Mutat Res Gen Tox En 839 (2019) 36–39

Contents lists available at ScienceDirect

Mutat Res Gen Tox En

journal homepage: www.elsevier.com/locate/gentox

Genotoxicity of (+)- and (−)-usnic acid in mice

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ARTICLE INFO

Keywords:
Usnic acid
Enantiomers
Genotoxcity
DNA-comets
Oxidative stress
Mice

ABSTRACT

Usnic acid, which is the most widespread and well-studied secondary lichen compound, has antibacterial and cytotoxic effects. Usnic acid is present in lichens as the (+)- and (-)-enantiomers, which have different biological activities. We used a DNA-comet assay to determine the genotoxic effect of (+)- and (−)-usnic acid in the liver and kidney cells of mice. The genotoxic effect of usnic acid was only observed 1 h after oral administration. Usnic acid doses of 100 and 50 mg/kg resulted in DNA damage in the liver and kidney cells. The genotoxic effect of usnic acid is associated with oxidative stress in cells. There were no significant differences in the effects of (+)- and (−)-enantiomers.

1. Introduction

At present, there are 854 known compounds related to secondary lichen compounds [1]. Usnic acid (UA) is the most common and well-studied lichen compound. The thalli of lichens contain (+)- and (−)-UA, which have *R* and *S* configurations, respectively (Fig. 1). Moreover, certain lichen species contain one main UA enantiomer, whereas the other enantiomer is either absent or only present in trace amounts [2].

UA enantiomers show a range of biological activities. (+)-UA has greater antibacterial activity against some anaerobic gram-negative bacteria than (−)-UA [3], whereas (-)-UA exhibits higher antiviral activity than (+)-UA [4]. *In vitro* studies of the cytotoxicity of UA enantiomers against the V79 and A549 cell lines and peripheral blood lymphocytes have shown that (+)-UA has more higher cytotoxic effects than (−)-UA [5,6].

A study of the genotoxic effects of (+)- and (−)-UA in peripheral blood lymphocytes did not reveal a significant increase in the frequency of micronucleation in cells [5]. However, the more sensitive DNA-comet method revealed that in lymphocytes, (+)- and (−)-UA caused DNA fragmentation and the genotoxicity of (-)-UA was twice as high as that of (+)-UA [6].

To date, there is only information about the genotoxic effect *in vivo* of (+)-UA. Al-Bekairi et al. [7] observed a slight increase in the frequency of micronuclei in polychromatic normoblasts in mice given single doses of 100 and 200 mg/kg (+)-UA. However, further study of the genotoxicity of (+)-UA in the mice, both with a micronucleus test and the DNA-comet method, did not reveal significant differences from the control [8].

In this work, we examine the genotoxic effects of (+)- and (−)-UA in the liver and kidneys of mice using a DNA-comet assay.

2. Materials and methods

2.1. Lichen material

(+)- and (−)-UA were extracted from *Cladonia arbuscula* and *Cladonia stellaris* lichens, respectively. Lichens were collected in June 2015 in the Aldan District of the Republic of Sakha (Yakutia), Russia.

2.2. Isolation of (+)- and (−)-UA

To obtain UA, milled air-dry lichen thalli (50 g) were extracted for 12 h with petroleum ether using a Soxhlet apparatus (fraction: 0.4–0.5 g). After cooling, fine yellow crystalline sediment precipitated from the partially evaporated extracts. The UV and IR spectra of the isolated fraction matched those of UA [9]. Measurements of the turning angle of the polarization plane confirmed that (+)- and (-)-UA were extracted from *C. arbuscula* and *C. stellaris* lichens, respectively ([α] +420 and –500, $CHCl_3$, *c* 0.2). The purity of the compounds was determined by the melting point and by TLC and was 97% and 96% for (+) and (-)-UA respectively.

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https://doi.org/10.1016/j.mrgentox.2019.01.010
Received 3 October 2018; Received in revised form 16 January 2019; Accepted 21 January 2019
Available online 21 January 2019
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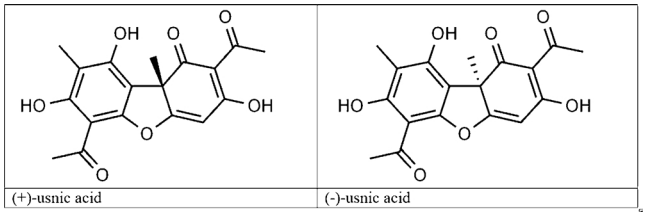

Fig. 1. Chemical structures of usnic acid enantiomers.

2.3. Test animals

Experiments were performed on male CD-1 mice (20–22 g) obtained from "Pushchino" animal breeding facility. The animals were housed (10–12 per cage in conventional polycarbonate 1290D cages) in the facility with room temperature maintained at 22 ± 2 °C under an automatically controlled cycle of 12 h light/dark. Standard laboratory animal feed (MEST Ltd, Russia) and water were provided *ad libitum*. Animals were quarantined for at least one week before the beginning of the experiment. During that time, the animals were observed daily for signs of illness and only healthy animals were used.

The study was approved by the Ethics Committee of Zakusov Institute of Pharmacology (Protocol No. 5.1).

2.4. Experimental design

To determine the optimal exposure time for the internal organs to UA during the genotoxicity assessment experiments, five experimental groups of mice and five negative control groups (corn oil) containing five animals each (total of 50 mice) were used. Each animal from the experimental group received a single intragavage dose of 100 mg/kg (+)-UA as a suspension in corn oil (0.1 ml). The animals were euthanized 1, 3, 6, 12, and 24 h after administering UA.

To determine the dose dependence of UA genotoxicity, the mice were divided into 10 groups with five animals in each: group 1, negative control; group 2, positive control (methyl methanesulfonate (MMS); groups 3–10, experimental groups, which were given 1, 10, 50, and 100 mg (+)- and (-)-UA per kilogram, respectively. The dose selection was based on the fact that UA begins to show a clear toxic effect on mice after a single oral ingestion of more than 180 mg/kg [10]. UA was given by gavage using soft 22 gauge feeding tube as a suspension in corn oil. MMS was given *via* intraperitoneal injection.

2.5. Determination of UA in tissues

To determine the UA content in the organs, 0.5 g of liver and 0.05 g of kidney were taken. Over 24 h, the organs were ground and extracted twice with acetone (4 ml). The extracts were centrifuged at 6000*g* for 10 min. The supernatant was evaporated and the resulting dry residue was dissolved in acetone (50 μL) and analysed by high-performance liquid chromatography with a microcolumn chromatograph (Milichrom A-02, EcoNova, Russia). Separation was performed using a ProntoSIL 120-5-C18 AQ reverse-phase column (2 × 75 mm). Mobile phase (A) was a 0.1% aqueous solution of acetic acid, and mobile phase (B) was acetonitrile. The elution gradient was an increase in (B) from 10% to 50% over 5 min and an increase from 50% to 100% over 20 min at a flow rate of 100 μL/min and a column temperature at 40 °C. Detection was carried out at a wavelength of 230 nm.

2.6. Comet assay

The liver and kidney tissues of the mice were homogenized in phosphate buffered saline (pH 7.5). The same buffer suspensions of cells were placed in tubes with a 1% solution of low-melting-point agarose and applied to microscope slides that were first covered with high-melting-point agarose. After agarose crystallization, the micropreparations were lysed with a cool buffer (10 mM Tris-HCl, pH 10, 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10% DMSO) for at least 1 h at 4 °C. After lysis, the micropreparations were placed in alkaline buffer for electrophoresis (300 mM NaOH, 1 mM EDTA, pH > 13) for DNA denaturation and transformation of alkali-labile sites into single-stranded breaks. Electrophoresis was performed for 20 min at an electric field intensity of 1 V/cm and electric current of ˜300 mA. The preparations were fixed in 70% ethanol solution and dried.

Before microscopy, preparations were stained with SYBR Green I dye (20 μg/mL) for 30 min. The analysis was carried out using a fluorescent microscope (MikMed-2, Lomo, Russia) with excitation and cutoff filters of 490 and 530 nm, respectively. The images of DNA comets were analysed with CASP 2.2.1 software. The percentage of the total comet DNA present in the tail of the comets was used as a measure of DNA damage. Atypical DNA comets with an absent or practically absent head and a wide diffuse tail (ghost cells) were calculated separately and excluded from the total analysis.

2.7. Determination of malondialdehyde (MDA)

The intensity of the processes of lipid peroxidation was determined from the accumulation of a colored MDA complex with thiobarbituric acid ($\varepsilon = 155\ mM^{-1}\ cm^{-1}$) at 532 nm. To determine the MDA content in the organs, 0.2 g of liver and 0.05 g of kidney were homogenized in 10 ml of a 1.2% KCl solution cooled to 0 °C. The extracts were centrifuged at 6000*g* for 10 min. For the protein precipitation 1 ml of the supernatant was mixed with the equal amount of 40% trichloroacetic acid. The resulting precipitate was separated by centrifugation. To the supernatant were added 1 ml of 0.8% thiobarbituric acid and incubated at 100 °C for 15 min. The absorbance was measured on a spectrophotometer (UV-2600, Shimadzu, Japan).

2.8. Statistical analysis

The results are presented as the arithmetic mean and standard deviation (M ± SD). Mean values were compared using one-factor dispersive analysis of variance (ANOVA), the significance of difference using Newman-Keuls test for multiple comparisons or Dunnett's test for multiple comparisons from the control, at a level of $p \leq 0.05$. The calculation was carried out using the StatPlus statistical analysis program in AnalystSoft v. 2007.

3. Results and discussion

The genotoxic effect of a single 100 mg/kg dose UA in the kidney and liver of mice was measured at various times after administration. In the liver, after 1 and 3 h, a statistically significant increase in DNA damage (percentage of DNA in the comet tail) was observed and in the kidneys the effect was observed only after 1 h (Fig. 2). Based on these results, the dose dependence experiments on the genotoxic effects of (+)- and (-)-UA on the internal organs of the mice were conducted 1 h after administration.

After administration of various doses of (+)- and (-)-UA, the UA content in the liver and kidneys of mice was determined. The UA content in the liver and kidney tissues was 2–18 μg/g wet mass 1 h after the mice were administered doses of 10, 50, and 100 mg/kg UA (Table 1). The accumulation of (+)- and (−)-UA in the liver and kidney tissues was not statistically significant and depended only on the dose. Previous studies have shown that intraperitoneal injection in rats resulted in a UA content in the liver 3–4 times higher than that in the kidneys [11]. This difference may be due to the differences in routes of UA administration.

The genotoxicity of the UA enantiomers at doses from 1 to 100 mg/kg *in vivo* in the liver and kidneys of mice was studied. At doses of 1 and 10 mg/kg, neither enantiomer induced DNA damage in either organ. At

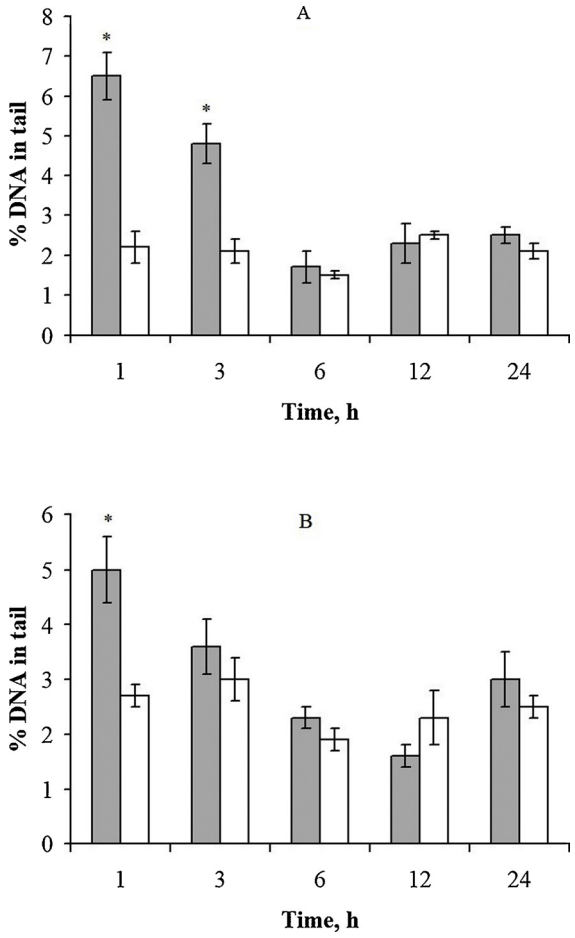

Fig. 2. Percentage of DNA in the comet tail for liver (A) and kidney (B) cells of mice as a function of time after oral administration of (+)-UA. Grey bars: 100 mg/kg UA; white bars: control (corn oil); asterisk: statistically significant difference compared with the control at $p \leq 0.05$ (*t*-test); results are presented as M ± SD (n = 5 for each group).

Table 1
Content of UA enantiomers in organ tissues.

Dose, mg/kg body weight	Content, μg/g wet weight	
	Liver	Kidney
(+)-UA		
1	ND	ND
10	2.2 ± 0.7[a]	3.5 ± 0.8[a]
50	6.8 ± 1.5[b]	8.3 ± 1.2[b]
100	15.8 ± 1.2[c]	17.7 ± 3.5[c]
(–)-UA		
1	ND	ND
10	2.1 ± 0.5[a]	3.1 ± 0.6[a]
50	6.6 ± 1.3[b]	6.9 ± 1.0[b]
100	13.8 ± 2.4[c]	15.2 ± 3.4[c]

The mean values with the same superscript letter are not statistically significant at $p < 0.05$ (ANOVA, Newman-Keuls test). ND - Not Detected.

doses of 50 and 100 mg/kg, the DNA damage in the liver and kidneys was 2–3 times greater than in the control (Table 2). However, there were no significant differences in the effects of (+)- and (−)-UA.

We observed "ghost cells" in addition to typical DNA comets. The formation of "ghost cells" indicates early-stage chromatin fragmentation during cell destruction or the induction of oxidative stress in cells [12,13]. The proportion of liver and kidney cells with atypical DNA comets in mice administered doses of 50 and 100 mg/kg (+)- and

Table 2
Genotoxicity of UA enantiomers in liver and kidney cells after oral administration.

Dose, mg/kg body weight	Liver		Kidney	
	% DNA in comet tail	Frequency of "ghost cells", %	% DNA in comet tail	Frequency of "ghost cells", %
Control (corn oil)	2.2 ± 0.4	2.2 ± 0.3	2.7 ± 0.3	2.9 ± 0.6
MMS, 40 mg/kg	15.5 ± 2.9*	3.3 ± 1.0	18.3 ± 1.7*	4.6 ± 0.9
(+)-UA				
1	3.3 ± 0.6	2.7 ± 0.9	3.4 ± 0.6	3.6 ± 0.5
10	3.9 ± 0.7	3.4 ± 0.8	3.5 ± 0.5	4.7 ± 0.7
50	5.7 ± 1.0*	5.4 ± 1.1*	4.7 ± 0.4*	6.5 ± 1.0*
100	6.5 ± 0.6*	6.0 ± 0.6*	5.0 ± 0.6*	6.9 ± 0.8*
(–)-UA				
1	3.4 ± 0.6	2.0 ± 0.6	3.4 ± 0.6	3.3 ± 0.7
10	3.7 ± 0.3	3.1 ± 0.4	3.4 ± 0.7	4.1 ± 0.5
50	5.2 ± 1.3*	5.3 ± 0.8*	4.9 ± 0.4*	5.8 ± 0.5*
100	5.8 ± 0.7*	6.0 ± 0.8*	5.1 ± 0.6*	7.0 ± 0.4*

* Differences are statistically significant ($p < 0.05$) compared with a negative control (ANOVA, Dunnett's *t* − test).

(-)-UA was 3–5 times higher than that in the control.

Current data on the genotoxicity of UA are ambiguous. In Ames tester strains TA98 and TA100, UA did not show mutagenic activity [14], whereas in *Drosophila melanogaster*, UA showed recombinogenic, mutagenic, and potential carcinogenic activity [15]. In most studies, UA did not induce chromosomal aberrations and micronuclei *in vitro* and *in vivo* [5,8,16]. *In vitro* experiments demonstrated that UA damaged DNA in V79 cells and human lymphocytes [6,8], whereas *in vivo* UA did not show genotoxic effects in the liver of mice at doses of 25–200 mg/kg [8]. In contrast, in the present study, both UA enantiomers induced DNA damage in the liver and kidneys of mice at doses of 50 and 100 mg/kg. The discrepancies in UA genotoxicity are likely related to differences in the experimental conditions, such as the model, exposure time, and doses or concentrations. For example, although Leandro et al. [8] did not observe genotoxicity, they estimated the DNA damage 24 h after UA was administered. We found that the genotoxic effect of UA on DNA in the liver occurred after 1 and 3 h, and in the kidneys after 1 h; the effect was not detected after longer periods. This result indicates the rapid induction and repair of DNA damage, which is typical for agents that cause oxidative stress. This inference is supported by the observed formation of atypical DNA comets.

The mechanism of action of UA is associated with the effect of UA on the functional activity of mitochondria [17]. At low concentrations, UA stimulates the respiration in mitochondria, but at high concentrations UA causes oxidative phosphorylation separation, suppression of ATP synthesis, and hyperproduction of reactive oxygen species (ROS) [18,19].

The hyperproduction of ROS in cells initiates the lipid peroxidation (LPO) in membranes [16]. One of the most important markers of LPO is MDA – a stable product of free radical reactions. At doses of 50 and 100 mg/kg UA the MDA content in the liver and kidney cells was higher than in the control (Table 3). An increase in the MDA content in cells may indicate that genotoxic effect of UA is associated with oxidative stress.

The oxidative effect of UA and associated genotoxicity depends on the state of the antioxidant system in the cell, which may explain the differences in the sensitivities of various cell types *in vitro* to the damaging effect of UA [5,6,8].

Our results together with literature data indicate genotoxic and carcinogenic risk of UA. Although UA demonstrated a wide range of bioactivities, including antimicrobial, antiprotozoal, antiviral and other

Table 3
Content of malondialdehyde (MDA) in organ tissues.

Treatment (mg/kg b.w.)	MDA, nM/g wet weight	
	Liver	Kidney
Control (corn oil)	487.7 ± 33.7	248.3 ± 7.5
(+)-UA		
1	502.0 ± 28.7	240.7 ± 9.1
10	511.3 ± 35.8	254.6 ± 4.5
50	631.7 ± 71.3*	280.3 ± 19.0*
100	600.8 ± 55.3*	301.5 ± 59.5*
(−)-UA		
1	490.2 ± 22.6	238.1 ± 10.3
10	525.8 ± 43.7	259.4 ± 7.2
50	639.3 ± 89.5*	292.3 ± 12.4*
100	621.5 ± 12.5*	354.5 ± 48.5*

* Differences are statistically significant ($p < 0.05$) compared with a negative control (ANOVA, Dunnett's *t*–test).

[20], these potential adverse effects restrict development of UA as pharmacological agents. At the same time UA is considered like a promising antitumor agent that has shown good antiproliferative and cytotoxic activity against various cancer cell lines [20,21]. In this regard, our results on the study of the effects of various doses of enantiomers of the UA in the tissues of the organs of mice may be useful in subsequent studies of its antitumor activity *in vivo*.

4. Conclusion

Doses of 50 and 100 mg/kg (+)- and (-)-UA show genotoxicity in the liver and kidneys of mice *in vivo*. The effects were observed after 1 and 3 h in the liver and after 1 h in the kidneys, and were not detected subsequently. An increase in the MDA content in cells may indicate that genotoxic effect of UA is associated with oxidative stress. The genotoxic effect of UA did not depend on the enantiomer.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the framework of the state assignment of the Institute for Biological Problems of Cryolithozone, Siberian Branch of the Russian Academy of Sciences for 2017–2020 (Nos. AAAA-A17-117020110056-0 and AAAAA17-117020110055-3).

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