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HYPERFLAV — PERSPECTIVE PHOTOSENSITIZER FOR PDT: CELL STUDIES

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***Background*: Application of hypericin (an alkaloid from *Hypericum perforatum* plants) as photodynamic agent may become the next successful step in photodynamic therapy of malignant tumors. Hyperflav — is a purified Hypericum extract designed for the purpose of photodynamic diagnosis. *Aim*: Present studies investigated the effectiveness *in vitro* of Hyperflav application as a photosensitizer for photodynamic therapy. *Methods*: Hyperflav photodynamic activity was assessed in phototoxic cell tests on Jurkat, MT-4 and Namalwa leukemic cell lines. Spectroscopic measurements of Hyperflav solutions were performed. *Results*: Hyperflav aqueous solubility was maintained in presence of polyvinylpyrrolidone with the most pronounced photodynamic activity at 1:5 (w/w) Hyperflav-PVP ratio. Hyperflav fluorescence spectrum in ethanol exhibits two main peaks around 597 and 647 nm, in accordance with the spectrum of pure hypericin. Fluorescence spectrum of aqueous solution exhibits peaks at 604 and 655 nm and indicates decreasing in fluorescence intensity. Hyperflav at drug dose range of 5 –25 μg/ml and light dose 15 J/cm^2 showed a dose-dependent cytotoxicity on tested cell cultures, while dark cytotoxicity was not observed. Light irradiation of cell samples preincubated with 15 μg/ml Hyperflav resulted in 69.9, 76.0 and 78.3% cell death of Jurkat, MT-4 and Namalwa cultures, respectively. Combined preparation of Hyperflav with gold nanoparticles showed low photocytotoxicity (24.2%) in comparison with Hyperflav alone (99.6%) on Namalwa cells. *Conclusion:* Hyperflav being solubilized in nontoxic aqueous media exhibits *in vitro* photodynamic activity at doses that do not have dark toxicity, and therefore it meets requirements as a perspective photosensitizer. Further studies, particularly *in vivo,* are warranted to fully evaluate photodynamic potential of Hyperflav.**

***Key Words:* photodynamic therapy (PDT), Hypericum extract, hypericin, gold nanoparticles, antitumor effect.**

Antitumor photodynamic therapy (PDT) comprises systemic administration of a photosensitizer and subsequent visible light delivery to the tumor lesion. Sufficient oxygenation of targeted tissue enables photochemical reactions with generation of reactive oxygen species and/or free radicals leading to oxidative damage and destruction of sensitized cells [1]. Promising clinical results obtained with photodynamic therapy stimulate searching of novel photosensitizers with better chemophysical properties. One of such promising substances is hypericin, a natural product which was found together with other naphthodianthrone derivatives such as pseudohypericin, protohypericin, protopseudohypericin, in a number of plants of the genus *Hypericum*, *H.perforatum* and *H.maculatum* for instance [2].

Nowadays drugs based on Hypericum extract is widely used for the treatment of mild and moderate depression [3], although there is some evidence that indicates that not hypericin but hyperforin is responsible for the antidepressant activity. Also because of antiviral activity of hypericin [4] its possible application as photodependent blood sterilizer was investigated [5]. Hypericin is under investigation as photoinhibitor of the progression of proliferative vitreoretinopathy in ophthalmology [6].

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Abbreviations used: PDT – photodynamic therapy; PVP – polyvinylpyrrolidone.

Due to comparatively high singlet oxygen and superoxide anions generation rate, triplet quantum yield, potent light-dependent antineoplastic and antiviral activities hypericin is under investigation as a photosensitizer for PDT treatment of superficial bladder tumor [7], recurrent mesothelioma, basal and squamous cell carcinoma [8] and for inhibition of the growth of malignant glioma [9, 10], pituitary adenoma, and cutaneous T-cell lymphoma. Moreover, hypericin is successfully applied as a diagnostic tool for the fluorescent detection of flat neoplastic lesions in bladder [8, 11, 12].

Hypericin is soluble in polar solvents such as ethanol, methanol, acetonitrile, tetrahydrofuran, cyclohexane, acetone, dimethylsulfoxide and insoluble in nonpolar solvents [13]. At physiological pH hypericin forms organic and inorganic monobasic salts in organic solvents. Dissolved in organic solvents sodium hypericinate exhibits bright red fluorescence (absorption λ_{max} = 592 nm, emission λ_{max} = 594 nm in ethanolic solutions) [14, 15]. Unfortunately hypericin is insoluble in water under physiological conditions and becomes barely soluble in pure water if pH rises above 8 [13]. But hypericin can be solubilized in biological media, if formation of complexes with biomacromolecules is possible [14].

Nanoparticles being from several dozens to hundreds nanometers in size and from about one hundred to ten thousand times smaller than human cells, possess unique abilities in interaction with biomolecules, what may be used to improve anticancer drugs and diagnostic agents. Among the most studied nanoparticles are colloid gold, quantum dots, carbon nanotubes, silicon and paramagnetic nanoparticles

[16, 17]. Main fields of gold nanoparticles application in biomedicine are drug delivery, photothermal and photodynamic therapy, diagnostics.

MATERIALS AND METHODS

Cell lines. In our studies we used MT-4 and Jurkat T-cell leukemic lines and B-cell Burkitt lymphoma cell line Namalwa obtained from the Bank of Cell Lines from Human and Animal Tissue NAS of Ukraine. Cells were cultured in RPMI-1640 medium (FarmBiotek, Ukraine) supplemented with 10% fetal cow serum (Sigma, Germany) at 37 °C in presence of 5% CO_2. Cells were reseeded three times a week.

Hyperflav preparations. Hyperflav was provided by SIC "Borshchahivskiy Chemical-Pharmaceutical Plant". Hyperflav dried powder was dissolved in 0.9% sodium chloride with addition of various concentrations (0.5; 1.25; 2.5; 5 and 10%) of polyvinylpyrrolidone and then diluted with sodium chloride to final concentration of 500 μg/ml. Hyperflav solutions were prepared *ex tempore* for every experiment.

Nanocomposite Hyperflav-colloid gold was prepared in Scientific Research Institute of Nanotechnological Industry by conjugation of commercial of commercial Hyperflav with gold nanoparticles. Dried substance was dissolved in 0.9% sodium chloride with 2.5% polyvinylpyrrolidone and further diluted with sodium chloride to obtain the concentration of 500 μg/ml.

K-30 polyvinylpyrrolidone (BASF, China) with approximate molecular weight of 40 kDa was used to prepare Hyperflav solutions.

Fluorescence measurements. Fluorescence emission spectra of Hyperflav solutions were recorded with ND-3300 cuvetteless spectrofluorometr (Nanodrop Technologies, USA) connected to PC. Measurements were conducted in 1 mm fluid column formed between surfaces of pedestal and receiving optical fiber.

Radiation sources. As the light source for irradiation of cell samples we used an experimental device (Photonika Plus, Ukraine) based on incandescent lamp (Philips, USA) equipped with appropriate broadband filter. The device emitted light in spectral range of 560–700 nm. Light beam was delivered to test tubes with cells by a fiber-optical probe.

Photodynamic procedure. Each experimental sample contained 2 ml of 2 x 10^6 cells/ml suspension. Cell samples were preincubated in Hanks solution with addition of appropriate Hyperflav preparations in sodium cloride for 1 hour and then washed once to remove non-absorbed photosensitizer. After subsequent resuspending in Hanks solution, samples were irradiated with a power density of 150 mW/cm^2 and a dose of 15 J/cm^2. Irradiation dose was controlled with the help of energy and power meter (3A-p thermal head, Ophir Optronics, USA). Irradiated cells were incubated in RPMI-1640 media for 24 h, and then a phototoxic effect was assessed by trypan blue dye exclusion test.

Statistical analysis. Per cent values of dead cells was determined by counting of 200 cells five times for each sample, and were presented as a mean ± standard deviation. One-way ANOVA analysis was used to determine significance of difference between means.

RESULTS AND DISCUSSION

The first part of the study was devoted to elaboration of the preparation dissolved forms which would be suitable for biological investigations. Several reports on photodynamic properties of Hypericum extracts have been published earlier [18, 19]. Hyperflav, that is actually a dried St. Johns wort (*H. perforatum*) extract, proposed as a photodiagnostic agent, contains about 2% of hypericin and pseudohypericin. Hyperflav like other similar extracts and pure hypericin, is insoluble in water, being well soluble in ethanol and other polar solvents. It is known that hypericin in aqueous buffers is present in a form of colloidal high molecular weight aggregates. In such aggregated form hypericin loses its photodynamic activity as well as suffers decrease in fluorescence yield [14]. Due to its lipophilic properties it is possible to prevent aggregation of hypericin by addition of albumin and plasma lipoproteins that adsorb hypericin in aqueous environment [14]. Some macromolecular substances such as polyethyleneglycol [14], N-methylpyrrolidone [20] and polyvinylpyrrolidone [21, 22] can be used instead of serum proteins to solubilize hypericin in aqueous media.

Thus, to obtain aqueous solutions of Hyperflav for cell tests, we used polyvinylpyrrolidone (PVP). A correlation exists between the amount of nonaggregated hypericin and its fluorescence yield in a solution [14]. Fluorescence emission profile of Hyperflav solubilied in water with PVP is almost similar (except its much lower intensity) to that in ethanol and specifies hypericin as the major photoactive constituent (Fig. 1). Results of fluorescent spectroscopy measurements show significant decrease in fluorescence intensity of water-PVP solubilized Hyperflav compared to ethanolic solution. Nevertheless they show presence of some nonaggregated, thus bioavailable and photodynamically active hypericin in solution.

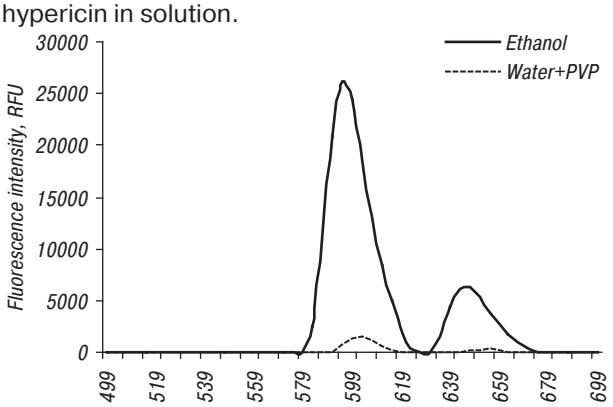

Fig. 1. Fluorescence emission spectra of ethanolic and polyvinylpyrrolidone-aqueous Hyperflav solutions. Concentration of Hyperflav 500 μg/ml. Peak fluorescence emission at 597 nm in ethanolic solution, and 604 nm in water-PVP solution

In the next stage of the study cytotoxicity cell tests were performed to determine influence of Hyperflav to PVP ratio on photodynamic activity of solutions (Fig. 2). Samples containing Hyperflav in concentration of 10 μg/ml with 20, 50, 100, 200 or 400 μg/ml of PVP in 0.9%

sodium chloride were tested. Maximal activity under light irradiation was shown by the solution with 1:5 ratio (10 μg/ml Hyperflav and 50 μg/ml PVP). No statistically significant cytotoxicity in dark conditions was observed.

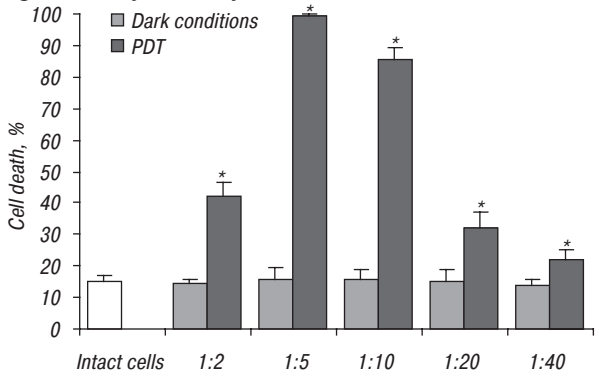

Fig. 2. Photodynamic treatment of Jurkat cells. Hyperflav-polyvinylpyrrolidone (w/w) ratios 1:2, 1:5, 1:10, 1:20 and 1:40. *Significantly different from control at $p < 0.05$

Next, using the most effective Hyperflav: polyvinylpyrrolidone ratio 1:5, we assessed photodynamic activity of Hyperflav on three different cell cultures. Under light irradiation Hyperflav showed dose dependent cytotoxicity. Data obtained in tests with Jurkat, Namalwa and MT-4 cell lines are presented in Fig. 3–5. As it follows from the figures, 69.9, 78.3 and 76% cell death was observed in respective culture samples preincubated with Hyperflav in concentration of 15 μg/ml, and the preparation in concentration of 20–25 μg/ml was able to induce near total death of tested cells. No significant cytotoxicity was observed in dark conditions.

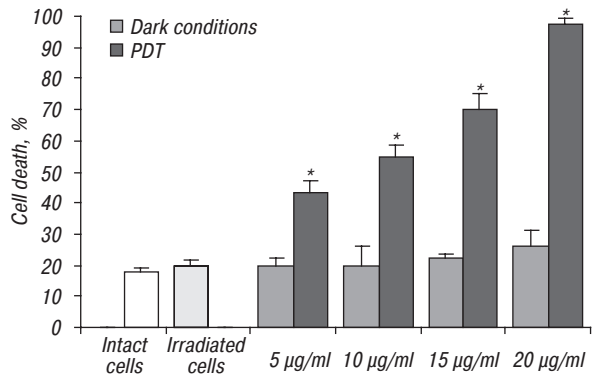

Fig. 3. Photodynamic treatment of Jurkat cells with Hyperflav in different concentrations. *Significantly different from control at $p < 0.05$

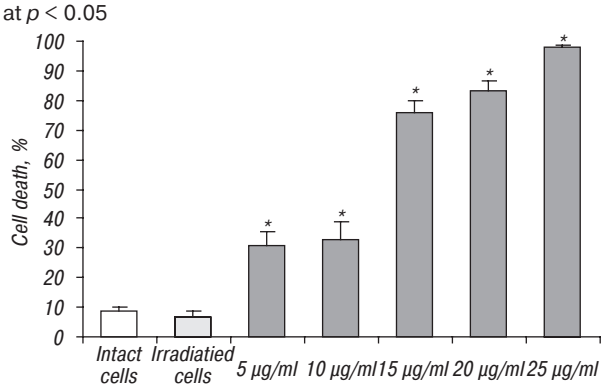

Fig. 4. Photodynamic treatment of MT-4 cells with Hyperflav in different concentrations. *Significantly different from control at $p < 0.05$

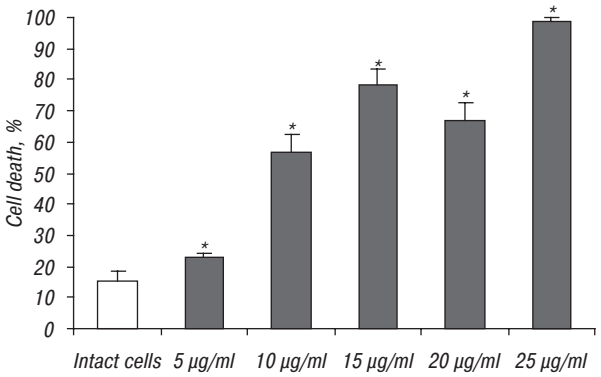

Fig. 5. Photodynamic treatment of Namalwa cells with Hyperflav in different concentrations. *Significantly different from control at $p < 0.05$

Among different nanotechnology products gold nanoparticles draw a special attention as perspective drug delivery agents for cancer therapy [16, 17, 23]. Similar to the Hyperflav solubility problem, nanoparticles can be stabilized in colloidal state by biocompatible polymers which are able to inhibit colloid aggregation in physiological conditions [16].

Therefore a conjugated preparation of Hyperflav with nanogold, stabilized by PVP, was obtained and preliminarily tested using Namalwa cell culture. Contrary to expectations phototoxic tests revealed decrease in phototoxicity of nanocomposite preparation in comparison with Hyperflav alone (Fig. 6).

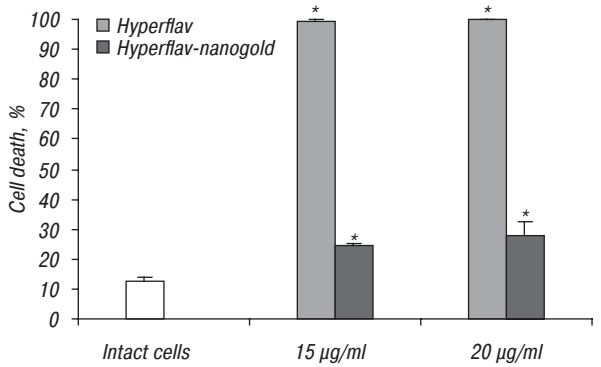

Fig. 6. Effect of photodynamic treatment with Hyperflav and Hyperflav-nanogold on Namalwa cells. *Significantly different from control at $p < 0.05$

The preliminary results obtained may be explained by the fact that Hyperflav is polycomponent substance containing not only the prooxidant constituent (hypericin) but antioxidative ones (flavonoids, quercetin) as well [24]. It could interfere with the cell oxidative damage by PDT. Also, the influence of gold nanoparticles on bioavailability and distribution of prooxidative and antioxidative components of the preparation in cell culture system is still to be studied.

CONCLUSION

Hyperflav can be maintained in aqueous solution in presence of polyvinylpyrrolidone. Hyperflav in dose of 15 μg/ml shows photodynamic activity in tests with leukemic cell cultures Jurkat, Namalwa and MT-4, inducing approximately 70% cell death and does not show dark toxicity. Conjugation with gold nanoparticles causes decrease in phototoxic activity of Hyperflav.

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