and 14%, respectively (Table 2), and in protein biosynthesis of 16 and 11%, respectively, (Fig. 4). Isosilibinin and silidianin exhibit similar inhibitory effects in all three test systems.

It was also discovered in the case of liver cells (Andres, 1996) that silibinin and silicristin increase RNA and protein biosynthesis, whereas the remaining silidianin and isosilibinin exhibit little or no inhibitory effects. With regard to our research on the binding of silibinin to an effector site of the RNA-polymerase I (Zetl, 1985; Sonnenbichler and Zetl, 1988), it was revealed after molecular modeling studies in our laboratory, that indeed only silibinin and silicristin satisfy the requirements (suitable distance between functional groups, partial planarity, and resemblances between aromatic groups) for the binding to the receptor.

Because silibinin is capable of compensating for numerous forms of toxic damage in liver cells, it seemed appropriate to investigate whether flavonolignans can also prevent or reduce the detrimental effect of toxicants on kidney cells. This is of particular significance in the clinical application of cytostatics, but also for paracetamol, as its possible side effects include kidney damage. McMurtry et al. (1978) demonstrated that a high dose of paracetamol induces acute proximal tubular necroses in rats and in humans.

Cisplatin (cis-diamminedichloroplatinum) is a chemotherapeutic agent for the treatment of malignant tumors in humans (Prestayko et al., 1980), although its toxicity is high. Its dose limit can be attributed to its nephrotoxicity (Madias and Harrington, 1978, Erlanger and Cutler, 1992). As reported by Tay et al. (1988), DNA biosynthesis is inhibited by cisplatin. Various attempts have been made to reduce cisplatin-induced nephrotoxicity (Walker and Gale, 1981). These involved the use of various chemoprotectors, e.g., diethyl dithiocarbamate (Bodenner et al., 1986) and Ebselen (Baldew et al., 1992) for the purpose of lessening the cisplatin toxicity.

Vincristine, an alkaloid isolated from the evergreen Vinca rosea Linn., is also a cytostatic agent used in the treatment of chronic lymphatic leukemias, Hodgkin’s disease, non-Hodgkin’s lymphomas, neuroblastomas, and Wilms’ tumors (Hahn et al., 1968; De Vita, 1981). Vincristine inhibits cells in the mitotic metaphase (Pouillart, 1993). Its biological activity is explained by its specific ability to bind to tubulin, thereby preventing polymerization of proteins to microtubules (Böhmarn et al., 1988; Owellen et al., 1976). Carcinoma patients exhibited acute renal insufficiency after administration of vincristine (Jackson et al., 1984), e.g., increased excretion of renal enzymes was demonstrated by Dreiler (1988) in patients with malignant melanomas during cytostatic perfusion therapy. According to Sharma et al. (1995), polyuria, enlarged kidneys, and renal insufficiency are the side effects of vincristine in the treatment of non-Hodgkin’s lymphomas.

With these problems in mind, the effect of silibinin on damaged kidney cells was examined by administration of silibinin before and after the chemical-induced injury. In any case, the time kinetics of the selected parameters were measured, but for clear representation, only the values 72 h after silibinin addition are listed in the tables. The concentrations of paracetamol, cisplatin, and vincristine, capable of causing damage, were tested in preliminary experiments and subsequently selected so as to result in an approximate 20 to 30% inhibition of proliferation after 72 h.

The administration of silibinin 1 h after paracetamol intoxication shows distinct improvements in proliferation, protein biosynthesis, and LDH activities (Table 3). This effect becomes more pronounced if the relative improvements with respect to the damaged status are calculated: 16 to 26%. This also applies to previous administration of the flavonolignan.
24 h before paracetamol-induced damage (Table 4), where relative improvements of 22 to 33% were reached. With values of 26 to 28%, the effect of silibinin on Vero kidney cells previously damaged by cisplatin shows similar improvements in proliferation, protein biosynthesis, and LDH activity previously reduced by the toxicant (Table 5). In these experiments, it can be seen that the inhibitory effects of cisplatin on proliferation are considerably more pronounced than those on protein biosynthesis and LDH activities. As described, the cell volumes increase appreciably, possibly a compensation reaction. The disproportionately large growth inhibition can be attributed to the fact that, although RNA and protein biosynthesis are stimulated by silibinin, the inhibition of DNA synthesis (Tay et al., 1988) cannot be largely compensated by silibinin. The relative improvements, however, are similar for all three parameters. In the case of administration of silibinin before cisplatin damage, the relative stimulatory effects amount to 20 to 26%.

The effects of silibinin on parameters of cells damaged by vincristine are distinctly less marked. The relative improvements are 6 to 16% for subsequent and 10 to 22% for silibinin administration in advance.

With these experiments, it was possible to demonstrate that the flavonolignans isolated from the milk thistle, silibinin and silicristin, stimulate kidney cells in much the same way as they do liver cells. Considering the results of the experiments with kidney cells damaged by taxicants, the data warrant corresponding in vivo evaluations.

References
Koch H and Tscherny J (1983) Bioavailability of silymarin, 3: Splitting of silybin and silychristin, stimulate kidney cells in much the same way as they do liver cells. Considering the results of the experiments with kidney cells damaged by taxicants, the data warrant corresponding in vivo evaluations.
Koch H and Tscherny J (1983) Bioavailability of silymarin, 3: Splitting of silybin and silychristin, stimulate kidney cells in much the same way as they do liver cells. Considering the results of the experiments with kidney cells damaged by taxicants, the data warrant corresponding in vivo evaluations.

Send reprint requests to: Dr. Johann Sonnenbichler, Max Planck Institut für Biologische Klopfspermat, D-82152 Marbach, Germany.