Hepatotoxic effect of (+)usnic acid from *Usnea siamensis* Wainio in rats, isolated rat hepatocytes and isolated rat liver mitochondria

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Abstract

Hepatotoxic effect of (+)usnic acid, the active constituent of *Usnea siamensis* Wainio was studied in rats, isolated rat hepatocytes and isolated rat liver mitochondria. In rats, after treatment with high dose of (+)usnic acid (200 mg/kg per day, i.p.) for 5 days, there was no significant change in serum transaminase activity (serum AST, ALT) while the electron micrographs showed apparent morphological damage of mitochondria and endoplasmic reticulum. (+)Usnic acid at high dose (1 mM) as well as carbon tetrachloride (CCl4, the reference hepatotoxin) induced loss of cell membrane integrity in isolated rat hepatocytes by increasing the release of cellular transaminases (AST, ALT). Increase in lipid peroxidation, decrease in glutathione (GSH) content and increase in aniline hydroxylase activity (CYP 2E1) were also found. Combination of (+)usnic acid and CCl4 showed the additive results. (+)Usnic acid (0.15–6 μM) possessed uncoupling activity in isolated rat liver mitochondria. It stimulated respiration by mitochondria respiring with glutamate plus malate or succinate as substrates and activated ATPase activity. Increasing concentration of (+)usnic acid (>6 μM) exhibited loss of respiratory control and ATP synthesis. In conclusion, hepatotoxic effect of high dose (+)-usnic acid may involve its reactive metabolite(s), causing loss of integrity of membrane like structures, resulting in destruction of mitochondrial respiration and oxidative phosphorylation.

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Keywords: (+)-usnic acid, Hepatotoxic effect

1. Introduction

(+)-Usnic acid is the normal component of lichen cells and is one of the most common and abundant lichen metabolites. This natural monobasic acid (Fig. 1) exists as (+) and (−) enantiomers which found to be more effective than the synthetic derivatives. Usnic acid is well known as antibiotic and endowed with several biological and physiological activities including antiparasitic, antimitotic, antiproliferative, anti-inflammatory, analgesic and antipyretic (Campanella et al., 2002; Cocchietto et al., 2002; Ghione et al., 1988). This natural compound has shown a great relevance in pharmacology and clinics. To be further developed, more research is needed especially the details of mechanism of its different actions and its toxicity. In the present investigation, hepatotoxic effect of (+)-usnic acid was studied in rats, isolated rat hepatocytes and isolated rat liver mitochondria using carbon tetrachloride as the reference hepatotoxin. (+)-Usnic acid is derived from *Usnea siamensis* Wainio, known in Thai as Foi-lom.

2. Materials and methods

2.1. Materials

(+)-Usnic acid was kindly provided by Nijsiri Ruangrungsi, Associate Professor in the Department of Pharmacognosy, Chulalongkorn University.
2. Test animals

Male Wistar albino rats weighing 200–250 g were obtained from National Laboratory Animal Center, Mahidol University. They were allowed free access of standard rodent food and tap water throughout the investigation.

2.3. Hepatotoxic study in rats

Rats were divided into four groups of eight animals each. The control groups were given saline (0.1 ml per day, i.p.) or DMSO (0.1 ml per day, i.p.) for 5 days. The test groups were treated with (+)-usnic acid (dissolved in DMSO) at the doses of 50 or 200 mg/kg per day, intraperitoneally for 5 days. At the end of 5 days treatment, blood sample were taken for the measurement of serum transaminase activity (serum AST, ALT), livers were exposed for histopathological examination.

2.3.1. Microscopy

Electron micrographs were performed using transmission electron microscope (TEM).

Liver slices (in 1 mm³) were fixed with 1–2% OsO₄ in 0.1 M phosphate buffer, pH 7.3 for 2 h. After washing with phosphate buffer, liver cubes were dehydrated with ethanol (from 35 to 100%) then infiltrated with propylene oxide and plastic mixture for 2–3 days. The embedded liver cubes were sectioned with ultramicrotome and dyed with uranyl acetate and lead citrate prior to photomicrography.

2.4. Hepatotoxic study in isolated rat hepatocytes

2.4.1. Preparation of isolated rat hepatocytes

Isolation of rat hepatocytes was routinely prepared at 9.00 a.m. using the method of Berry and Friend (1969) as modified by Stacey and Priestly (1978) and Pramyothin (1986). Under ether anesthesia, liver was perfused with Ca²⁺-free physiological solution (96 mM NaCl, 1.4 mM KCl, 0.7 mM MgSO₄, 2.5 mM KH₂PO₄, 30 mM NaHCO₃ and 21.7 mM sodium glutonate, equilibrated with 95% O₂/5% CO₂ at pH 7.4) via the portal vein. When the perfusion of all hepatic lobes was rapid and complete, 100 ml of 0.5% collagenase in the same Ca²⁺-free physiological solution was added and allowed to digest under the recirculating condition. Flow was maintained at 30–35 ml/min with the pressure head of 20 cm water, and temperature was maintained at 37°C throughout the procedure. After perfusion with collagenase (10–15 min), liver was dispersed with blunt spatula in 50 ml of 0.5% fresh collagenase buffer, and incubated at 37°C in a shaker water bath for 10 min. Bovine serum albumin (BSA) was added to give a final concentration of 12 mg/ml and cells were harvested through nylon mesh (250–61 μm). Hepatocytes were separated from other cells and cellular debris by differential centrifugation (50 g, 1 min). The cell pellet was washed twice with this fresh physiological medium containing 12 mg/ml BSA and once with the incubation medium. Hepatocytes were finally suspended in Eagle’s basal medium containing 12 mg/ml BSA at the concentration of approximately 40 mg wet weight cells per milliliter or approximately 4.5 × 10⁶ cells per milliliter. Trypan blue exclusion index was routinely performed. Preparation with the trypan blue exclusion index lesser than 90% was never used.

2.4.2. Wet weight calibration

Duplicate aliquots of 1 ml cell suspension were put into preweighted tubes, and centrifuged at 5000 rpm for 5 min. Supernatant was discarded, the upside down tubes were allowed to stand in room temperature for 2 h. Then tubes were weighed and cell pellet was calculated as milligram of wet weight cells per milliliter of cell suspension.

2.4.3. Incubation of isolated rat hepatocyte suspension

In every experiment, the aliquot of 3 ml cell suspension was incubated in 25 ml Erlenmeyer flask in the metabolic shaker bath, under the atmosphere of carbogen (95% O₂/5% CO₂) at 37°C for 1 h.

2.4.4. Cytotoxicity study

Cell suspension isolated from four control rats was used. For each cell preparation, triplicate samples were used for control and treated groups including DMSO, (+)-usnic acid (0.01, 0.1, 1 mM), carbon tetrachloride (10 μl), and (+)-usnic acid plus carbon tetrachloride. After 1 h incubation, aliquot of 0.5, 1.5 and 0.5 ml of each sample were used for the determination of transaminase activity, malondialdehyde (MDA) formation, and glutathione content.

2.4.5. Determination of transaminase activity (AST, ALT)

Aliquot of 0.5 ml cell suspension was centrifuged at 1000 rpm for 1 min, the supernatant was measured for transaminase activity using the method of Reitman and Frankel (1957).

2.4.6. Lipid peroxidation and glutathione (GSH) determination

Aliquot of 1.5 ml cell suspension was used in the thiobarbituric acid assay for the formation of malondialdehyde by the method of Buege and Aust (1978). Glutathione was measured using 0.5 ml cell suspension by the method of Ellman (1959) as modified by Jollow et al. (1974).
Fig. 2. Electron micrographs: (A) control (13,600×); (B) DMSO, same as control (13,600×); (C) (+) usnic acid 50 mg/kg per day, i.p., 5 days, slightly swelling of mitochondria (13,600×); (D) (+) usnic acid 200 mg/kg per day, i.p., 5 days, markedly swelling of mitochondria and endoplasmic reticulum (13,600×); enlargement from (D) of mitochondria in (E) (68,000×) and endoplasmic reticulum in (F) (68,000×). N = nucleus, M = mitochondria, E = endoplasmic reticulum.
2.4.7. Determination of aniline hydroxylase activity

Using cell suspension isolated from rats preadministered with isoniazid in drinking water (0.1%, w/v, pH 7.4) for 10 days. After 15 min incubation of 3 ml cell suspension with 0.1 ml 5 mM aniline and (+)Usnic acid (1 and 10 mM), all samples were used for the determination of aniline hydroxylase activity by the method of Gibson and Skett (1986).

2.5. Hepatotoxic study in isolated rat liver mitochondria

2.5.1. Preparation of rat liver mitochondria

Intact rat liver mitochondria was prepared by the method of Hogeboom (1955). The mitochondrial protein was determined by the method of Lowry et al. (1951) as modified by Miller (1959) using bovine serum albumin as standard.

2.5.2. Oxygen consumption measurements

The oxygen uptake by intact mitochondria was measured polarographically by Clarke-type oxygen electrode connected to an oxygen monitor (YSI model 53) and recorded on a strip chart recorder (Gibson model N2).

2.5.3. ATPase activity

The mitochondrial ATPase activity was measured by determining the amount of inorganic phosphate liberated at the end of incubation period. The reaction was terminated by adding 1 ml aliquot of reaction mixture to 1 ml of ice-cold trichloroacetic acid. After centrifugation, the amount of inorganic phosphate in the supernatant was determined by the method of Fiske and Subbarow (1925).

2.5.4. Experimental conditions

All experiments were performed at 37°C. The composition of reaction mixtures and other experimental conditions are described in the table legends. The mitochondria used in this study must have the RCI (respiratory control index) value not less than five with glutamate plus malate as substrate. The experimental results reported here were reproducible with at least four separate mitochondrial preparations.

2.6. Statistical analysis

Statistical analysis of the results were performed with Student’s t-test.

3. Results

After 5 days treatment of (+)Usnic acid in rats, there was no significant change in serum transaminase activity (serum AST, ALT) (Table 1) while the electron micrographs illustrated the signs of liver cell damage as shown by the markedly swelling of mitochondria and endoplasmic reticulum, especially at the 200 mg/kg dose of (+)Usnic acid (Fig. 2).

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose per day, 5 days</th>
<th>Serum AST* (S.F. units per millilitre)</th>
<th>Serum ALT* (S.F. units per millilitre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>0.1 ml, i.p.</td>
<td>162.5 ± 20.9</td>
<td>126.1 ± 19.5</td>
</tr>
<tr>
<td>Control (DMSO)</td>
<td>0.1 ml, i.p.</td>
<td>167.1 ± 27.7</td>
<td>134.3 ± 12.4</td>
</tr>
<tr>
<td>(+)Usnic acid</td>
<td>50 mg/kg, i.p.</td>
<td>199.0 ± 32.7</td>
<td>150.2 ± 5.7</td>
</tr>
<tr>
<td>(+)Usnic acid (0.1 mM)</td>
<td>200 mg/kg, i.p.</td>
<td>211.9 ± 29.9</td>
<td>164.0 ± 13.6</td>
</tr>
</tbody>
</table>

S.F. units per millilitre × 0.48 = IU/l.

* Values are mean ± S.E.

### Table 2

Cytotoxic effects of (+)Usnic acid, CCl4 and (+)Usnic acid plus CCl4 in isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Transaminase activity</th>
<th>MDA* (mole/g wet weight cells)</th>
<th>GSH (mole/g wet weight cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AST</td>
<td>ALT</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>215.2 ± 11.6</td>
<td>260.7 ± 27.6</td>
<td>17.1 ± 0.7</td>
</tr>
<tr>
<td>DMSO (10 μl)</td>
<td>238.6 ± 18.4</td>
<td>290.5 ± 29.1</td>
<td>15.0 ± 0.9</td>
</tr>
<tr>
<td>CCl4 (10 μl)</td>
<td>515.1 ± 51.1**</td>
<td>523.5 ± 59.8**</td>
<td>26.9 ± 1.2**</td>
</tr>
<tr>
<td>(+)Usnic acid (0.01 mM)</td>
<td>276.0 ± 20.2</td>
<td>310.4 ± 38.3</td>
<td>14.3 ± 0.1</td>
</tr>
<tr>
<td>(+)Usnic acid (0.1 mM)</td>
<td>280.3 ± 20.1</td>
<td>333.8 ± 27.2</td>
<td>19.1 ± 0.7**</td>
</tr>
<tr>
<td>(+)Usnic acid (1 mM)</td>
<td>361.8 ± 35.2**</td>
<td>498.8 ± 19.7**</td>
<td>26.4 ± 1.5**</td>
</tr>
<tr>
<td>(+)Usnic acid (0.01 mM) + CCl4</td>
<td>866.4 ± 127.5***</td>
<td>1010.3 ± 264.2***</td>
<td>35.6 ± 1.3***</td>
</tr>
<tr>
<td>(+)Usnic acid (0.1 mM) + CCl4</td>
<td>990.3 ± 160.7***</td>
<td>1229.0 ± 264.8***</td>
<td>46.2 ± 3.7***</td>
</tr>
<tr>
<td>(+)Usnic acid (1 mM) + CCl4</td>
<td>1144.3 ± 342.7***</td>
<td>1758.1 ± 235.9***</td>
<td>47.9 ± 3.31***</td>
</tr>
</tbody>
</table>

S.F. units per millilitre × 0.48 = IU/l.

* Values are mean ± S.E.

** Significantly different from control group, p < 0.05.

** Significantly different from DMSO group, p < 0.05.

*** Significantly different from CCl4 group, p < 0.05.
DMSO (10 μM) to dissolve (+) usnic acid for 1 min before substrates were added. The rates of oxygen consumption were increased, the glutathione content was decreased. The study of cytotoxic effect of (+) usnic acid in isolated rat hepatocytes demonstrated the dose related pattern in the release of cellular transaminases (AST, ALT) and malondialdehyde formation (index of lipid peroxidation). DMSO, at the volume used (10 μl) to dissolve (+) usnic acid had no effect on these criteria. When increasing the dose of (+) usnic acid, transaminase activity (AST, ALT) and lipid peroxidation were increased, the glutathione content was decreased. CCl₄ (10 μl) was used as the reference hepatotoxin, similar results were obtained as shown in the high dose (+) usnic acid group (1 mM). Coadministration of (+) usnic acid and CCl₄ exerted the additive effects on the release of cellular transaminases, the formation of MDA and GSH content (Table 2). The aniline hydroxylase activity was also found to be increased with high doses of (+) usnic acid (1, 10 mM) (Table 3). (+) usnic acid stimulated respiration in isolated rat liver mitochondria using glutamate plus malate and succinate as substrates. The rate of oxygen consumption in state 4 respiration increased when increased doses of (+) usnic acid starting from 0.15, 4.5 and 6 μM doses showed the highest rate of mitochondrial respiration, resiping with succinate and glutamate plus malate, respectively. After the highest rate, there was a slow down in respiration even when increased the doses of (+) usnic acid (Table 4). Mitochondrial ATPase activity was also stimulated by (+) usnic acid, with the dose starting from 0.3 μM. At 2 μM dose of (+) usnic acid showed the maximal stimulation. Doses higher than 2 μM illustrated the slower rate of ATPase stimulation (Table 5).

Table 3: Effect of (+) usnic acid on aniline hydroxylase activity in isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Aniline hydroxylase activity a (μmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.4 ± 1.4</td>
</tr>
<tr>
<td>DMSO (10 μM)</td>
<td>8.0 ± 1.3*</td>
</tr>
<tr>
<td>(+) usnic acid (1 mM)</td>
<td>32.8 ± 1.7**</td>
</tr>
<tr>
<td>(+) usnic acid (10 μM)</td>
<td>46.5 ± 2.3**</td>
</tr>
</tbody>
</table>

a Values are mean ± S.E.

**Significantly different from control group, p < 0.05.

4. Discussion

In the present investigation, the hepatotoxicity induced by (+) usnic acid in rats requires both high dose and time of exposure using serum AST, ALT and histopathological changes as the criteria. (+) usnic acid seems to be a lesser hepatotoxin due to its lesser effect on the transaminase activity and liver cell injury. Membrane like structures such as mitochondria and endoplasmic reticulum are affected by high dose of (+) usnic acid as seen clearly in the electron micrographs.

In order to search further for hepatotoxic mechanisms of (+) usnic acid, isolated rat hepatocytes were used, together with carbon tetrachloride (CCl₄) as the reference hepatotoxin. CCl₄ is well known for its hepatotoxicity. It is metabolized in rats by the cytochrome P450 system, especially CYP 2E1 to a highly reactive metabolite, trichloromethyl-free radical (CCl₃•). This free radical may react with oxygen and generate the trichloromethylperoxy radicals (OOCCL••). Both radicals may attack lipids on cell membrane and membrane like structures such as mitochondria and endoplasmic reticulum.


