Food and Chemical Toxicology 120 (2018) 112–118

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Usnic acid reactive metabolites formation in human, rat, and mice microsomes. Implication for hepatotoxicity

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ARTICLE INFO

Keywords:
Usnic acid
Liver failure
Reactive metabolites
Drug toxicity
Hepatotoxicity

ABSTRACT

Usnic acid is a lichen compound which is extensively studied due to its cytotoxic, antiproliferative, antimicrobial, antiviral, antiprotozoal, and anti-inflammatory activities. Despite a broad spectrum of biological properties, usnic acid is a hepatotoxic agent, thus its potential use as a drug is limited. Certain hepatotoxic drugs may act by generating reactive metabolites that damage the liver. The aim of the study was to predict the biotransformation of usnic acid enantiomers to reactive products using a trapping assay with glutathione in human, rat, and mice liver microsomes. Our results indicate that each enantiomer forms two reactive metabolites; in turn, these metabolites form adducts with glutathione, which may partially explain the toxicity of usnic acid. In silico analysis indicated structural alerts for the generation of reactive metabolites in usnic acid formula. This study proposes a novel mode of the hepatic toxicity of usnic acid enantiomers; it also provides some useful suggestions for designing safer usnic acid derivatives.

1. Introduction

Usnic acid (Fig. 1) is a lipophilic and optically active dibenzofuran derivative that is naturally found in lichens, especially within the *Cladonia*, *Usnea*, *Lecanora*, *Ramalina*, *Evernia* and *Parmelia* genera (Araújo et al., 2015). Both enantiomers of usnic acid have been reported to have cytotoxic, antiproliferative (Galanty et al., 2017), antimicrobial (Maciag-Dorszyńska et al., 2014; Studzińska-Sroka et al., 2015), antiviral (Sokolov et al., 2012), antiprotozoal (Si et al., 2016) and anti-inflammatory (Su et al., 2014) properties. Right-handed usnic acid has been studied more often and seems to be more active compared to its left-handed form, but exceptions exist (Bazin et al., 2008; Yilmaz et al., 2004).

Despite such a broad spectrum of interesting biological activities, usnic acid is a known hepatotoxic agent (Araújo et al., 2015). This property has been reported both *in vitro* (Chen et al., 2017; Han et al., 2004; Pramyothin et al., 2004; Sonko et al., 2011) and *in vivo* in rodents (Pramyothin et al., 2004). Hepatotoxicity due to usnic acid has also been observed in humans ingesting weight-reducing supplements containing this compound (Brown, 2017), therefore its potential application in medicine is limited.

One of the mechanisms of action of hepatotoxins is their metabolic modification, which results in the formation of reactive, usually electrophilic metabolites which covalently bind intracellular nucleophiles (e.g. glutathione) and macromolecules such as proteins and DNA, leading to their dysfunction. The toxicity of reactive metabolites may be also associated with the production of immunogenic complexes (Cho and Uetrecht, 2017). This mode of hepatotoxicity is distinctive for drugs like acetaminophen (paracetamol) (Hinson et al., 2010), as well as a number of phytoconstituents such as safrole (Yang et al., 2017), myristicin (Yang et al., 2015) and bakuchiol (Chi et al., 2016).

In order to identify possible new metabolites from usnic acid, we utilized a trapping assay; this is a standard procedure for detecting reactive metabolites in which a tested compound is subjected to incubation with a mixture of metabolizing enzymes and a trapping nucleophilic agent such as glutathione. The potential reactive compounds that are generated during metabolism are captured by the trapping agent and form adducts, which can then be detected by mass spectrometry (Yang et al., 2015).

In this study, usnic acid enantiomers were subjected to a trapping assay in human, rat, and mice microsomes. The *in silico* method allowed structural alerts of usnic acid for the generation of reactive metabolites

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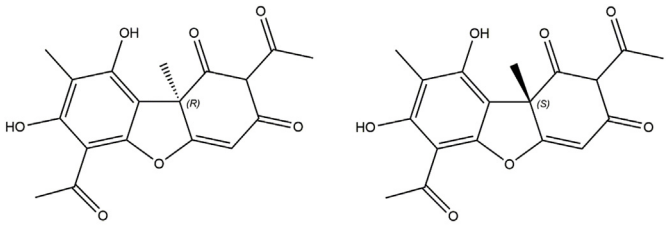
https://doi.org/10.1016/j.fct.2018.07.005
Received 2 March 2018; Received in revised form 10 May 2018; Accepted 2 July 2018
Available online 04 July 2018
0278-6915/



(+)-usnic acid (-)-usnic acid

Fig. 1. Usnic acid enantiomers in keto forms.

to be indicated. The results allow us to propose a novel mechanism of usnic acid hepatotoxicity and provide some new implications for the process of developing safer usnic acid derivatives.

2. Materials and methods

2.1. Chemicals

(+)-Usnic acid, reduced glutathione, human, rat, and mice liver microsomes, $NADP^+$, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, buffers, LC/MS-grade acetonitrile, LC/MS-grade methanol, formic acid puriss. p.a., HPLC-grade chloroform were from Sigma-Aldrich. HPLC-grade water was obtained from an HLP 5 (HYDROLAB Poland) apparatus and was filtered through a 0.2 μm filter before use.

2.2. Isolation and identification of (-)-usnic acid

(-)-Usnic acid was isolated from *Cladonia uncialis* subsp. *uncialis* (L.) Weber ex F.H. Wigg., collected in northern Poland in dry, non-coastal European Scots pine forests in July 2008; its identity was verified by one of the authors (AG, a specialist in pharmacognosy and lichenology). The voucher specimen was deposited in the herbarium of Jagiellonian University Department of Pharmacognosy (Ref. No. KFg/2008/L2). Isolation of the compound was performed as described previously (Galanty et al., 2017) using a chloroform extract of dried lichen material (80 g). The identity of the compound was assessed by measuring the melting point and by TLC and HPLC analyses, according to previously published methods (Galanty et al., 2017). The enantiomeric form of isolated usnic acid was confirmed using a Jasco P-2000 polarimeter.

2.3. Microsomal incubations

A usnic acid primary stock solutions were prepared using acetonitrile (7.27 mM), which was further diluted in a phosphate buffer (second stock) to a concentration of 1.25 mM.

The assay was performed as previously described (Yang et al., 2015), with small modifications. The reaction mixtures consisted of (+) or (-)-usnic acid (50 μM), glutathione (5 mM), and human, rat, or mice microsomes (1 mg protein/mL) in a phosphate buffer (pH = 7.40; 0.1 M); they were preincubated for 10 min on an Eppendorf Thermoblock (37 °C, 350 rpm). Next, a NADPH-regenerating system ($NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase in phosphate buffer) was added to initiate the reaction. The total volume of the reaction mixture was 250 μL. Control reactions were performed without glutathione, microsomes, or the NADPH-regenerating system. The final concentration of acetonitrile did not exceed 0.68%. After 1 h, the reaction was terminated by adding an equal volume of ice-cold methanol and the tubes were transferred for 1 h to −20 °C. Samples were then centrifuged (13.000; 10 min) and the resulting supernatants were transferred to new microcentrifuge tubes and subjected to UPLC-MS/MS analyses. Three separate repeats of the experiments were conducted.

2.4. UPLC-MS/MS analyses

The UPLC-MS/MS system consisted of a Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI and tandem quadrupole). Chromatographic separations were carried out using the Acquity UPLC BEH (bridged ethyl hybrid) C_{18} column (2.1 × 100 mm, and 1.7 μm particle size) equipped with Acquity UPLC BEH C18 VanGuard pre-column (2.1 × 5 mm, and 1.7 μm particle size). The column was maintained at 40 °C and eluted under linear gradient conditions using 95%–0% of eluent A over 10 min, followed by isocratic elution using 100% of B over 2 min and linear gradient elution from 0% to 95% of A over 0.5 min, at a flow rate of 0.3 mL min^{-1}. Eluent A was water/formic acid (0.1%, v/v); eluent B was acetonitrile/formic acid (0.1%, v/v).

Chromatograms were recorded using a Waters eλ PDA detector. Spectra were analysed in the range 200–700 nm with 1.2 nm resolution and a sampling rate of 20 points/s.

MS detection settings of the Waters TQD mass spectrometer were as follows: source temperature 150 °C, desolvation temperature 350 °C, desolvation gas flow rate 600 L h^{-1}, cone gas flow 100 L h^{-1}, capillary potential 3.00 kV, cone potential 20 V. Nitrogen was used for both nebulizing and drying gas. The data were obtained in a scan mode ranging from 50 to 1000 *m/z* at 0.5 s intervals; 8 scans were summed to get the final spectrum. Collision activated dissociation (CAD) analyses were carried out with an energy of 20 eV and 50 eV using argon as the collision gas. Consequently, the ion spectra were obtained by scanning from 50 to 660 *m/z* range.

Data acquisition software was MassLynx V 4.1 (Waters).

2.5. Determination of structural alerts in ToxAlert

ToxAlert web database (http://ochem.eu/alerts) (Sushko et al., 2012) was used to examine usnic acid for structural alerts in relation to the formation of reactive metabolites. For this purpose, SMILES formulas of both usnic acid enantiomers and tautomers were introduced to the software and the structures were correctly generated. Analysis was performed with “Idiosyncratic toxicity (RM formation)” as an endpoint, while Molecule Preprocessing parameters were default.

3. Results

3.1. (-)-Usnic acid isolation

The applied isolation procedure yielded 108 mg of 95% pure (-)-usnic acid with a melting point of 202 °C; $[\alpha]_D^{24}$ - 375° ($CHCl_3$). The expected values for a reference substance were 203 °C for melting point and $[\alpha]_D^{24}$ - 495° for optical rotation [Huneck and Yoshimura, 1996].

3.2. Assessment of usnic acid structural alerts in silico

ToxAlerts generated two structural alerts of usnic acid: the presence of “ortho- or para-alkylphenols” and “Michael acceptors” (Kalgutkar and Soglia, 2005). There were no differences between the enantiomeric and tautomeric forms.

3.3. Trapping assay of usnic acid enantiomers

Incubation of usnic acid enantiomers with the tested microsome systems resulted in the formation of several metabolites. In the experiments performed in the absence of glutathione, two metabolites were detected: products of usnic acid hydroxylation (*m/z*: 361; retention time: 4.47 min) and dehydrogenation (*m/z* = 343; retention time: 6.33 min). A different metabolic profile was observed in the samples containing reduced glutathione. The peaks which appeared in the chromatograms of samples with (+)-usnic acid and glutathione were eluted in 4.58 min (M1) and 4.74 min (M2) (Fig. 2). The same eluting