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# ANTITUMOR ACTIVITY OF CONJUGATES OF THE ONCOFETAL PROTEIN ALPHA-FETOPROTEIN AND PHTHALOCYANINES *IN VITRO*

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## SUMMARY

The several conjugates of aluminium and cobalt complexes of phthalocyanines with human alpha-fetoprotein have been synthesized. Their cytotoxic activity against tumor cells and human peripheral blood lymphocytes was studied. The experimental data demonstrate that the cytotoxic activity of alpha-fetoprotein-phthalocyanine conjugates against three types of tumor cells of various origin is much higher (for aluminium and cobalt complexes more than 1000 and 50 times, respectively) in comparison with phthalocyanines themselves. The application of phthalocyanines as conjugates with alpha-fetoprotein makes it possible to markedly enhance the selective toxicity of phthalocyanines against human tumor cells.

*Key words*: alpha-fetoprotein, phthalocyanine, conjugates, tumor targeting, photodynamic therapy

## INTRODUCTION

One of the central problems of modern-day chemotherapy of cancer is the design of medicinal drugs displaying high selectivity against tumor cells. Earlier we have demonstrated [1] that the cytotoxic activity (CTA) of a broad spectrum of antitumor agents against cancer cells increases drastically when these drugs are used as conjugates with the oncofetal protein, alpha-fetoprotein (AFP) whose receptors are expressed by the majority of tumor cells [2,3]. In this case, the toxic activity of the conjugates (used in identical concentrations) against normal human lymphocytes was much lower than that for tumor cells.

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Much attention has been presently given to a new class of activated by visible light antitumor agents pertaining to the phthalocyanine (PC) series; their mechanism of action is similar to that of porphyrins. The principle of photodynamic therapy consists in accumulation by tumor tissues of a photosensitizer which displays no toxic activity under normal conditions as well as irradiation with red light that is not absorbed by cells or their components. Irradiation following the photosensitizer accumulation induces a photochemical reaction of the second type [4] which leads to the formation of singlet oxygen, eventually resulting in the destruction of biomolecules and intracellular organelles. Therefore, sensitizers designed for photodynamic therapy must possess at least two specific properties: firstly, the ability to be predominantly accumulated in tumor cells and, secondly, the ability to intensively absorb the red light. Both capabilities must provide the minimal side effect. In case of photodynamic therapy the side effect manifests itself in the damage of tissues surrounding the tumor as well as in skin and eye tissue injuries caused by natural light [5]. Photodynamic therapy employs the red light from laser sources which can be focused on the tumor and thus permits to avoid the damage of normal tissues.

Studies of intracellular localisation of phthalocyanines by using laser scanning fluorescent and electron microscopy methods revealed that phthalocyanine accumulation as an aluminium complex with different content of $SO_3H$-groups occurs in lysosomes, mitochondria and, in some cases, the nucleus [6-8].

Using fluorescent and radiolabelling techniques, it has been demonstrated that phthalocyanine accumulation in tumor cells largely prevails in comparison with normal cells [9-12]. However, in the skin, spleen and some other tissues [10,11] phthalocyanine accumulation appears to be as intense as in tumor cells. Hence a problem arises as to how to increase the selective activity of the drug.

Because of technical reasons photodynamic therapy can be used for localising tumors in the skin or hollow organs, to which the light can be conducted either in a natural way or with the help of flexible wavebeam guides. The use of dark (catalytic) therapy based on the generation of free oxygen species by a combined influence of water-soluble cobalt phthalocyanine and the reductive agent (e.g., ascorbic acid) expands the potentialities of the methods used for the treatment of tumors of more profound localisation [13,14]. However, in this case the role of directed delivery increases further in comparison with PDT when the formation of singlet oxygen can take place only in the laser irradiation zone. To increase the selectivity of phthalocyanine action, we have synthesized several conjugates of aluminium (PCAl) and cobalt (PCCo)

complexes of phthalocyanine with the alpha-fetoprotein and studied their cytotoxic activity against reinoculated lines of human tumor cells and human peripheral blood lymphocytes.

## MATERIALS AND METHODS

*Synthesis of AFP conjugates with phthalocyanines (PC) (AFP-PC).* Mono- or disulfochlorides of PC dissolved in DMSO (1-4 mg/ml) were added to an AFP solution in 0.2 M carbonate buffer (0.6-1.0 mg/ml) pH 9.2. The original sulfochlorides were synthesized by sulfochlorination of the corresponding unsubstituted PC with chlorosulfonic acid using slightly modified procedures earlier described for copper PC [15]. The mixture was incubated for 1 hour at room temperature (+20°C) and applied to a G-25 column (0.6 x 10 cm) equilibrated with PBS pH 7.2-7.4. The first, brightly stained peak was collected. PC concentration was measured by absorption at 690 nm. Protein concentration was determined by the method of Lowry [15].

*Cell lines.* Human T-lymphoblastoma QOS cells and human ovarian carcinoma CaOv cells were cultured in plastic cultural flasks (Costar) in the RPMI medium (Sigma) supplemented with 10% fetal calf serum (Sigma), 100 u/ml of penicillin and 100 μg/ml of streptomycin in a humidity atmosphere containing 5% $CO_2$ at 37°C. Human neuroblastoma IMR-32 cells were cultured under identical conditions except that the DMEM medium (Sigma) was used.

*Isolation of mononuclear leucocytes from peripheral human blood.* Human mononuclear leucocytes (ML) were isolated from whole blood by the method of [16]. The blood was centrifuged through a Ficoll-Paque solution (Pharmacia). ML was collected at the plasma/Ficoll-Paque interface, washed 3 times with a phosphate buffered saline (PBS), suspended in the same nutrient medium and cultured under the conditions used for the cultuvating of lymphoblastoma QOS cells. Lymphocyte proliferation was induced by the T-cell mitogen, phytohaemagglutinin P (PHA, 10 μg/ml) (Gibco).

*Determination of cytotoxic acitivity of AFP-PC conjugates (as complexes with aluminium(PCAl) or cobalt (PCCo)).* To examine the cytotoxic activity of the conjugates, the cells were placed into 96-well microtitration plates (Costar) (10,000 cells per well for lymphoblastoma QOS and 50,000 cells per well for neuroblastoma IMR-32), to which phthalocyanine (as PCAl or PCCo) was added either in the free form or as conjugates with AFP at the PC concentration varying from 1 nM to 10 μM. The cells were further incubated under standard conditions (2 hrs, 37°C) in a $CO_2$ incubator, after which the cultural medium was completely removed and replaced with a free portion containing no phthalocyanines or conjugates. In case of PCAl the cells were irradiated for 10 min with an incandescent lamp (60 Wt) with an emission maximum in the visible region of the spectrum. The distance from the light source was 15 cm. In experiments designed to study the cytotoxicity of PCCo-containing conjugates the cultural medium was also removed after incubation with the drugs and replaced with a free portion containing ascorbic acid whose concentration exceeded 10-fold that of PCCo. The cells were then placed into a $CO_2$ incubator for 72 hours, after which cell survival rates were established.

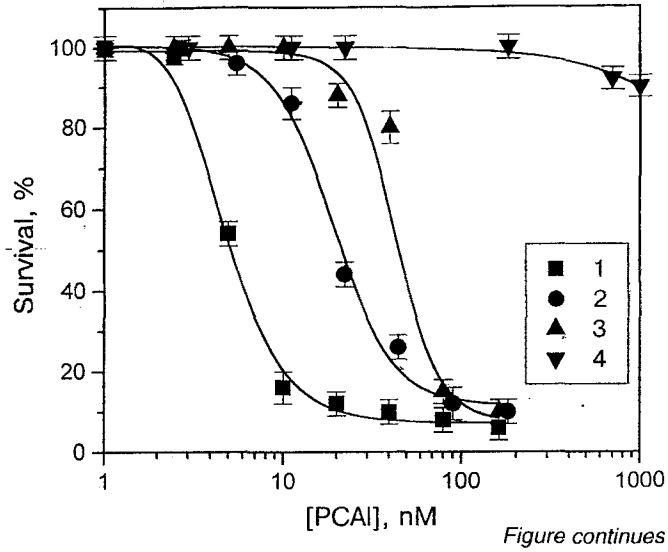
*Cytotoxic assay.* For quantitative estimation of cell survival rates we used the MTT test [17]. Two to four hours prior to the end of incubation, 50 μl of a MTT (3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide (Sigma) solution (1 mg/ml) dissolved in the medium used for cell culturing were added to each well. After staining the medium was removed, formazan crystals were dissolved in 150 μl of dimethylsulfoxide, and the intensity of staining was measured by absorption at 540 nm using a multiwell scanning spectrophotometer (Labsystem). Cell surival rates were evaluated as percentage of the corresponding control.

## RESULTS AND DISCUSSION

### *Cytotoxic activity of AFP-PCAl and AFP-PCCo conjugates*

AFP-PCAl conjugates (with AFP-PC ratios of 1-1, 1-2, 1-8, 1-20) and AFP-PCCo conjugates (with AFP-PC ratios of 1-2, 1-4, 1-9, 1-14) against human T-cell lymphoblastoma QOS, human neuroblastoma IMR-32 cells and human ovarian carcinoma CaOv cells were assayed for cytotoxic activity. It was found that the cytotoxic activity of the conjugates against tumor cells was much higher than that of free phthalocyanines (Tables 1, 2 and 3; Figures 1 and 2). The inhibition of the viability of tumor cells was especially strong when AFP conjugates with PCAl were used (Figure 1). This is apparently due to the fact that after receptor-mediated endocytosis induced by AFP interaction with its specific receptor on the surface of tumor cells, a significant part of the protein (in our case, the AFP-PC conjugate) enters into lysosomes. The formation of singlet oxygen as a result of irradiation of cells with visible light leads to the decay of lysosomal membranes and a resulting release of proteolytic enzymes "ingesting" tumor cells from inside. Inhibition of proliferation of tumor cells occurs in a dose-dependent manner. The cytotoxicity of the conjugates estimated from $IC_{50}$ (the concentration causing a 50% decrease in survival) depended on the origin of tumor cells and the AFP-PCAl ratio in the conjugate. The $IC_{50}$ values for AFP-PCAl conjugates are listed in Table 1.

The survival of tumor cells also depended on the PCCo concentration in the PCCo conjugates. Figure 2 shows the results of experiments designed to investigate the effects of

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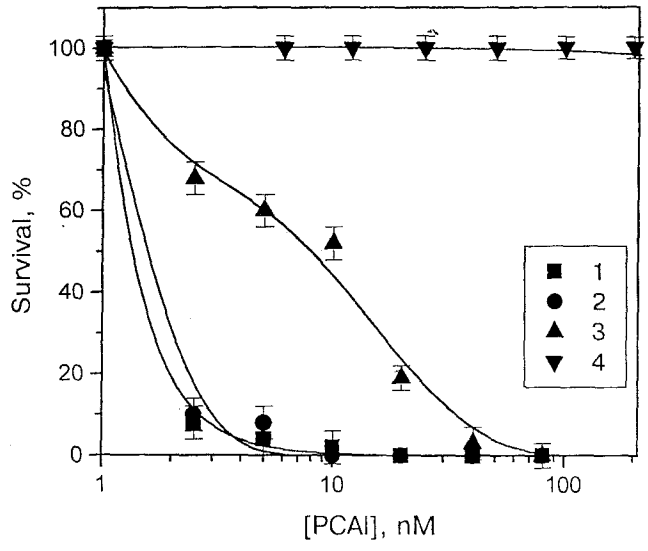


**Figure 1.** Cytotoxic activity of AFP-PCAl conjugates against human T-cell lymphoblastoma QOS a); AFP-PCAl ratio is 1-1 (1), 1-2 (2), 1-8 (3), PCAl (4); the same against human neuroblastoma IMR-32 b); AFP-PCAl ratio is 1-1 (1), 1-2 (2), 1-8 (3); PCAl (4).

**Table 1.** $IC_{50}$ values for AFP-PCAl conjugates at various AFP-PCAl ratios

| AFP-PCAl ratio | $IC_{50}$, nm QOS | $IC_{50}$, nm IMR-32 |
|---|---|---|
| 1-1 | 12 | 1.5 |
| 1-2 | 22 | 1.5 |
| 1-8 | 100 | 7.0 |
| 1-20 | - | 30 |
| free PCAl | >10 μM | >10 μM |

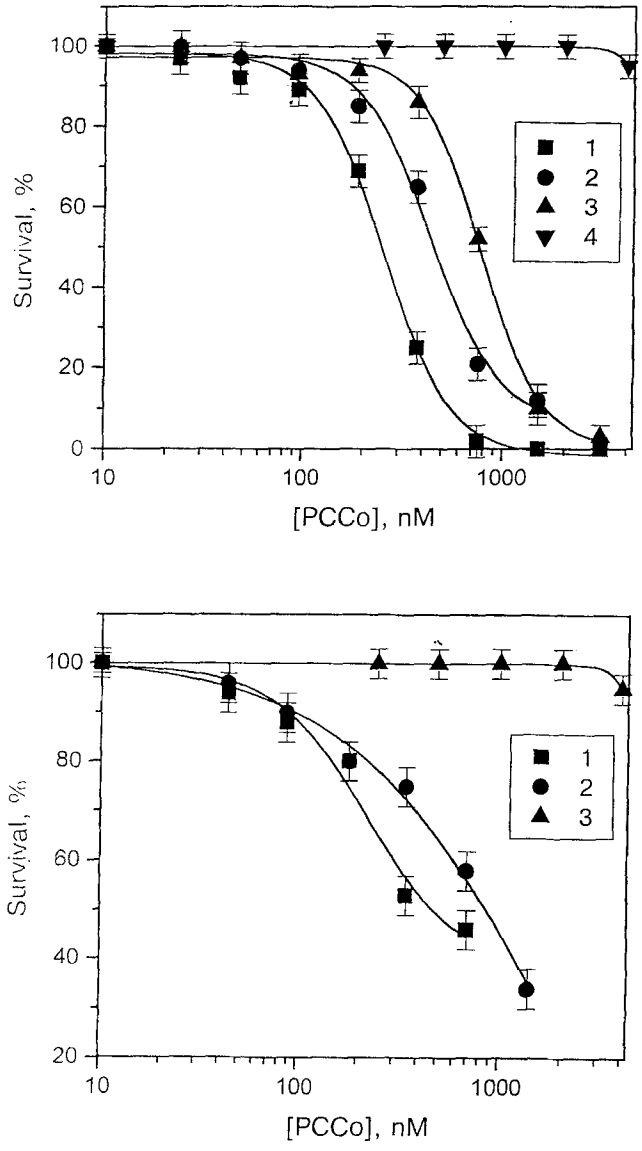


**Figure 2.** Cytotoxic activity of AFP-PCCo conjugates against human T-cell lymphoblastoma QOS a); AFP-PCCo ratio is 1-2 (1), 1-4 (2), 1-9 (3), PCCo (4); the same against human neuroblastoma IMR-32 b); AFP-PCCo ratio is 1-2 (1), 1-4 (2); PCCo (3).

various concentrations of three AFP-PCCo conjugates on survival rates of T-cell lymphoblastoma QOS and neuroblastoma IMR-32 cells after their 4-hr incubation with the conjugates and PCCo along. The cytotoxicity of conjugate AFP-PCCo (ratio 1-4) was 100 times higher in comparison with PCCo for ovarian carcinoma CaOv cells.

The $IC_{50}$ values for AFP-PCCo conjugates are given in Tables 2 and 3.

Thus, the experimental results unambiguously demonstrate that the cytotoxic activity of AFP-PC conjugates used as aluminum or cobalt complexes against three types of tumor cells of various origin is much higher than that of free PC complexes themselves.

***Selectivity of cytotoxic activity of AFP-PCCo conjugates in vitro***

To evaluate the selectivity of action of AFP-PCCo conjugates against human tumor cells, we compared the cytotoxic activity of the conjugates used at the AFP-PCCo ratio of 1-2 against human T-lymphoblastoma QOS cells and human peripheral blood lymphocytes. According to literary data, AFP-specific receptors are absent on the surface of unstimulated lymphocytes but

**Table 2**. $IC_{50}$ values for AFP-PCCo conjugates at various AFP-PC ratios

| AFP-PCCo ratio | $IC_{50}$, μM QOS | $IC_{50}$, μM IMR-32 |
|---|---|---|
| 1-2 | 0.2 | 0.7 |
| 1-4 | 0.4 | 0.8 |
| 1-9 | 0.7 | - |
| free PCCo | >10 | >10 |

**Table 3**. $IC_{50}$ and $IC_{90}$ values for AFP-PCCo conjugate (ratio 1-4) and free PCCo for ovarian carcinoma CaOv cells.

| | $IC_{50}$, μM | $IC_{90}$, μM |
|---|---|---|
| AFP-PCCo | 0.1 | 0.5 |
| PCCo | 10 | 50 |

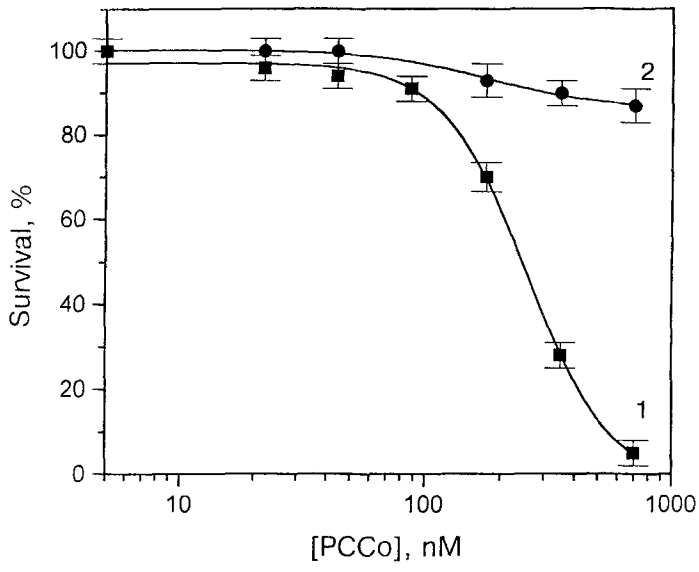


**Figure 3.** Cytotoxic activity of AFP-PCCo conjugates against human T-cell lymphoblastoma QOS (1) and human peripheral blood lymphocytes (AFP-PCCo ratio 1-2).

do occur on the surface of stimulated lymphocytes; however, they are much fewer in number (more than 10 times) in comparison with tumor cells [18]. The experimental results are depicted in Figure 3. It has been found that at the AFP-PCCo concentration causing complete decay of lymphoblastoma cells only a slight (about 10%) decrease in human lymphocyte survival rates is observed.

The summarized experimental date allow to make a conclusion that the application of PC as a conjugate with AFP makes it possible to markedly enhance the selective toxicity of PC against human tumor cells *in vitro*.

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