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Effects of cycloartane saponins from hairy roots of *Astragalus membranaceus* Bge., on human tumor cell targets

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ABSTRACT

For the first time three different natural compounds, isolated from hairy roots of *Astragalus membranaceus*, cultivated in airlift bioreactor were tested for their cytotoxic potential and apoptosis induction in a panel of human tumor cell lines. Root cultures, cultivated in bioreactor gave 18.5 $g l^{-1}$ dry wt roots with the highest astragaloside production in vitro up to now — 1.64% (astragaloside I), 1.12% (astragaloside II) and 1.08% (astragaloside III). In this manner the production in airlift bioreactor can be used as means of reliable supply of cycloartane saponins to extend the research to human clinical studies.

1. Introduction

The roots of *Astragalus membranaceus* Bge. (Fabaceae) is a very old and well-known drug in traditional Chinese medicine, and is used as an immunostimulant, diuretic, anti-cancer or a general tonic [1,2]. *Astragalus* root of many species contains a series of cycloartane triterpene glycoside denoted astragalosides (saponins), that are based on the aglycone cycloastragenol and contain one to three sugar residues attached at the 3-, 6-, and 25-positions [3,4]. Several saponins have also been reported that are based on the oleanene skeleton [5]. *A. membranaceus*, has demonstrated a wide range of potential therapeutic applications, including cancer therapy [6–8].

The limited supply (endemic plant species for East Asia) together with the over-exploitation of this species constitutes the most important hurdles for the optimal production of natural compounds, found in this plant. In the coming decades, several new enabling biotechnologies will be required to develop the next generation of advanced plant-based pharmaceuticals. With modern biotechnology, it has become feasible to use plant cells for the production of specific pharmaceuticals. To extend the research to human clinical studies, we needed to find a reliable supply of astragalosides and develop a transformed "hairy root" (HR) culture as a means of biomass production [1].

One of the goals of cancer chemotherapy and prevention is the discovery of compounds that are relatively selective to tumor cells and, therefore, have reduced effects on normal cell growth [9,10]. In the current study, three different natural compounds, isolated from transformed hairy roots of *A. membranaceus*, cultivated in airlift bioreactor were tested for their cytotoxic potential and apoptosis induction in a panel of human tumor cell lines, which is representative for some common human malignancies.

2. Experimental

2.1. Plant material

The seeds of *A. membranaceus* Bge. were provided by the botanical garden of Dusseldorf (Germany).

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2.2. Hairy-root induction and cultivation in airlift bioreactor

Leaf segments from sterile grown plants, were wounded with a sterile needle and the bacteria from the media were spread onto the leaves. After four days, the explants were transferred to an MS medium without phytochormones, containing 500 mg l^{-1} sodium cefotaxim (Claforan, Hoechst AG, Frankfurt) to remove the excess of bacteria. The cultures were kept in dark, at 25 °C. Roots developed after 3–4 weeks of incubation. After completely removing free-living Agrobacteria, the hairy roots are cultivated in the usual manner. Single roots (ca. 10–20 mm long) were transferred to a liquid MS medium without phytochormones. For the time course of growth and saponin production, 2–3 cm (0.5 g fresh weight) root tips were inoculated in 50 ml medium in 300 ml flasks and cultivated on a gyratory shaker at 120 rpm in darkness at 25 ± 2 °C and subcultured at a four-week interval.

A rapidly growing root line of *A. membranaceus*, transformed with *Agrobacterium rhizogenes*, LBA 9402 was selected for cultivation in airlift bioreactor for 45 days. After four weeks of cultivation in Erlenmeyer flasks, the hairy roots were scaled up to a 2-L bioreactor. Upon this subculture, the harvested roots were inoculated in airlift bioreactor after being cut to about 1.0 cm length.

2.3. Extraction and quantification of astragalosides

Hairy roots were lyophilised and extracted as described earlier [1]. Silica gel 60 F254 plates (20×20 cm) were used and developed with $CHCl_3$–MeOH–CH_3CHOCH_3–H_2O (80:50:10:10). After development and drying, the plates were sprayed with p-dimethylaminobenzaldehyde reagent, followed by heating at 105 °C for 10 min. The spots were measured with a TLC Scanner (Shimadzu CS - 9000) at 483 nm. Calibration graphs were prepared with standard methanol solutions of astragalosides I, II and III. The values of the saponins were calculated from the integration of spots corresponding to three different concentrations of astragalosides and three spots of solutions of unknown concentrations.

2.4. Drugs and solutions

Stock solutions of all agents under investigation were freshly prepared in DMSO and promptly diluted serially with RPMI-1640 medium to the desired extend. At the final dilutions obtained the concentration of the solvent never exceeded 1%.

2.5. Chemicals and reagents

RPMI-1640 growth medium, fetal calf serum, L-glutamine were purchased from Sigma. (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) and DMSO were supplied form Merck Co. 'Cell Death Detection' ELISA kit was purchased from Roche Applied Science.

2.6. Cell lines and culture conditions

The cell lines used in this study, namely SKW-3, HL-60, HL-60/Dox, MDA-MB-231 and HT-29 were obtained from DSMZ GmbH (Braunschweig, Germany). They were maintained in a controlled environment: RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine, at 37 °C in a 'Heraeus' incubator with 5% CO_2 humidified atmosphere. In order to keep cells in log phase the cultures were re-fed with fresh RPMI-1640 medium two or three times every week.

2.7. Cytotoxicity assay

Cell viability was assessed using the standard MTT-dye reduction assay as previously described [11] with minor modifications [12]. Exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 μl/well) at a density of 1×10^5 cells per ml and after 24 h incubation at 37 °C they were exposed to various concentrations of the tested compounds for 72 h. For each concentration at least 8 wells were used. After the incubation with the test compounds MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide — Sigma) solution (10 mg/ml in PBS) was added (10 μl/well). Microplates were further incubated for 4 h at 37 °C and the quantity of formazan product obtained was determined spectrophotometrically using a microprocessor controlled multiplate reader (Labexim LMR-1) at 580 nm. Cell survival fractions were calculated as percentage of the solvent-treated control (exposed to 1% DMSO for 72 h; set as = 100% viable). In addition IC_{50} values were derived from the concentration–response curves. All tests were run in triplicate with cisplatin used as referent positive control.

2.8. Apoptosis assay

The characteristic for apoptosis oligonucleosomal DNA fragmentation was examined using a commercially available 'Cell-death detection' ELISA kit (Roche Applied Science). This method allows semi-quantitative determination of the characteristic for the apoptotic process histone-associated mono- and oligonucleosomal DNA fragments using 'sandwitch' ELISA. Exponentially growing cells were exposed to varying concentrations of the tested compounds and thereafter cytosolic fractions of 1×10^4 cells per group (treated or untreated) served as antigen source in a sandwich ELISA, utilizing primary anti-histone antibody-coated microplate and a secondary peroxidase-conjugated anti-DNA antibody. The photometric immunoassay for histone-associated DNA fragments was executed according to the manufacturers' instructions at 405 nm, using ELISA reader (Labexim LMR-1). The results are expressed as the oligonucleosomal enrichment factor (representing a ratio between the absorption in the treated vs. the solvent-treated control samples (exposed to 1% DMSO)).

2.9. Statistical analysis

All statistical analyses were performed by the independent sample Student's *t*-test, with $p \leq 0.05$ set as significance level.