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Redox characterization of usnic acid and its cytotoxic effect on human neuron-like cells (SH-SY5Y)

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ABSTRACT

Usnic acid (UA) is the most common and abundant lichenic secondary metabolite with potential therapeutic application. Anti-inflammatory and antitumour properties have already been reported and UA-enriched extracts are widely used to treat several diseases in the folk medicine. First, we performed *in silico* evaluation of UA interactions with genes/proteins and important compounds for cellular redox balance and NO pathway. Then, we assessed UA redox properties against different reactive species (RS) generated *in vitro*, and evaluated its action on SH-SY5Y neuronal like cells upon hydrogen peroxide (H_2O_2), since no *in vitro* neurotoxicological data has been reported so far. Total reactive antioxidant potential index (TRAP) showed a significant antioxidant capacity of UA at the highest tested concentration; UA was also effective against hydroxyl radicals and reduced the formation of nitric oxide. *In vitro*, lipoperoxidation was enhanced by UA and changed the cellular viability at highest concentration of 20 μg/mL for 1 and 4 h, as well as 2 and 20 μg/mL for 24 h of treatment, according to MTT reduction assay. Moreover, UA did not display protective effects against H_2O_2-induced cell death in any case. Evaluation of intracellular RS production by the DCFH-based assay indicated that UA was able to induce changes in basal RS production at concentration of 20 μg/mL for 1 h and from 2 ng/mL to 20 μg/mL for 4 and 24 h. In conclusion, UA could display variable redox-active properties, according to different system conditions and/or cellular environment. Moreover, our results suggest that potential neurotoxicological effects of UA should be further studied by additional approaches; for instance, *in vivo* and clinical studies.

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1. Introduction

Therapeutic properties displayed by some species of lichens are related to the production of secondary metabolites such as depsides, depsidones, dibenzofuranes, xanthenes, anthraquinones, aliphatic acids, atranorin and usnic acid (Ingólfsdóttir, 2002). Usnic acid (UA) is one of the most abundant secondary lichen metabolites and has been extensively studied. It has several biological activities, such as antibiotic (Cocchietto et al., 2002), antiviral (Campanella et al., 2002; Scirpa et al., 1999), analgesic and antipyretic (Okuyama et al., 1995), as well as anti-inflammatory properties (Vijayakumar et al., 2000). UA was reported to induce apoptosis of murine leukemia L1210 cells in a dose- and time-dependent manner (Bezivin et al., 2004), and exhibited anti-proliferative action against MCF7 breast cancer cells (Mayer et al., 2005). These results highlighted UA as a potentially new precursor for novel chemotherapic agents.

Already, UA has shown a dualistic effect *in vivo* when compared to *in vitro* data. For instance, it exerts a significant gastroprotective effect in indomethacin-induced gastric ulcer rats, by reducing the formation of reactive species, and therefore, oxidative damage (Odabasoglu et al., 2006); but also it has shown to be a potent hepatotoxic agent that disrupts electron transport in mitochondria, inducing oxidative stress in cells (Han et al., 2004; Joseph et al., 2009). However, there exist many controversies about the toxic activities undertaken by this compound. All in all, better comprehension about UA-induced cellular mechanism would significantly contribute towards a safer therapeutic use.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in the pathogenesis of numerous diseases such as cancer, inflammatory diseases and neurodegenerative disorders (Seifried et al., 2007). Under normal physiological conditions, ROS/RNS participate as intracellular messengers and regulatory molecules. They are tightly regulated by balancing systems formed

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by different antioxidants, antioxidant enzymes, and proteins (Kowaltowski et al., 2009). Non-enzymatic antioxidants coming from diet or other processes regulate oxidative and nitrosative reactions in the body, which prevents oxidative stress by removing both ROS and RNS (Maes et al., 2011).

Main actions of secondary metabolites in biological systems have also been linked to their redox properties. Our group has been studying the redox properties of atranorin, another lichenic secondary metabolite with potential therapeutic activity, similar to that exerted by UA. In that study, atranorin increased lipoperoxidation and displayed general antioxidant activity (Melo et al., 2011). The potential health-promoting effects of naturally occurring compounds are traditionally ascribed to a general antioxidant action (Aravindaram and Yang, 2010). In fact, the properties of UA are generally associated to its widespread antioxidant action, found in most phenolic compounds, since many therapeutic properties attributed to lichen extracts (and to UA itself) are intimately associated to oxidative stress; together with an unbalanced free radical production, mutagenicity, and inflammation (Halliwell and Gutteridge, 2007). However, only few works have studied the potential pro- or antioxidant properties of UA (Carlos et al., 2009; Jayaprakasha and Rao, 2000; Toledo Marante et al., 2003; Valencia-Islas et al., 2007). Interestingly, a number of phenolic compounds and other naturally derived substances, that were initially observed to act as antioxidants in mammalian cells, have later been described to unbalance the cellular redox state towards pro-oxidant states, depending on specific conditions (i.e., higher or lower drug concentrations) (Halliwell, 2008).

Since there are some studies showing that UA prevents oxidative stress by removing both ROS and RNS, we decide to deeper characterize *in silico* the general landscape of interactions of UA with genes/proteins, and compounds generally involved in both redox and NO pathways, by using system biology tools. Thereafter, we performed an *in vitro* characterization of the redox properties of UA against different reactive species, and evaluated its potential effects on cellular viability by using SH-SY5Y cells neuronal like cells since no *in vitro* neurotoxicological reports have been published yet.

2. Materials and methods

2.1. Chemicals

AAPH (2,2′-Azobis(2-methylpropionamidine)dihydrochloride), Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), 2-deoxyribose, glycine, Griess reagent, SNP (sodium nitroprusside), TBA (2-thiobarbituric acid), (4,6-dihydroxypyrimidine-2-thiol), H_2O_2 (hydrogen peroxide), adrenaline, catalase, SOD (superoxide dismutase), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), DMSO (Dimethyl sulfoxide), DCFH-DA (2′,7′-dichlorohydrofluorescein diacetate) and (+)-Usnic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Materials used in cell culture were acquired from Gibco®/Invitrogen (São Paulo, SP, Brazil) and from Rio de Janeiro Cell Bank (BCRJ, Rio de Janeiro, Brazil). The rest of reagents used in this study were of either analytical or HPLC grade. For each assay, UA (20 mg mL^{-1}) was dissolved in DMSO (100%) and serial dilutions were obtained from this stock solution. Therefore, at the highest concentration of UA in these assays (20 μg mL^{-1}), concentration of the vehicle DMSO would correspond to 0.1%.

2.2. Interaction networks of compounds and gene/proteins

In order to develop a model network for gene/protein and UA interaction, we first selected a number of gene/proteins involved in redox and NO-related pathways and then, by using STITCH 2.0 (Kuhn et al., 2008, 2010) we screened the possible protein–protein and protein-compound interactions based on experimental knowledge and database (confident score = 0.4, medium). A list with gene symbols and Ensembl protein IDs is additionally provided (Supplementary Table 1).

The network connected 61 proteins and 5 compounds together, based on their possible interaction through "activation", "catalysis", "binding", "inhibition", and "reaction"; giving rise to a model for UA interactions through redox/NO pathways (MUA network).

2.3. Total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR)

Total reactive antioxidant potential (TRAP) is utilized to estimate the non-enzymatic antioxidant capacity of samples *in vitro*. This method is based on the quenching of luminol-enhanced chemiluminescence (CL) derived from the thermolysis of AAPH as the free radical source (Lissi et al., 1992). Briefly, we prepared AAPH solution, added luminol (AAPH + luminol, radical generating system) and then, we waited for the system to stabilize for 2 h before the first reading. Different concentrations of UA were added and the luminescence produced by the free radical reaction was quantified in a liquid scintillator counter (Wallac 1409, Perkin–Elmer, Boston, MA, USA) for 60 min. The results were transformed in percentage and area under curve (AUC), and calculated by software (GraphPad software.®San Diego, CA; version 5.00) as previously described (Dresch et al., 2009).

Total antioxidant reactivity (TAR) was analyzed by using the same samples utilized for TRAP readings. TAR results were calculated as the ratio of light intensity in absence of samples (I_0)/light intensity right after UA addition. Although TAR and TRAP evaluations are obtained in the same experiment, they represent different observations, since the TAR is more related to the antioxidant quality (reactivity, the scavenging capacity in a short-term period) and TRAP is more related to the antioxidant amount and kinetic behavior (Lissi et al., 1995).

2.4. Hydroxyl radical-scavenging activity

The formation of ·OH (hydroxyl radical) from Fenton reaction was quantified by using the 2-deoxyribose oxidative degradation assay. The principle of the assay is the incubation of 2-deoxyribose together with a hydroxyl radical generation system, which produces malondialdehyde (MDA). This system is then incubated with 2-thiobarbituric acid (TBA), which reacts with MDA and forms a chromophore quantifiable by spectrophotometry (Lopes et al., 1999). Briefly, typical reactions were started by the addition of Fe^{2+} ($FeSO_4$ 6 μM final concentration) to solutions containing 5 mM 2-deoxyribose, 100 mM H_2O_2 and 20 mM phosphate buffer (pH 7.2).

To measure UA antioxidant activity against hydroxyl radicals, different concentrations of UA were added to the system before Fe^{2+} addition. Reactions were carried out for 15 min at room temperature and were stopped by the addition of 4% phosphoric acid (v/v) followed by 1% TBA (w/v, in 50 mM NaOH). Solutions were boiled for 15 min at 95 °C, and then cooled at room temperature. The absorbance was measured at 532 nm and results were expressed as percentage of TBARS formed.

2.5. Nitric oxide (NO·) scavenging activity

Nitric oxide was generated from spontaneous decomposition of sodium nitroprusside in 20 mM phosphate buffer (pH 7.4). Once it is generated, NO interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction (Basu and Hazra,

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