Suillus luteus methanolic extract inhibits proliferation and increases expression of p-H2A.X in a non-small cell lung cancer cell line

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ABSTRACT

Methanolic extract of Suillus luteus was previously shown to inhibit proliferation of colon cancer cells with mutant p53. The effect of the same extract was further investigated here in a wildtype (wt) p53 non-small cell lung cancer cell line. Treatment with the extract increased the levels of p-H2A.X and the number of p-H2A.X foci/cell, indicating a possible increase in deoxyribonucleic acid (DNA) damage. Nevertheless, it did not cause alterations in wt p53 levels nor in programmed cell death. The extract caused inhibition of cellular proliferation and an increase in the % of cells in the G0/G1 phase of the cell cycle. In conclusion, even though there is evidence of DNA damage being caused by this extract, there is no induction of cell death in this p53 wt cell line.

1. Introduction

Mushrooms and their extracts are generally well-tolerated with few, if any, side-effects. Anti-tumor effects of mushrooms and their extracts appear to hold potential health benefits (Roupas, Keogh, Noakes, Margetts, & Taylor, 2012). These benefits have been attributed to their polysaccharide content (beta-glucans or polysaccharide–protein complexes) and evidences for the anti-tumor role of low molecular weight compounds (e.g., phenolic acids) are scarce.

Suillus luteus (L.: Fries) Gray is a common edible mushroom and several studies have been carried out in order to elucidate the chemical composition of this species as well as its antioxidant potential (Pinho et al., 2008; Reis et al., 2011; Ribeiro, Pinho, Andrade, Baptista, & Valentão, 2009; Ribeiro et al., 2006). In particular, the methanolic extract of this mushroom...
revealed the presence of protochatechuic and cinnamic acids (Reis et al., 2011).

The cytotoxic potential of *S. luteus* has also been investigated; its methanolic extract was shown to present cytotoxic activity against the L1210 cell line (murine lymphocytic leukemia) (Tomasi, Lohezic-Le Devehat, Sauleau, Bezivin, & Bous-tie, 2004). In addition, treatment of a human melanoma cell line (SK-MEL-1) with sulforamide, a phytosphingosine-type ceramide isolated from *S. luteus*, caused cell growth inhibition (Leon et al., 2008). More recently, it was shown that a metha-nolic extract of *S. luteus* presented cytotoxicity towards several human tumor cell lines, namely MCF-7 (breast), NCI-H460 (non-small cell lung cancer), AGS (gastric) and HCT-15 (colon) (Santos et al., 2013). Most interestingly, this methano-lic extract, which was not cytotoxic towards primary cultures of porcine hepatocytes, reduced the proliferation of HCT-15 cells, caused an increase in the % of cells in the G1 phase of the cell cycle and increased the levels of p-H2A.X which was suggestive of DNA damage in these cells. Nevertheless, those studies were carried out in cells with mutant p53 and it was not known if the effect would be the same in cells present-ing wt p53. This was particularly interesting since p53 is considered the guardian of the genome, providing an important link between DNA damage and apoptosis (Wang, 2001). Therefore, in the present study, the effect of *S. luteus* metha-nolic extract was further investigated in a wt p53 non-small cell lung cancer cell line (NSCLC).

2. Materials and methods

2.1. Extracts

Methanolic extract from *S. luteus* (L.: Fries) Gray (edible mush-room) had been previously prepared from samples collected in Bragança (Northeast Portugal) in autumn 2009, as previously published (Santos et al., 2013). Extracts were kept in dimethyl sulfoxide (DMSO) and stored at −20 °C.

2.2. Cell culture

The non-small cell lung cancer cell line NCI-H460 was rou-tinely cultured in RPMI-1640 with Ultraglutamine I medium (Lonza) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (PAA Laboratories) and incubated at 37 °C in a humidified atmosphere containing 5% CO2.

2.3. Cellular proliferation analysis using the BrdU incorporation assay

Cells were plated at 1.5 × 10^5 per well in 6-well plates and incubated for 24 h. Cells were then treated with complete medium (blank), with *S. luteus* methanolic extract (at 30.33 µg/mL (corresponding to the previously determined concentration that inhibited growth in 50% - GI50 (Santos et al., 2013)), or at 60.66 µg/mL (corresponding to 2 × GI50) or with DMSO (solvent of the extract, at the highest concentration tested corresponding to 2 × GI50). Following 24 h treatment, cells were given a 1 h pulse of bromodeoxyuridine (BrdU) (10 µM, Sigma), washed and fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline solution (PBS). Cytospins were prepared and incubated in 2 M HCl for 20 min. Following incubation with mouse anti-BrdU (1:10, Dako), cells were fur-ther incubated with fluorescein-labeled rabbit anti-mouse antibody (1:100, Dako), as previously described (Palmeira et al., 2010). Slides were mounted in Vectashield Mounting Media with DAPI (Vector Laboratories). Cells were observed in a DM2000 microscope (LEICA) and a semi-quantitative evaluation of proliferation levels was obtained by counting a minimum of 500 cells per slide.

2.4. Cell cycle profile analysis

For the analysis of cell cycle profile, NCI-H460 cells were plated at 1.5 × 10^5 cells/well in 6-well plates and incubated for 24 h. Cells were then incubated with complete medium (blank), with *S. luteus* methanolic extract at the GI50 or at 2 × GI50 concentrations or DMSO (control, corresponding to the highest concentration used). Following 24 or 48 h incubations, cells were fixed in ice-cold 70% ethanol and kept at 4 °C for at least 12 h. Prior to analysis, cells were incubated with propidium iodide (5 µg/mL) and RNase A in PBS (100 µg/mL) for 30 min on ice. Cellular DNA content was analyzed using a FACS Calibur (BD Biosciences) flow cytometer (Neves et al., 2011; Vasconcelos et al., 2000). Analysis of cell cycle profile was carried out using the FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR, USA) after cell debris and aggregates exclusion.

2.5. Programmed cell death analysis by TUNEL assay

Cells were plated at 7.5 × 10^4 per well in 12-well plates and incubated for 24 h. Cells were then further treated with complete medium (blank), with *S. luteus* methanolic extract (at its GI50, or 2 × GI50), with DMSO (solvent of the extract, at the highest concentration tested corresponding to 2 × GI50), or with 100 nM of doxorubicin (used as positive control).

Following a 24 or 48 h treatment, cells were fixed in 4% PFA in PBS and further stored in PBS at 4 °C until analysis. Cyto-spins were prepared and cells were permeabilized in ice-cold 0.1% Triton X-100 in 0.1% sodium citrate for 2 min. Programmed cell death was assessed using the “in situ cell death detection, fluorescein kit” (Roche, Basel Switzerland) according to manufacturer’s instructions and, as previously de-scribed, using a 1:20 enzyme dilution in the TUNEL reaction mix (Lima, Martins, Guimarães, Sambade, & Vasconcelos, 2006). Slides were mounted in Vectashield Mounting Media with DAPI (Vector Laboratories). Cells were observed in a DM2000 microscope (LEICA) and a semi-quantitative evaluation of the levels of programmed cell death was performed by counting a minimum of 500 cells per slide.

2.6. Protein expression analysis

Cells were plated at 1.5 × 10^5 per well in 6-well plates and incubated for 24 h. Cells were then treated with complete medium (blank) with *S. luteus* methanolic extract (at its GI50 or 2 × GI50), or with DMSO (control, corresponding to both concentrations used). Following 48 h incubation, cells were lysed in Winman’s buffer (1% NP-40, 0.1 M Tris–HCl pH 8.0, 0.15 M
NaCl and 5 mM ethylenediamine tetraacetic acid (EDTA) with EDTA-free protease inhibitor cocktail (Roche). Protein quantification was carried out with the DC Protein Assay kit (Bio-Rad). Proteins (30 μg) were electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitro-cellulose membrane (GE Healthcare). Membranes were incubated with the following primary antibodies: p53 (1:200, NeoMarkers), p-H2A.X (1:200, Santa Cruz Biotechnology) and actin (1:2000, Santa Cruz Biotechnology). They were then incubated with the respective secondary antibodies – horseradish peroxidase (HRP) conjugated (1:2000, Santa Cruz Biotechnology). The signal was detected with the Amersham ECL kit (GE Healthcare) in Hyperfilm ECL (GE Healthcare) using the Kodak GBX developer and fixer (Sigma), as previously described (Lima, Seca, Soares, Nascimento, & Vasconcelos, 2011). Densitometric analysis of the bands obtained in each film was further carried out using the software Quantity One – 1D Analysis (Bio-Rad).

2.7. Immunofluorescence for p-H2A.X

Cells were plated at 1.5×10^5 per well in 6-well plates and incubated for 24 h. Cells were then treated with complete medium (blank), with S. luteus methanolic extract (at its GI\textsubscript{50} or 2×GI\textsubscript{50}) or with DMSO (control). Following 48 h incubation, cells were fixed in 4% PFA and after cytopsin, incubated for 10 min in 50 mM of ammonium chloride in PBS. Cells were then permeabilized in ice-cold 0.2% Triton X-100 in PBS for 10 min. After blocking with 2% bovine serum albumin (BSA) in PBS for 20 min, slides were incubated for 1 h at room temperature with a mouse antibody for phospho-histone H2A.X (Ser139) (1:450, Milipore) diluted in 2% BSA in PBS. Cells were then washed with 2% BSA in PBS and incubated with a secondary antibody (mouse IgG conjugated to fluorescein isothiocyanate (FITC), 1:100; Dako) for 1 h at room temperature. Slides were mounted in Vectashield mounting media (ZIESS Axio Imager.Z1 coupled with ApoTome Imaging System). Images were decompressed with Irfanview (4.35, Irfan Skiljan, Vienna, Austria) and analyzed with ImageJ (version 1.46r, http://rsbweb.nih.gov/ij/index.html), using a module written by Dr. Niklas Schultz at GMT Department, Stockholm University, Sweden (Markova, Schultz, & Belyaev, 2007). At least 100 cells for each treatment were analyzed.

2.8. Statistical analysis

Statistical significance was determined with a two-tailed paired Student’s t-test except for data concerning the expression of p-H2A.X protein by Western blot, in which the unpaired t-test was used. Indicates p < 0.05 when compared to DMSO treatment.

3. Results and discussion

The chemical characterization of S. luteus was previously reported (Reis et al., 2011). This mushroom presented α-tocopherol (19.14 μg/100 g), β-tocopherol (15.34 μg/100 g), γ-tocopherol (366.77 μg/100 g), δ-tocopherol (78.51 μg/100 g), mannitol (1.29 g/100 g), trehalose (1.35 g/100 g), polyunsaturated fatty acids (52.75%, with linoleic acid – 52.31% – as the main fatty acid), monounsaturated fatty acids (32.93%, with oleic acid – 31.24% – as the main fatty acid), and saturated fatty acids (14.32%, with palmitic acid – 10.57% – as the main fatty acid) (Reis et al., 2011). In particular, the methanolic extract used in the present work revealed the presence of protocatechuic acid (0.47 mg/100 g dry weight) and cinnamic acid (0.41 mg/100 g) (Fig. 1).

S. luteus methanolic extract had previously been reported to exhibit cytotoxic activity against a panel of human tumor cell lines (Santos et al., 2013). In addition, the mentioned study focused on the effect of this extract on the HCT-15 colon cancer cells, which have mutant p53 (O’Connor et al., 1997). Results from that work indicated that the effect of this mushroom was p53 independent. In the present study, we intended to further investigate the effect of this methanolic extract in a cell line with wildtype (wt) p53. Therefore, we selected a cell line (NCI-H460) with wt p53 and in which the extract was quite potent, presenting a GI\textsubscript{50} concentration of 30.3 μg/mL. Two concentrations of the extract were used throughout this study: the previously published GI\textsubscript{50} (Santos et al., 2013) and twice this concentration (2×GI\textsubscript{50}).

3.1. Effect of S. luteus methanolic extract in p-H2A.X, a protein involved in DNA damage

It was previously suggested that S. luteus methanolic extract induced DNA damage in colon carcinoma cells (Santos et al., 2013). Therefore, in the present study it was intended to confirm if DNA damage was possibly induced in a different cell line (NCI-H460), by analyzing the protein levels of p-H2A.X by Western blot and the DNA repair foci formation by immunofluorescence for p-H2A.X. Results showed a statistically significant increase in the expression levels of p-H2A.X following treatment of the NCI-H460 cells with the S. luteus methanolic extract at its 2×GI\textsubscript{50} concentration (Fig. 2A). Furthermore, an
Fig. 2 – Increase in DNA damage in NCI-H460 cells treated with the methanolic extract of S. luteus. Cells were treated for 48 h with medium (blank), GI50 or 2 × GI50 of S. luteus methanolic extract or with DMSO. (A) p-H2A.X expression analyzed by Western blot. Actin was used as loading control. Image is representative of 3 independent experiments. The respective densitometric analysis is expressed after normalization of the values obtained for each protein with the values obtained for actin (relative to control cells) and represent the mean ± SE from 3 independent experiments. (B) p-H2A.X foci analyzed by immunofluorescence. The upper panel shows representative fluorescence microscopy images of p-H2A.X foci (green) and DAPI stained nuclei (blue). Lower panel shows the quantification of these foci and are the mean ± SE of three independent experiments. Bar corresponds to 20 µm. *p ≤ 0.05 between each treatment and DMSO control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
increase in the total number of p-H2A.X foci/cell was also verified in NCI-H460 cells treated with the 2 GI50 concentration of S. luteus methanolic extract (when compared to control cells, Fig. 2B). Together, these results suggest that S. luteus methanolic extract also causes DNA damage in this cell line.

3.2. Effect of the S. luteus methanolic extract in p53 protein expression

It was previously shown (Santos et al., 2013) that the S. luteus methanolic extract had surprisingly caused an increase in p53 levels of the HCT-15 cells (although p53 is mutated in this cell line). In the current study it was intended to further verify if, when p53 is functional, the extract caused cellular alterations in p53 wt levels, which could lead to p53 dependent increase in cell death and cell cycle arrest. Results (Fig. 3) showed no alteration in the wt p53 expression levels following treatment with the two concentrations of the methanolic extract, thus confirming that the activity of the methanolic extract of S. luteus does not alter wt p53 levels.

3.3. Effect of S. luteus methanolic extract in programmed cell death

It was also intended to verify if the methanolic extract of S. luteus increased programmed cell death in a cell line presenting functional p53, since p53 provides an important link between DNA damage and apoptosis (Wang, 2001). Therefore, the levels of programed cell death were analyzed in NCI-H460 cells with the TUNEL assay, 24 and 48 h following treatment.

<table>
<thead>
<tr>
<th>Time</th>
<th>Blank</th>
<th>DMSO</th>
<th>GI50</th>
<th>2xGI50</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>1.0 ± 0.4</td>
<td>0.9 ± 0.2</td>
<td>1.2 ± 0.4</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>48 h</td>
<td>1.2 ± 0.1</td>
<td>0.8 ± 0.03</td>
<td>1.2 ± 0.06</td>
<td>1.4 ± 0.1</td>
</tr>
</tbody>
</table>

Results, determined by TUNEL assay, are the mean ± SE of at least 3 independent experiments, performed in duplicate. Doxorubicin was used as a positive control, showing a percentage of programmed cell death of 1.4 ± 0.5% and 12.6 ± 2.8% following 24 or 48 h treatment, respectively.
Results (Table 1) showed no alterations in the levels of TUNEL positive labeled cells following treatment with the S. luteus methanolic extract when compared with blank or control cells. This was in agreement with what had been published in the above mentioned study with cells having mutant p53 (Santos et al., 2013), which confirms that this extract does not interfere with programmed cell death even in the presence of wt p53.

3.4. Effect of S. luteus methanolic extract in cellular proliferation and cell cycle profile

In order to verify if the S. luteus methanolic extract interfered with the proliferation of NCI-H460 cells, the levels of BrdU incorporation were analyzed following 24 and 48 h treatment (Fig. 4). Results clearly indicate that cells treated with the extract have lower proliferation than blank or control cells. Moreover, this effect seemed to be dose- and time-dependent, being statistically significant for the higher concentration of extract used, at both time points analyzed. Indeed, 24 h following incubation, the proliferation levels decreased from 42% (blank) or 37% (control) to 32% and 20%, following treatment with GI50 and 2 x GI50, respectively. At 48 h, the decrease was from 21% (blank) or 26% (control) to 12% and 8%, following treatment with GI50 and 2 x GI50, respectively, showing clearly the mentioned behavior.

Since a decrease in the proliferation levels of NCI-H460 cells was observed following treatment of the extract, possible alterations in their cycle profile were analyzed by flow cytometry (Fig. 5). As expected, DMSO treatment caused no alteration in the cell cycle profile. Treatment with the extract caused an increase in the percentage of cells in G0/G1 from 53% and 51% in blank and control cells, respectively, to 64% and 75% in cells treated with the GI50 and 2 x GI50 concentrations, respectively. This was associated with a decrease in the % of cells in the S phase of the cell cycle, from 34% and 37% in blank and control cells, respectively, to 25% and 16% in cells treated with the GI50 and 2 x GI50 concentrations, respectively.

These effects caused by the S. luteus methanolic extract in the NCI-H460 cellular proliferation and cell cycle profile were in agreement with those previously published for the HCT-15 cells which presented mutant (mut) p53 (Santos et al., 2013), further indicating that p53 was not relevant for the mechanism of action of this extract.

In summary, this study confirmed that the methanolic extract of S. luteus induces an increase in p-H2A.X and in the number of p-H2A.X foci/cell, indicating an increase in DNA damage in the NCI-H460 cell line. In addition, this extract does not increase p53 levels, does not induce cell death but reduces proliferation and increases the % of cells in the G0/G1 phase of the cell cycle. These results are in agreement with the previously published ones in a cell line with mut p53 (Santos et al., 2013), thus indicating that the presence of cellular wt p53 does not alter the mechanism of action of this extract. Nevertheless, it is possible that treatment periods longer than 48 h would alter p53 levels and programmed cell death levels. The present study also gives evidence for the bioactive potential of mushroom extracts enriched in phenolic acids (in this case, protocatechuic and cinnamic acids, Reis et al., 2011) highlighting the role of these low molecular weight compounds and not only of polysaccharides that have been extensively reported as anti-tumor molecules in mushrooms.

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