Exp Oncol 2010
32, 1, 44–47

Experimental ONCOLOGY

PHOTODYNAMIC ACTIVITY OF HEMATOPORPHYRIN CONJUGATES WITH GOLD NANOPARTICLES: EXPERIMENTS *IN VITRO*

*N.F. Gamaleia[1,] *, E.D. Shishko[1], G.A. Dolinsky[1], A.B. Shcherbakov[2], A.V. Usatenko[2], V.V. Kholin[3]*
[1]R.E.Kavetsky Institute for Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv 03022, Ukraine
[2]Research Institute for Nanotechnological Industry, Kyiv 03055, Ukraine
[3]"Photonika Plus", Cherkasy 18023, Ukraine

Aim: **To synthesize a conjugate of hematoporphyrin with gold nanoparticles, and to evaluate its photodynamic activity in experiments on cultures of transformed cells.** ***Methods:*** **nanosized gold particles and nanocomposites synthesis methods, cell culture methods, photobiology methods, trypan blue dye exclusion test, chemiluminescence assay.** ***Results:*** **Various hematoporphyrin-gold nanocomposites were obtained, which contained similar hematoporphyrin concentration (5 μg/ml) and varied concentrations (0.5–5 μg/ml) of gold nanoparticles with a diameter of 15 nm or 45 nm. It was established by chemiluminescence method that nanocomposites synthesized induce more efficiently the formation of photo-oxidative products than original photosensitizer. The experiments with transformed cell lines showed that photodynamic *in vitro* activity of synthesized hematoporphyrin-nanogold composites is much higher than that of the original photosensitizer. The better activity of the nanocomposites with gold particles of 45 nm *vs* such of 15 nm which was demonstrated in the experiments, can be apparently connected with the fact that bigger particles are able to transport more porphyrin molecules into malignant cells.** ***Conclusion:*** **The results obtained warrant the necessity of further studies with hematoporphyrin-gold nanocomposites *in vivo* on transplanted tumors of animals which have to define the real perspectives of the nanocomposites application in PDT.**
Key Words: **hematoporphyrin-nanogold composite, photodynamic effect, reactive oxygen species, chemiluminescence, malignant cell cultures.**

Photodynamic therapy of tumors (PDT) is a modern low-traumatic method which proved to be effective in the treatment of a number of oncological conditions [1–3]. Main advantages of the PDT are selectivity of tumor tissue damage and low invasiveness of the procedure that allows in certain cases to avoid a surgical intervention [4–6]. Besides, an actual absence of serious side effects distinguishes favourably the PDT from conventional chemotherapy and radiotherapy of oncological patients.

However, PDT has an important drawback connected with innate properties of the light radiation — a small depth of light penetration into biological tissues that limits drastically practical applications of the method. One possible way of overcoming the drawback is employment of photosensitizers which are accumulated by tumors in greater quantity making them sensitive to the light of even low intensity. The goal may be pursued combining principles of PDT and nanotechnology when nanoparticles are used as a vector for photosensitizer transportation [7, 8].

The objectives of the study were to synthesize a conjugate of the most extensively studied photosensitizer hematoporphyrin with gold nanoparticles, and to evaluate its photodynamic activity in experiments on cultures of transformed cells. It was shown that nanosized gold particles has an ability to selectively accumulate in tumor tissues [9], thus adding to the porphyrins own tumor selectivity. To our knowledge, there are no data published on the PDT application of porphyrin conjugates with gold nanoparticles.

Received : February 12, 2009.
*Correspondence: Fax: +38 (044) 258-16-56
E-mail: gamaleia@onconet.kiev.ua
Abbreviations used: PDT – photodynamic therapy; ROS – reactive oxygen species.

MATERIALS AND METHODS

Gold nanospheres (also known as gold colloids) of 15 nm and 45 nm in diameter were synthesized by controlled reduction of an aqueous $HAuCl_4$ solution with citrate [10], stabilizing the colloid by polyvinylpyrrolidone. The conjugated photosensitizer was obtained with addition of hematoporphyrin to polyvinylpyrrolidone-nanogold solution in calculated molar ratio.

T-cell lines MT-4 and Jurkat (human T-cell leukemia) were obtained from culture bank of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology and were cultured in standard RPMI-1640 medium supplied with 10% fetal bovine serum. For photodynamic treatment with photosensitizing nanocomposite under investigation, cell suspensions (2×10^6/ml) in Hanks balanced salt solution (pH = 7.2) were prepared from cultures of the leukemic cell lines in log phase of growth. The cells were incubated (37 °C) for 1.5 h in Hanks' solution with the photosensitizer in concentrations specified, the photosensitezer was washed off, and then the cells were irradiated.

For samples irradiation, the diode laser with radiation wavelength of 635 nm ("Photonica Plus", Cherkasy, Ukraine) and dose rate 50 J/cm^{2}w was used. The irradiated cells viability was evaluated by trypan blue dye exclusion test.

To investigate the ability of the composite to induce free radicals and reactive oxygen species (ROS) formation, two systems were used: the first one, containing solution of liposomes, produced from lecithin preparation Lipin® ("Biolek", Kharkiv, Ukraine), and the second one, the suspension of human leukemic cells mentioned above. Lipid peroxidation rate in the model solution system was assessed by means of the chemiluminescence intensity measuring after the coumarin

C-334 ("Sigma-Aldrich", USA) addition as an enhancer (10 µl of 0.05% ethanol solution to 2 ml of the liposome suspension, 0.2 mg of phospholipids her 1 mL of 20 mM Tris-buffer with 100 mM KCl, pH = 7.4). The measurements were made according to the technique of the pharmaceutical substances oxidation activity investigation in phospholipid liposome suspensions [11, 12] under the constant temperature conditions (37 °C) throughout all the observation period.

In cell samples, the production of peroxide compounds, which are considered to be the end products of ROS interaction with biological substrates, was revealed by enhancement of the Luminol chemiluminescence, following the sample injection into the test Luminol solution (0.5 ml of the cells suspension in Hanks solution, 10^6 cells per ml to 2 ml of 10 µM Luminol solution in 60 mM carbonate buffer, pH = 10.5, with 5 µM cobalt chloride) [13].

The chemiluminescence intensity was recorded by means of multiplier photocell-equipped unit with digital pulse counter ("ISKRA" Research Institute, Lugansk, Ukraine), communicated to PC for datamation.

To assess free radicals production rate, the chemiluminescence curves showing photon counts per 1 s (I, s^{-1}) were plotted, and the integral parameter of the luminescence intensity during 500 s after the sample injection (ΣI_T, T = 500 s) was calculated.

For the statistical analysis the Student's test was used.

RESULTS AND DISCUSSION

Chemiluminescence tests. Before starting to investigate the photodynamic effects of the hematoporphyrin-nanogold composite on cultured malignant cells, it was reasonable to elucidate if the composite is capable of reactive oxygen species production, which are the immediate cause of the biological substrate alteration by PDT. There are two types of the photo-oxidative reactions that underlie the photodynamic destruction of tumors [14]. Type I reactions involve electron or hydrogen atom transfer, producing radical forms of the photosensitizer or the substrate. These intermediates may react with oxygen to form peroxides, superoxide ions and hydroxyl radicals, which initiate free radical chain reactions.

Type II mechanism is mediated by the energy transfer process with the transformation of the ground state oxygen to the exited singlet oxygen ($^1O_2$) and with the return of the sensitizer to its ground state. The *in situ* generation of singlet oxygen via type II reactions appears to play the central role in photodynamic cytotoxicity because of the highly efficient interaction of the $^1O_2$ species with different biological molecules.

In our chemiluminescence tests, the photo-oxidative activity of the hematoporphyrin-nanogold composite in the phospholipid model system turned out rather high as it is evidenced by free radical oxidation records (Fig. 1). The chemiluminescence intensity in liposome suspensions containing the composite, was greater than with addition of the pure hematoporphyrin (luminescence total ΣI_{500} exceeded the background level by 39.0% and 17.6%, respectively, $p < 0.05$). That may be, presumably, explained by the gold nanoparticles catalytic activity resulting in the peroxide formation with further substrate oxidizing [15, 16]. Hence, enhanced cell-membrane phototoxicity can be expected with the nanocomposite as compared to the original photosensitizer, and the modified ratio in the induction of oxidative reactions type I and type II [17].

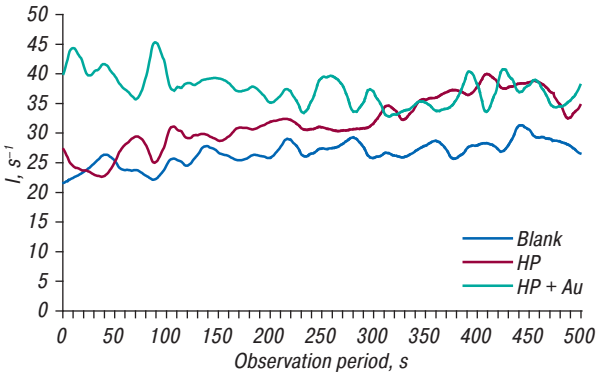

Fig. 1. The chemiluminescence kinetics in phospholipid liposomes suspension following its irradiation by red laser light (635 nm, 50 J/cm²): without photosensitizer (Blank), and with hematoporphyrin (HP) or the nanocomposite photosensitizer (HP + Au)

The investigations exploiting cell suspensions showed similar patterns in nanocomposite photosensitizer effects upon both Jurkat and MT-4 cell lines (Fig. 2). The main trends in cellular reactions to the nanocomposite *vs* the original hematoporphyrin photosensitizer were decreased "dark" free-radical formation rate (ΣI_{500} amounted 38.2% *vs* 56.6% above the background level) and increased free- radical formation rate as a result of photodynamic effect (ΣI_{500} amounted 438.6% *vs* 119.2% above the background level) (Fig. 3).

Cell experiments. In experiments with transformed cell cultures the nanocomposites with gold particles of two sizes (15 nm and 45 nm) were used in which the ratio hematoporphyrin : nanogold varied: the hematoporphyrine content was constant (5 µg/ml) but gold concentrations amounted to 5 µg; 2.5 µg; 1.0 µg or 0.5 µg. The hematoporphyrin in concentration 5 µg/ml had no "dark" cytotoxicity but under light irradiation it led to the death of 60–70% of cells. The activity of the nanocomposites was quite different. In Table 1 the data on the "dark" cytotoxicity and photodynamic activity of the 15 nm nanocomposites tested are given.

As it follows from the Table 1, the nanocomposites with hematoporphyrin concentration 5 µg/ml (№ 1–4) have some "dark" cytotoxicity: after 1.5 h incubation the death of 70–90% cells was observed. Hence, in further experiments the preparations diluted 10-fold were tested (№ 5, 6). The preparations had no "dark" cytotoxicity but their photodynamic activity remained rather high: cell irradiation induced the death of 77–88% cells. After the further 5-fold dilution the photodynamic activity still made 68% (№ 7) and only under additional twofold dilution it was actually absent (№ 8).

In Table 2 the data obtained with nanocomposites of hematoporphyrin and gold particles of 45 nm are shown. It is evident that such nanocomposites also have some "dark" cytotoxicity (№ 1–4) but lesser than the preparations with nanoparticle diameter of 15 nm. Ten fold dilution of them practically eliminated

the "dark" cytotoxicity while their photodynamic activity was higher than in analogical 15 nm composites (№ 5–8). They remained photodynamically active even after further 50-fold dilution (№ 9), and only preparations with 100-fold less concentration (№ 10) revealed the low activity. In Fig. 4 the comparative photodynamic efficiency of hematoporphyrin nanocomposites with gold particles of 15 nm and 45 nm is presented.

Table 1. "Dark" cytotoxicity and photodynamic activity of hematoporphyrin-gold nanocomposites with particles diameter of 15 nm (dead cells% in MT-4 cell line)

#	Nanocomposites (HP μg/ml : Au μg/ml)	"Dark" cytotoxicity	Photodynamic activity
1	5 : 5	90.1 ± 0.5	100.0 ± 0.0
2	5 : 2.5	69.3 ± 2.7	100.0 ± 0.0
3	5 : 1	86.2 ± 4.0	100.0 ± 0.0
4	5 : 0.5	81.9 ± 3.6	94.1 ± 2.8
5	0.5 : 0.1	0.0 ± 0.0	77.5 ± 3.8
6	0.5 : 0. 05	5.2 ± 0.4	88.5 ± 0.4
7	0.1 : 0.01	1.9 ± 0.4	67.5 ± 1.0
8	0.05 : 0.005	0.7 ± 0.01	12.5 ± 1.3

Table 2. "Dark" cytotoxicity and photodynamic activity of hematoporphyrin-gold nanocomposites with particles diameter of 45 nm (dead cells% in MT-4 cell line)

#	Nanocomposites (HP μg/ml : Au μg/ml)	"Dark" cytotoxicity	Photodynamic activity
1	5 : 5	41.3 ± 0.9	100.0 ± 0.0
2	5 : 2.5	22.2 ± 1.2	100.0 ± 0.0
3	5 : 1	81.7 ± 4.0	100.0 ± 0.0
4	5 : 0.5	79.5 ± 2.8	100.0 ± 0.0
5	0.5 : 0.5	10.7 ± 0.2	76.9 ± 2.3
6	0.5 : 0.25	10.9 ± 0.2	100.0 ± 0.0
7	0.5 : 0.1	5.9 ± 0.1	100.0 ± 0.0
8	0.5 : 0. 05	3.7 ± 0.4	100.0 ± 0.0
9	0.1 : 0.01	1.1 ± 0.2	69.1 ± 2.3
10	0.05 : 0.005	0.7 ± 0.01	15.4 ± 1.3

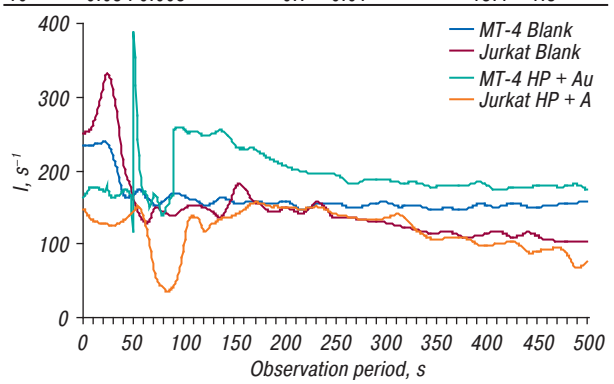

Fig. 2. The chemiluminescence kinetics in Luminol solution following the injection of different cell suspensions irradiated by red laser light (635 nm, 50 J/cm²): MT-4 without photosensitizer (MT-4 Blank), Jurkat without photosensitizer (Jurkat Blank), MT-4 with nanocomposite photosensitizer (MT-4 HP + Au), Jurkat with nanocomposite photosensitizer (Jurkat HP + Au)

In conclusion, we established that photodynamic *in vitro* activity of synthesized hematoporphyrin-nanogold composites is much higher than that of the original photosensitizer. The better activity of the nanocomposites with gold particles of 45 nm *vs* such of 15 nm, which was demonstrated in the experiments, can be apparently connected with the fact that bigger particles are able to transport more porphyrin molecules into malignant cells. The results obtained warrant the necessity of further studies with hematoporphyrin-gold nanocomposites *in vivo* on transplanted tumors of animals which have to define the real perspectives of the nanocomposites application in PDT.

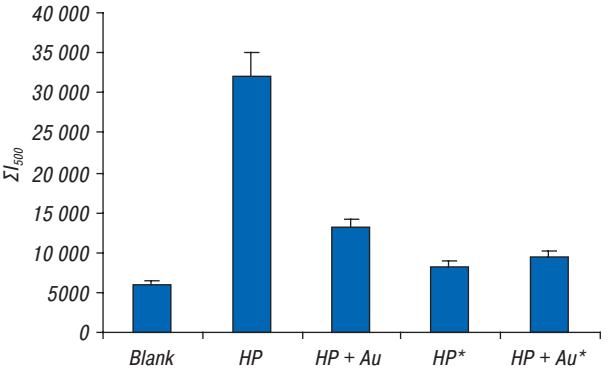

Fig. 3. The total intensity of the Luminol solution luminescence during 500 s following the injection of different Jurkat cell suspensions: irradiated by red laser light (635 nm, 50 J/cm²) without photosensitizer (Blank), hematoporphyrin-treated without irradiation (HP), nanocomposite-treated without irradiation (HP + Au), irradiated by red laser light (635 nm, 50 J/cm²) after hematoporphyrin-treating (HP*), irradiated by red laser light (635 nm, 50 J/cm²) after nanocomposite-treating (HP + Au*)

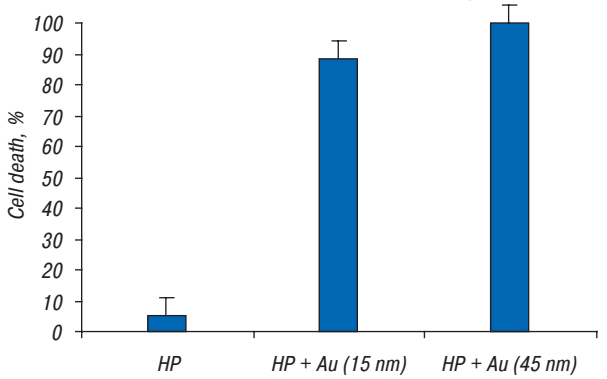

Fig. 4. Photodynamic activity of hematoporphyrin (HP) and its gold nanocomposites (HP + Au) (MT-4 cells, irradiation dose of 50 J/cm²)

REFERENCES

11. **Sibata CH, Colussi VC, Oleinick NL, *et al.*** Photodynamic therapy in oncology. Expert Opin Pharmacother 2001; **2**: 917–27.
12. **Allison RR, Bagnato VS, Cuenca R, *et al.*** The future of photodynamic therapy in oncology. Future Oncol 2006; **2**: 53–71.
13. **Palumbo G.** Photodynamic therapy and cancer: a brief sightseeing tour. Expert Opin Drug Deliv 2007; **4**: 131–48.
14. **Oleinick NL, Evans HH.** The photobiology of photodynamic therapy: cellular targets and mechanisms. Radiat Res 1998; **150**: S146–56.
15. **Chen B, Pogue BW, Hasan T.** Vascular and cellular targeting for photodynamic therapy. Crit Rev Eukaryot Gene Expr 2006; **16**: 279–305.
16. **Calzavara-Pinton PG, Venturini M, Sala R.** Photodynamic therapy: update2006. Part 1: Photochemistry and Photobiology. J Eur Acad Dermatol Venereol 2007; **21**: 293–302.
17. **Chaterjee DK, Fong LS, Zhang Y, *et al.*** Nanoparticles in photodynamic therapy: an emerging paradigm. Adv Drug Deliv Rev 2008; **60**: 1628–37.
18. **Stavropoulos NE, Kim A, Nseyo UU, *et al.*** Hypericum perforatum L. extract — novel photosensitizer against human bladder cancer cells. J Photochem Photobiol B: Biol 2006; **84**: 64–9.
19. **Mukherjee P, Bhattacharya R, Wang P, *et al.*** Antiangiogenic properties of gold nanoparticles. Clin Cancer Res 2005; **11**: 3530–4.
20. **Cai W, Gao T, Hong H, *et al.*** Applications of gold nanoparticles in cancer nanotechnology. Nanotechnology Science and Applications 2008; **1**: 17–32.
21. **Vladimirov YA, Arroyo A, Taylor JM, *et al.*** Quinolizin-coumarins as physical enhancers of chemiluminescence during

lipid peroxidation in live HL-60 cells. Arch Biochem Biophys 2000; **384**: 154–62.

22. **Klebanov GI, Liubitskiy OB, Vasilieva OV, *et al.*** Antioxidant properties of 3-oxipiridine derivatives: mexidol, emoxipin and proxipin. Voprosy Med Chem 2001; **3**: 76–81 (In Russian).

23. **Moliner-Martinez Y, Meseguer-Lloret S, Tortajada-Genaro LA, *et al.*** Influence of water sample storage protocols in chemiluminescence detection of trace elements. Talanta 2003; **60**: 257–68.

24. **Luksiene Z.** Photodynamic therapy: mechanism of action and ways to improve the efficiency of treatment. Medicina 2003; **39**: 1137–50.

25. **Wang CM, Cui H.** Electrogenerated chemiluminescence of luminol in neutral and alkaline aqueous solutions on a silver nanoparticle self-assembled gold electrode. Luminescence 2007; **22**: 35–45.

26. **Safavi A, Absalan G, Bamdad F.** Effect of gold nanoparticle as a novel nanocatalyst on luminol–hydrazine chemiluminescence system and its analytical application. Analyt Chim Acta 2008; **610**: 243–8.

27. **Vladimirov YuA, Potapenko AYa.** Physicochemical background of photobiological processes. Moskow: Vysshaya shkola, 1989. 199 p (In Russian).