Agarikon.1 and Agarikon Plus Affect Cell Cycle and Induce Apoptosis in Human Tumor Cell Lines

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Dr Myko San – Health from Mushrooms
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INTRODUCTION

• Continuation of the study on 6 blended mushroom products and 3 single extracts on 4 human tumor cell lines (Durgo, Jakopovich 2013)

• Neutral Red and MTT proliferation assays demonstrate that blended extracts cause increased tumor cell membrane and mitochondria damage

• This study concentrates on the mechanisms; effects on the cell cycle and inducing apoptosis
Medicinal mushroom metabolites can interfere and modulate all processes related to the 8 steps of carcinogenesis (Petrova 2012):

- inflammation
- cancer cell proliferation
- adhesion
- apoptosis
- angiogenesis
- gene expression
- invasiveness
- metastasis
Apoptosis - programmed cell death - is a crucial tumor suppression mechanism

- eliminates cells with extensive DNA damage (potentially leading to cancer)

- differentiation - cell growth – apoptosis balance
Purpose of the Study

Investigate:
A. proliferation (cell cycle disturbance)
B. induction of apoptosis

– medicinal mushroom extract blends Agarikon Plus and Agarikon.1
– concentration response
– 24 and 48 hour response
– 2 human tumor cell lines: H460 (lung carcinoma) and Caco-2 (colon carcinoma)
– Camptothecin, referent antitumor compound; cytotoxic dose 10 μM used
MATERIALS AND METHODS

Cell lines
- H460 (large cell lung carcinoma)
- Caco-2 (colorectal adenocarcinoma)

Tested extract blends
- Agarikon.1 tablets
- Agarikon Plus

Experimental methods
- Proliferation Assay by MTT
- Cell Cycle Analysis by flow cytometry
- Annexin V Assay for Apoptosis Induction Detection
- Western Blot Analysis
TESTED PRODUCTS

- Agarikon.1 tablets (AG.1)
- proprietary mushroom extract blend from Dr Myko San company
- *Ganoderma lucidum, Lentinus edodes, Grifola frondosa, Pleurotus ostreatus, Agaricus brasiliensis*
- registered med. mushroom supplement
- recommended treatment dose: ~0.1 g/kg bodyweight per day of soluble polysaccharides
• Agarikon Plus extract blend (AG+)

• proprietary mushroom extract blend from DMS

• 10 medicinal mushroom species (inc. *G. lucidum*, *L. edodes*, *G. frondosa*, *P. ostreatus*, *A. brasiliensis*)

• in liquid form

• Recommended treatment dose: ~0.16g/kg BW per day of soluble polysaccharides
1 Proliferation Assay

• Cells cultured as monolayers, plated in parallel on day 0, at $3 \times 10^3$ cells/well (H460) and $7 \times 10^3$ cells/well (Caco-2), depending on doubling times

• AG.1 and AG+ added at 0.001, 0.01, 0.1, 1 and 10 mg/ml concentrations (stock solution for both 40 mg/ml, and $4 \times 10^{-3}$ M/DMSO for camptothecin)

• We used MTT assay to evaluate cell growth rate after 72 hours (absorbance was measured at 570 nm)
2 Cell Cycle Analysis

- seeded at $1 \times 10^5$ cells/well (H460) and $2 \times 10^5$ cells/well (Caco-2), depending on the doubling times

- After 24 hours, AG.1 and AG+ applied at concentrations 0.1 mg/ml and 1 mg/ml; camptothecin (10 μM) for positive control

- After the incubation period, cells were trypsinized, washed with Phosphate Buffer Saline (PBS); stained with propidium iodide (PI) and analyzed on FACScalibur flow cytometer

- Ratio of cells in each cell cycle phase was determined by analyzing the DNA histograms using ModFit LT™ software
3 Annexin V Assay for Apoptosis Induction Detection

- same concentrations used; 0.1 and 10 mg/ml
- cell populations were gated into regions corresponding to live, early apoptotic and late apoptotic/necrotic cells

<table>
<thead>
<tr>
<th>Annexin V</th>
<th>PI</th>
<th>Cell Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>Live cells</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>Early apoptotic</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Late apoptotic /necrotic</td>
</tr>
</tbody>
</table>
4 Western Blot Analysis

• mushroom extracts (0.1 and 1 mg/ml) were added to well plates after 24 hours

• total proteins were measured using BCA Protein Assay Reagent, separated by SDS-polyacrylamid gel electrophoresis and transferred to PVDF membrane → probing with anticaspase 3, anti-p53, and anti-p21 primary antibodies

• equal loading confirmed using anti-tubulin primary antibody
RESULTS

Proliferation Assay

• Agarikon Plus (strong effect at 10 mg/ml, $\text{GI}_{50} \approx 2-3 \text{ mg/ml}$) and Agarikon.1 inhibit the growth of both tumor cell lines

• H460 cells are more resistant to Agarikon.1 (approaching $\text{GI}_{50}$ above 10 mg/ml mass concentration)

<table>
<thead>
<tr>
<th>Test agent</th>
<th>Cell lines</th>
<th>$\text{GI}_{50}^{a}$ (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caco-2</td>
<td>NCI-H460</td>
</tr>
<tr>
<td>Agarikon Plus</td>
<td>1.9 ± 0.1</td>
<td>3.4 ± 1</td>
</tr>
<tr>
<td>Agarikon.1</td>
<td>1.6 ± 0.3</td>
<td>$\geq 10$</td>
</tr>
</tbody>
</table>

$^{a}$ $\text{GI}_{50}$: growth inhibition 50 - the concentration that causes 50% growth inhibition
• Concentration-response curves showing growth inhibition of H460 and Caco-2 cell in vitro after 72 hours after adding Agarikon.1, Agarikon Plus, and camptothecin.
• expected Camptothecin curve confirms valid measurements (positive control)
Cell Cycle Analysis

- Distribution of **H460** cells by cell cycle phase: G0/G1, S, G2/M; and sub G1 (dead/apoptotic cells)
- treated with Agarikon Plus, Agarikon.1 (at 0.1 and 10 mg/ml), camptothecin at 10 μM.
- measured by flow cytometry at 24 and 48 hours
• no significant influence on cell cycle
• moderate increase in apoptotic/dead cells for AG+ at higher concentrations
• AG+ and AG.1 (1mg/ml) induce accumulation of cells in G1, reduction in S, increase in sub G1 (apoptotic/necrotic)
• AG+ (1mg/ml): reduced G1, increased S
• no significant sub G1 influence
• AG+, AG.1 (1 mg/ml) reduced G1, increased S phase
• no significant sub G1 influence
Apoptosis Induction Detection by Annexin V Assay

- Ratio of H460 cells in early or late apoptosis, obtained by co-staining with FITC-labeled annexin V and propidium iodide (PI) and analyzed by flow cytometry.
• AG+ (1 mg/ml): moderate increase in early apoptotic cells
AG+ (1 mg/ml): larger increase in late apoptotic/necrotic cells
• no significant influence, AG+ (1mg/ml) moderate early apoptotic cell increase
• AG+ (1 mg/ml): moderate increase in induced early apoptosis
Apoptosis induction detection by caspase-3 cleavage assessment

- Appearance of the 17-kDa subunit (caspase-3 p17) – a major cleaved product of the 32-kDa zymogen pro-caspase-3 - confirms caspase-3 activation which marks the induction of apoptosis.
The effect of AG+ and AG.1 on cleavage of procaspase-3 in H460 cells (24; 48 hours)

A. AG+ at 0.1 mg/ml (lanes 1;6), 1 mg/ml (lanes 2;7).
B. AG.1 at 0.1 mg/ml (lanes 3;8), 1 mg/ml (lanes 4;9)
C. Camptothecin at 10μM (lanes 5;10)
The effect of AG+ and AG.1 on cleavage of procaspase-3 in **Caco-2** cells (24; 48 hours)

A. AG+ at 0.1 mg/ml (lanes 1;6), 1 mg/ml (lanes 2;7).
B. AG.1 at 0.1 mg/ml (lanes 3;8), 1 mg/ml (lanes 4;9)
C. **Camptothecin** at 10μM (lanes 5;10)
Influence of AG+ and AG.1 on p53 and p21 protein expression

- Agarikon.1, in both concentrations, induced mild p53 protein expression in H460 after 24 hours (A; lanes 3 and 4)
• Both Agarikon Plus (lane 2) and Agarikon.1 (lane 4) at the concentration of 1 mg/ml induced a moderate increase in the expression of both p53 and p21 after 24 hours in H460
• In Caco-2 cells (B), only minor upregulation of p21 protein expression is detected after 48 hour treatment.
CONCLUSIONS

• Agarikon Plus and Agarikon.1 possess antiproliferative, mainly cytostatic activity, on H460 and Caco-2 cells, in the concentration range 1-10 mg/ml

• Both induce cell cycle perturbations, by delaying the progress through the G1 and S phase

• This points to disturbances occurring before or during DNA replication (confirmed by increase in both p53 and p21 protein expression)
CONCLUSIONS (II)

- Although a modest induction of early (after 24 hours) and late (48 hours) apoptosis was noticed by annexin V test, no processing (cleavage) of caspase-3 was detected.
- More-pronounced antiproliferative activity (MTT) of tested agents towards Caco-2 line at maximal concentration (10 mg/ml) points to a non-specific cytotoxic effect.
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The percentage of growth (PG) expressions:

1. For \((A_t - A_0) \geq 0:\)
   \[ PG = 100 \times \frac{(A_t - A_0)}{(A_c - A_0)} \]

2. For \((A_t - A_0) < 0:\)
   \[ PG = 100 \times \frac{(A_t - A_0)}{A_0} \]

\(A_0\) … avg. absorbance before exposure

\(A_t\) … avg. absorbance after test (72 h)

\(A_c\) … avg. absorbance after 72 h, no exposure to test compound
Camptothecin Proliferation Assay

**Camptothecin**

Percentage of growth (%) vs. log concentration (M)

- Caco-2
- H 460