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BIOTECHNOLOGIES

Biological Activity of Agaricinic Acid Nanoparticles against Human Hepatoma HepG2 Cells

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A stable preparation of agaricinic acid nanoparticles was obtained. The mean hydrodynamic size of nanoparticles according to photon correlation spectroscopy was 200 nm and zeta potential was -57 mV. Cytotoxic activity of agaricinic acid nanoparticles against human HepG2 hepatoma cells was evaluated. Nanoparticles with a low concentration of agaricinic acid stimulated and with high concentration — suppressed metabolic activity and viability of hepatoma cells. The EC_{50} for the stimulating effect was 32.8 μg/ml, and the IC_{50}=602.1 μg/ml. The preparation of agaricinic acid nanoparticles can be used in medicine as a potential antitumor agent.

Key Words: *agaricinic acid; nanoparticles; cytotoxicity; EC_{50}; IC_{50}*

The fruiting bodies of basidiomycete *Fomitopsis officinalis*, along with other biologically active substances, contain agaricinic acid; biological activity of this substance is still poorly studied [13]. In early studies, the inhibitory effect of agaricinic acid on lipid metabolism and steroid biosynthesis was described [11]. Agaricinic acid can also affect permeability of large Ca^{2+}-dependent mitochondrial permeability transition pore (mPTP) that plays an important role in calcium exchange between the mitochondria and cytoplasm [2,4,6]. A key component of mPTP supplying ADP to the mitochondria is adenine nucleotide translocase (ANT) that acts as an ATP/ADP antiporter. Disruption of ANT function can lead to cessation of ATP synthesis in mitochondria and energy starvation of the cell [7,10]. Agaricinic acid binds to ANT through the citrate part of the molecule, while the aliphatic sequence $C_{16}H_{33}$ (Fig. 1) stabilizes its binding by anchoring in the phospholipid bilayer of the mitochondrial membrane [6]. By acting in this way, agaricinic acid induces pore opening in the inner membrane, which leads to Ca^{2+} release from the mitochondria, a drop in the membrane potential, massive influx of water and ions into the mitochondrial matrix, mitochondrial swelling, and rupture of the outer membrane. As a result, proteins that initiate the process of apoptosis, can be released from mitochondria [3,12,15]. The mechanism of the modulating effect of agaricinic acid on the mitochondrial pore can be more complex. It was demonstrated

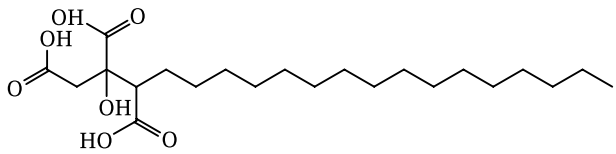

Fig. 1. Molecular structure of agaricinic (2-hydroxy-1,2,3-nonadecanetricarboxylic) acid.

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that although N-ethylmaleimide inhibits agaricinic acid-induced pore opening, it does not affect the adenine nucleotide exchange performed by ANT [5].

Compounds modulating activity of the mitochondrial pore are now considered as the basis for the development of new promising drugs for the treatment of cancer, coronary heart disease (CHD), neurodegenerative diseases, *etc*. [8,9,14]. Thus, due to the important role of agaricinic acid as mPMP modulator stimulating its opening and subsequent induction of apoptosis, this compound can be considered as a promising agent for the treatment of cancer and other diseases associated with impaired functional activity of mitochondrial pore.

The molecule of agaricinic acid, like phospholipids and fatty acids, is amphiphilic because it consists of a polar (three carboxyl groups) and non-polar parts (long hydrocarbon chain $C_{16}H_{33}$; Fig. 1). This molecular structure explains some features of the functional activity of agaricinic acid and attests to its low water solubility, which prevents full-scale study of its biological activity.

We studies the effect of agaricinic acid on HepG2 tumor cells. For studying agaricinic acid activity in biological media, a preparation of its nanoparticles was previously obtained.

MATERIALS AND METHODS

Agaricinic acid (2-hydroxy-1,2,3-nonadecane-tricarboxylic acid), PBS, glycerin, Sephadex G-50 (Sigma Chemicals Co.), arabinogalactan (Fluka) were used. All other reagents used in the study were of analytical grade.

To obtain nanoparticles, 50 mg agaricinic acid was added to a solution containing 1.5 ml 96% ethanol and DMSO. The solution was heated on a water bath until complete dissolution of agaricinic acid. Then, a 10-fold excess of an aqueous solution of the same temperature containing glycerin was added with vigorous stirring. The resulting mixture was cooled to 20°C with stirring, sonicated for 10 min using an Misonix sonicator S-4000 ultrasonic disintegrator and the nanoparticles were purified by gel filtration on a Sephadex G50 column. After measuring the concentration of agaricinic acid, the purified preparation was used in further experiments.

Particle size and zeta-potential were measured by dynamic light scattering method, or photon correlation spectroscopy, using Photocor Compact equipment (scattering angle 90°, laser 654 nm, 30 mW). The measurements were carried out at 25°C, the time of correlation functions accumulation was 60 sec.

Identification and determination of the concentration of agaricinic acid in the samples was carried out using reverse-phase HPLC. The samples were dissolved in 1 ml ethanol, stirred, centrifuged, and 20 μl of the supernatant were collected for HPLC analysis. The analysis was performed using an Agilent Technologies 1260 Infinity chromatograph with a C18 column for reverse phase chromatography at a flow rate of 1 ml/min. The mobile phase consisted of a mixture of acetonitrile and trichloroacetic acid (0.1% vol.). The eluate from the column was monitored at a wavelength of 206 nm. The retention time of the agaricinic acid contained in the samples on the reverse phase column was identical to the retention time of its standard sample. The concentration of agaricinic acid in the samples was determined according to a calibration curve constructed using a standard sample.

Evaluation of cytotoxic activity of agaricinic acid nanoparticles was carried out on a HepG2 human hepatoma cell culture (ATCC HB8065) obtained from the cryostorage of the Common Use Centre "Regenerative Medicine" of the I. M. Sechenov First Moscow State Medical University. Before use in the analysis, HepG2 cells were thawed and passaged. Cells were cultured in DMEM/F-12 (Gibco) medium supplemented with 5% fetal calf serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) in an incubator at 5% CO_2 and 37°C.

The effect of agaricinic acid nanoparticles on metabolic activity of HepG2 cells was analyzed using phase-contrast microscopy and colorimetric MTT test (Sigma-Aldrich) [1]. HepG2 cells were seeded in a 96-well plate (1.5×10^4 cells per well) and left for 12 h for adhesion. Then, the preparation of agaricinic acid nanoparticles was added in final concentrations of 2-1000 μg/ml. All measurements were carried out in 8 replicates. Negative control of cytotoxicity were cells incubated in the absence of the test substance (n=8). After 48 h of cultivation, in order to assess the morphology of the cells, their images were obtained in phase contrast using TE 2000-U Eclipse inverter microscope (Nikon) and an MTT analysis was performed. To this end, the medium was removed, 0.48 mM MTT reagent was added in a volume of 250 μl per well, and the plates were incubated for 4 h. Then, the supernatant was removed and the resulting formazan crystals were dissolved in 150 μl DMSO (Sigma). Absorbance was measured at λ=540 nm (A_{540}) on a Multiskan FC plate photometer (Thermo). The background value was measured in a sample containing the reagent and nutrient medium. Cell viability after incubation with agaricinic acid nanoparticles was calculated by the formula: $[A_{540}(\text{sample})-A_{540}(\text{background})/A_{540}(\text{control})-A_{540}(\text{background})]\times100\%$.

Statistical analysis was performed using GraphPad Prism 7.00; Gaussian distribution was verified by the Shapiro—Wilk test. Non-linear regression model

was used to calculate the half-maximal effective concentration (EC_{50}). The results are presented as $M \pm SD$. The differences were significant at $p<0.05$.

RESULTS

The mean size of the obtained nanoparticles of agaricinic acid was 200 nm. The nanoparticle preparation was a stable aqueous suspension (zeta potential -57 mV) without signs of precipitation for at least a month.

According to MTT test, agaricinic acid nanoparticles at low concentrations in the culture medium (from 2 to 62.5 μg/ml) had a weak stimulating effect on the viability of HepG2 tumor cells, while at higher concentrations (from 250 to 1000 μg/ml), they pronounced an inhibitory effect (Fig. 2). EC_{50} for the stimulating effect was 32.8 μg/ml, and IC_{50}=602.1 μg/ml.

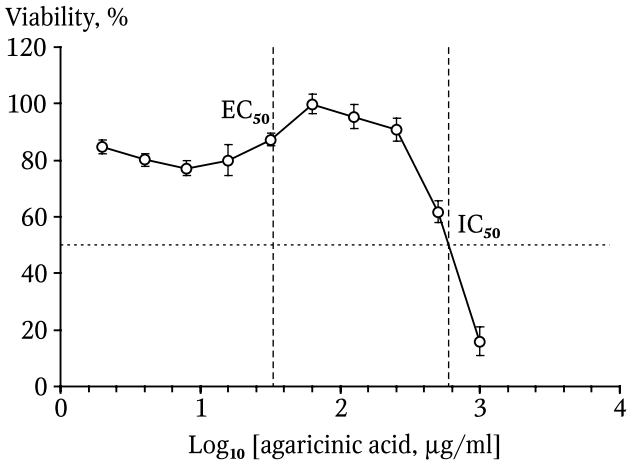

Fig. 2. The two-phase curve of the dependence of HepG2 tumor cell viability on the concentration of agaricinic acid nanoparticles in the culture medium (MTT test data). Dashed lines show the half-maximum effective concentration of stimulation (EC_{50}) and inhibition (IC_{50}) of HepG2 cell viability.

The effect of agaricinic acid nanoparticles on HepG2 tumor cells was studied using phase-contrast microscopy (Fig. 3). On phase-contrast images of HepG2 cells obtained after 48-h culturing with the tested concentrations of nanoparticles, no obvious changes in cell morphology in comparison with the control cells (without the addition of nanoparticles) were seen at low concentrations of the preparation in the incubation medium (31.3 μg/ml and 62.5 μg/ml): the cells were evenly spread on plastic. At higher concentrations (250, 500 and 1000 μg/ml), gradual changes in cell morphology and a decrease in cell density were observed, which is consistent with the MTT test data and may indicate a cytotoxic effect of agaricinic acid nanoparticles. Thus, suppression of tumor growth was achieved only at high concentrations of agaricinic acid nanoparticles, which can be due to the damaging effect of nanoparticles characterized by high free energy of surface atoms on cellular structures, and, to a lesser extent, due to the release of poorly soluble agaricinic acid from nanoparticles inside the cell. The cytotoxic effect of free agaricinic acid can be associated with its action on elements of the mitochondrial pores, leading to destabilization and rupture of the mitochondrial membrane with the subsequent release of cytochrome c and triggering of apoptotic cell death [15].

Agaricinic acid is characterized by low solubility in biological fluids, which is a serious obstacle to the study of its biological activity and the search for ways of its practical application. Our experiments demon-

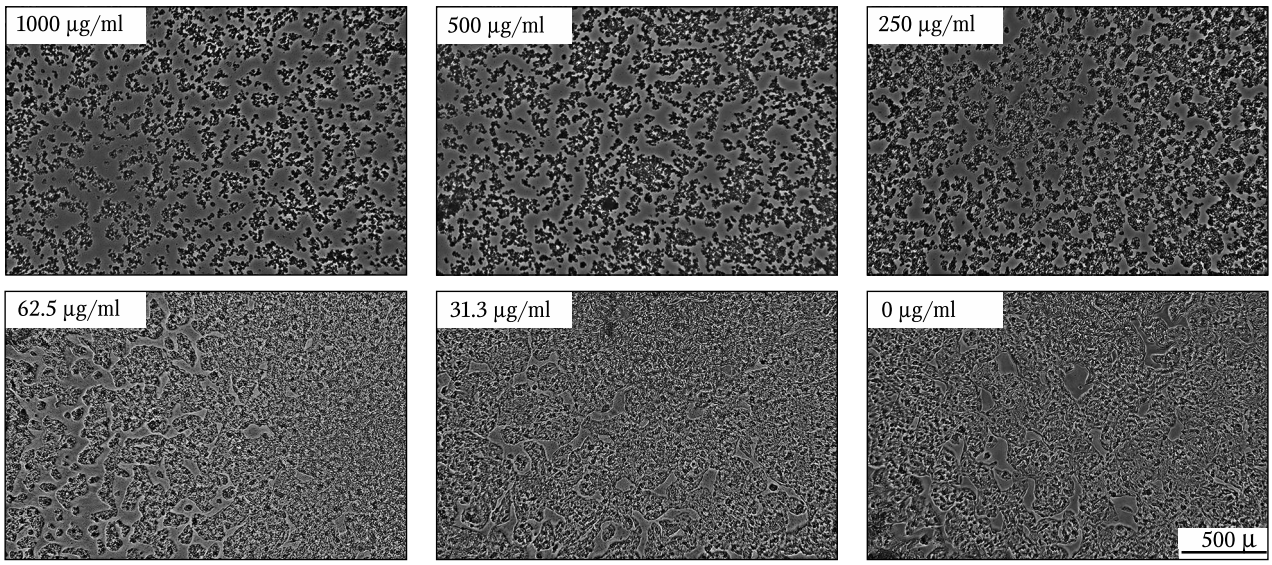

Fig. 3. HepG2 cells after 48-h incubation in the presence of various concentrations of agaricinic acid nanoparticles. Phase-contrast microscopy.

strated the possible of obtaining and using stable aqueous nanosuspension of agaricinic acid for evaluation of its cytotoxic and, possibly, other activities. Further studies will help to determine the place of agaricinic acid among biologically active compounds used in the biomedical field.

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