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# Oleuropein shows copper complexing properties and noxious effect on cultured SH-SY5Y neuroblastoma cells depending on cell copper content

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ABSTRACT

The secoiridoid oleuropein is a non-flavonoid polyphenol, found in the fruit, leaves and food derivatives from *Olea europea*. Like other polyphenols it shows a very low toxicity towards healthy tissues and a protective action against cancer or neurodegeneration, but its mechanism of action is not yet understood. In the present report we have used optical and ESR spectroscopy as well as molecular modelling to demonstrate that oleuropein forms a complex with the transition metal copper; the dysmetabolism of this metal is suspected to be involved in both cancer and neurodegeneration. Experiments carried out with the aglycon derivative of oleuropein, produced by β-glycosidase treatment of oleuropein glycoside, showed that also the aglycon forms copper-complexes, but with different spectroscopic features than the glycosidic form. Molecular modelling analysis confirmed that two oleuropein molecules (glycosidic or aglycon forms) can easily coordinate one copper ion. The relationship between oleuropein and copper was investigated in SH-SY5Y human neuroblastoma cells. When cells were depleted of copper by treatment with the copper chelator triethylenetetramine (Trien), that binds copper with higher affinity than oleuropein, oleuropein was less toxic than to copper-adequate cells. Conversely, incubation of SH-SY5Y cells with exogenous copper sulphate increased cell susceptibility to oleuropein. Furthermore SH-SY5Y cells differentiated by retinoic acid pre-treatment showed a lower level of copper, and were more resistant to oleuropein treatment. The oleuropein aglycon was not toxic towards SH-SY5Y cells. In conclusion, the copper-oleuropein complex may be involved in the toxicity of oleuropein towards tumour cells, depending on their copper level.

## 1. Introduction

Oleuropein, the ester of hydroxytyrosol (3,4-dihydroxyphenylethanol) with glycosylated elenolic acid, is a phenolic compound belonging to the group of secoiridoids, found in the leaves, fruits and food derivatives from the olive tree *Olea europea*. Epidemiological *in vivo* and *in vitro* studies report that secoiridoids from olive trees are beneficial for human health [1]. They show a protective action in several models of cardiovascular diseases, inflammation, viral or microbial infection, hypoglycemia, skin illnesses, osteoporosis and liver steatosis induced by high-fat diets [2–5]. Furthermore, epidemiological studies indicate that diets containing olive oil may delay cognitive decline, and reduce the severity of symptoms of neurodegenerative diseases such as Alzheimer's disease [6,7]. On this basis, the use of olive oil phenols in the prevention of neurodegenerative diseases was envisaged [8,9].

As far as anticancer properties are concerned, oleuropein has shown antiproliferative and pro-apoptotic effects on leukemia tumor cells, colorectal carcinoma cells, and breast cancer cell lines, and also *in vivo* cancer models [10–15]. Furthermore, since oleuropein showed high cell membrane permeability in breast-cancer-cell line SKBR3 [16] and is not toxic for healthy tissues [17] it may act as an anti-cancer molecule.

Several molecular mechanisms might explain the beneficial effects of these compounds in processes involved in neurodegeneration and cancer. Oleuropein contrasts *in vitro* aggregation of the Aβ42 peptide and protects cultured cells and model organisms against the toxic effects of Aβ-aggregates [18,19]. Furthermore, oleuropein also prevents Tau protein fibrillation *in vitro* [20]. Recently, oleuropein was found to be an efficient poison of topoisomerase II, a well-known target for anticancer drugs, by increasing enzyme-mediated DNA cleavage [21]. In addition, it has been suggested that the antiproliferative effect of oleuropein on a mesothelioma cell line may proceed through alteration of calcium homeostasis [22], and by authophagy stimulation *via* AMPK activation and mTOR inhibition [23].

Possible antioxidant activity have been ascribed to oleuropein or to its derivative hydroxytyrosol. They may act directly as radical

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scavengers [24] or, indirectly, they may modulate the antioxidant defence through the activation of nuclear factor (erythroyd-derived-2)-like 2 (Nrf2) and its target genes, including γ-glutamylcysteine synthetase, heme oxygenase-1 and NAD(P)H:quinone oxidoreductase 1 [2,25,26]. However, oleuropein and hydroxytyrosol can also give rise to pro-oxidant activity, both *in vitro* [27] and in cancer cell models [28], through the production of hydrogen peroxide. Whether its action can be linked to autoxidation or interaction with cell culture media components is still debated [29].

Interestingly, oleuropein can form complexes with transition metals such as iron [27], and such complexes may catalyze the production of reactive oxygen species, for instance through a Fenton-type reaction. Chelation of transition metals is one of the mechanisms through which polyphenols may act as either antioxidants or pro-oxidants. Polyphenols such as catechins or curcumin are known to bind copper or iron; in some conditions the resulting complexes decrease the redox activity of the metal and silence their possible redox-related toxicity, in other conditions complex formation promote their pro-oxidant activity, depending on the type of complex formed and on other components in their microenvironment [30–32].

Copper homeostasis deregulation is a feature of neurodegenerative diseases and tumours. The major neurological diseases, such as Alzheimer's disease, Parkinson's disease, prion diseases, or motor neuron diseases, all seems to be connected to this metal, in one way or another [33–37]. Cancer patients show higher copper levels in serum and in tumour tissues in comparison to healthy individuals [38,39]. Furthermore, copper seems to be involved in cell proliferation and cancer development; it is essential for angiogenesis, for the function of growth factors and other molecules involved in signalling pathways important for tumour growth and survival [40–43]. Deranged copper homeostasis leads to its uncontrolled redox activity, which elicits the production of reactive oxygen species and in turn damage to macromolecules, organelles and cells [44].

In the present report, we sought to investigate by optical spectroscopy, electron spin resonance (ESR) spectroscopy, and by molecular modelling whether oleuropein may form complexes with copper *in vitro* and whether copper or oleuropein may reciprocally modulate their toxicity towards the neuroblastoma cell line SH-SY5Y. We here show that oleuropein is able to form complexes with copper, both as the intact glycoside and in the aglycon form. By modelling analysis it has been demonstrated how two oleuropein molecules and one copper ion is likely to be assembled in a complex, and that a similar copper complex can be formed by two oleuropein aglycones. We also report that the cytotoxic effect of oleuropein glycoside depends on the intracellular copper levels, suggesting a novel mechanism for the anti-cancer effect of seicoiridoid polyphenols. Conversely, the aglycon form was not to be toxic to tumour cells.

## 2. Materials and methods

### 2.1. Materials

Oleuropein, 90% pure, was from Sequoia Research Products Ltd, UK. Triethylene tetramine tetrahydrochloride (Trien), DMEM F12 medium, $CuSO_4$, β-glucosidase from almonds, retinoic acid, 2′,7′-Dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) and propidium iodide were from Sigma-Aldrich (Saint Louis, MO, USA). Inactivated fetal calf serum, penicillin/streptomycin and L-glutamine were from Eurobio (Les Ulis, France), while (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium), inner salt (MTS) was from Promega, (Madison, WI, USA). The anti-GAPDH antibody SC32233 and the primary monoclonal antibody against GAP43 (clone 91E12) was from Santa Cruz Biotecnology (Dallas, TX, USA), whereas the anti-mouse secondary antibody was from Sigma-Aldrich. The Protease Inhibitor Cocktail was from Roche (Basel, Switzerland).

### 2.2. Optical spectroscopy

Optical spectra were carried out by a Beckman Coulter DU800 spectrophotometer. Oleuropein (20 mM stock solution) was dissolved in phosphate-buffered saline (PBS, 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4). $CuSO_4$ was dissolved at 25 mM concentration in $ddH_2O$. Oleuropein and $CuSO_4$ were added to PBS in the cuvette at the final concentrations reported in the figure captions.

### 2.3. ESR spectroscopy

Room temperature ESR spectra were recorded in $ddH_2O$ with an ESP 300 spectrometer (Bruker, Rheinstetten, Germany), using flat ESR cells in a $TE_{102}$ cavity operating at 9.79 GHz. ESR settings were: 800 G scan width, 10 G modulation, 20 mW microwave power, 64 ms time constant and 240 s scan time. To improve the signal-to-noise ratio, four consecutive scans were accumulated.

### 2.4. Preparation of oleuropein aglycon

Oleuropein deglycosilation was performed by incubation of 20 mM oleuropein glycoside with 25 Units of β-glucosidase, for 48 h at 37 °C, under shaking, followed by filtration through a Centricon Centrifugal Filter Units (cut off 10 kDa) (Amicon, MA, USA).

### 2.5. Cell cultures

Human neuroblastoma SH-SY5Y cells (European Collection of Cell Cultures, Salisbury, UK) were grown in DMEM F12 medium, supplemented with 15% inactivated fetal calf serum, 1% v/v penicillin/streptomycin; 1% v/v of L-glutamine at 37 °C in an atmosphere of 5% $CO_2$ in air. 24 h before treatments, cells were seeded at a density of $2 \times 10^5$ cells/ml.

### 2.6. Treatments

Cells were treated with oleuropein (from a 20 mM stock solution, in culture medium) for 24 h at the concentrations indicate in the figure legends. In some experiments, cells were pre-treated for 3 days with the specific copper chelator triethylene tetramine tetrahydrochloride (Trien, 125 μM final concentration) as previously described [45]. Cells were detached by trypsin treatment, seeded, and treated the day after with both Trien and oleuropein for an additional 24 h. Where indicated, cells were also treated with 150 μM $CuSO_4$, with or without 200 μM oleuropein. When required SH-SY5Y cells were differentiated by treatment with retinoic acid (final concentration 30 μM; from a stock solution in DMSO) [46]. The treatment was repeated every second day, for a total of 2 weeks. Differentiation was verified as described below. Differentiated cells were seeded at a density of $4 \times 10^5$ cells/ml, and treated with oleuropein 24 h later.

### 2.7. Measurement of cell viability

Viability of the cells was estimated by measuring their capability to reduce MTS to formazan. Cells were grown in 96-well plates and the formation of formazan was followed at 490 nm by a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA) [44,45].

### 2.8. Measurement of cell copper content

For the assay of the intracellular copper content cells were detached using a cell scraper, centrifuged, and the cell pellet washed with PBS. Then 65% nitric acid (1:1 v:v) was added to obtain whole cell lysates, and the samples were left at room temperature for at least 1 week to achieve complete digestion. The copper content was measured by atomic absorption spectrophotometry, using an AAnalyst 300 Perkin

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