ABSTRACT
Chlorin e$_6$ has been known to be attractive as photosensitizer (PS) for PDT for long. Its usefulness as a photosensitizing part of photoimmunotoxines for targeted PDT, as a synton for further chemical modification and as a promising PS has been widely recognized. There is a patented procedure at our disposal now allowing for preparation of a stable, well soluble and filtrating form of chlorin e$_6$ - "Photodithazine". The elaborated production technology includes 4 steps. "Photodithazine" s 1-octanol/phosphate buffer, pH 7.4 partition coefficient (Kp) has been found to be 1.4, indicating that it must be able to localize in the plasma membrane. This may be a partial explanation of its higher photodynamic efficacy in vitro, comparing to "Photosense" (Al-sulfophthalocyanine) (Kp 0.05) and its closeness to "Photogem" (HpD) (Kp 1.5). "Photodithazine" possesses an intensive absorption band in the long-wave red field of the spectrum ($\lambda_{\text{max}}$ 663 nm, $\varepsilon$=3.82x10$^4$ M$^{-1}$ cm$^{-1}$, borate buffer, pH 9.2 with albumine addition), and a high photocytotoxic efficiency in vitro - 5 $\mu$M for EC$_{65}$. Thus, it represents a promising second generation PS.

Keywords: photodynamic therapy, photosensitizer, chlorin e$_6$, chlorins, drug form, drug substance, photocytotoxicity, cytotoxicity.

INTRODUCTION
Chlorins represent an interesting class of tetapyrrole compounds as far as their plant origination and photophysical properties are concerned. They are known to combine high values of interconversion coefficient (and, consequently, high quantum yields of singlet oxygen) with an intensive absorption in the range of 650-680 nm, where biological tissues are transparent to a larger extent than in case of porphyrins (610-620 nm). The latter is advantageous as to their applicability in cancer photodynamic therapy (PDT) to be used instead of porphyrins as the second generation photosensitizers (PSs).

The most well-known chlorin photosensitizers (PSs) are derivatives of chlorin e$_6$. It has already been shown$^1$$^2$$^3$ that some of them favour the tumor-to-muscle ratios of c.a. 10 in experiments with tumor-bearing animals after i.v. injections of 5-200 mg kg$^{-1}$ doses.

Easily obtained from pheophorbide-a in alkaline conditions$^4$, chlorin e$_6$, was by itself well biologically studied$^5$$^6$$^7$. It has been known to be suitable for PDT for long. However, to our knowledge, there were no reports about clinical applications of chlorin e$_6$, mainly because of difficulty of its high-scale production from biological substrates. General problems have been known to be short shell lives of the ready drug forms in solutions, poor solubility of the dry sodium salt forms and a bad antibacterial filtration ability of such solutions$^8$. Nevertheless, its usefulness as a photosensitizing part of photoimmunotoxines for targeted PDT$^9$$^{10}$$^{11}$ as a synton for further chemical modification$^{16}$$^{21}$, and as a promising PS$^5$$^7$$^{21}$ has been widely recognized.

Chlorin e$_6$ drug substance was produced by JSC "Veta" Ltd, Moscow, Russia. Its 1% sterile solution containing 0.5% of N-methyl-D-glucosamine as solubilizing and stabilizing agent (drug form "Photodithazine") was prepared at the Laboratory of drug form preparation of the Research institute of experimental tumor diagnostics and treatment of N.N.Blokhin All-Russian Cancer Research Center of Russian Academy of Medical Sciences.
As references here were used the only two drugs permitted for the clinical use in Russia - oligomerized hetnatoporphyrin-IX mixture "Photogem" (Russia) prepared at Moscow state academy of fine chemical technology at the Department of chemistry and technology of fine organic compounds headed by Prof. A.F.Mironov, and sulfonated aluminium phthalocyanine complex "Photosense" (Russia) prepared at the State research center of organic semiproducts and dyes, both of them being supplied from the clinics of N.N.Blokhin All-Russian Cancer Research Center of Russian Academy of Medical Sciences.

PREPARATION OF A STABLE, WELL SOLUBLE AND FILTRATING FORM OF CHLORIN e₆

There is a patented procedure at our disposal now allowing for preparation of a stable, well water soluble and filtrating form of chlorin e₆ - "Photodithazine"² (Fig. 1). The main feature of the molecule is the type of its counter-ion - N-methyl-D-glucosamine - in accordance with our statement that to stabilize big ions of PSs in aqueous media as well big counter-ion(s) are needed.

The elaborated production technology allows for "Photodithazine" drug substance standard series derived from pure pheophorbide-a, where contents of the active component exceed 95%. The technology includes 4 steps: (1) acetone extraction of chlorophyll a from dry biomass of cyanobacteria Spirulina Platensis, its demetallation with diluted hydrochloric acid and purification of the resulting pheophytin a; (2) acid hydrolysis of the latter in organo-aqueous medium to pheophorbide-a and its purification; (3) saponification of pheophorbide-a to chlorin e₆, its conversion to its N-methyl-D-glucosamine complex, purification by gel-chromatography and (4) antibacterial Millipore filtration and preparation of drug form "Photodithazine".

The most characteristic features of this technology are centrifugal purification in each step and a careful approach to step 3, when pheophorbide-a is treated with alkali. It is crucial to provide such conditions for the reaction to avoid the oxidation (alleromerization) reactions at carbon C-132 of the exocyclic ring of pheophorbide-a. This last requirement must be met during the previous steps as well. Otherwise, another derivative of 17,18-dihydroporphyrin series is predominantly obtained, namely purpurin 18, transforming in alkaline conditions to only poorly soluble in water chlorin p₆.

The long-wave part of "Photodithazine's" electronic absorption spectrum is given in Fig.2.

Fig.2: A part of "Photodithazine's" absorption spectrum for concentration 26 µM/ml in 0.01 M borate buffer with human serum albumine addition (1%).
2-OCTANOL/PHOSPHATE BUFFER PARTITION COEFFICIENT DETERMINATION

The 2-octanol/phosphate buffer, pH 7.4 partition coefficient (Kp) determination is known to be a routine procedure allowing for preliminary estimation of PS amphiphilic properties. It is well known that namely amphiphilic PSs are most photocytotoxic and tumorotropic. This is why this simple procedure can help partly predict PS biological properties and behaviour. We substituted 2-octanol for more available 1-octanol without losing the advantages of the procedure (Table 1).

Table 1: 1-Octanol/phosphate buffer, pH 7.4 partition coefficients.

<table>
<thead>
<tr>
<th>Photosensitizer</th>
<th>Kp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosensitizer</td>
<td>1.4</td>
</tr>
<tr>
<td>Photoditazine</td>
<td>1.5</td>
</tr>
<tr>
<td>Photosense</td>
<td>0.05</td>
</tr>
</tbody>
</table>

CYTOTOXICITY AND PHOTOCYTOTOXICITY ASSAYS

METHODS

Cytotoxic and photocytotoxic studies of "Photodithazine" have been performed in vitro using PC 12 cells (rat pheochromocytoma).

In PC12 test cells were seeded on 48 well dishes and maintained in 0.3 ml RPMI 1640 medium (Flow, UK) without phenol red, supplemented with 10% fetal calf serum, and gentamycin. All the dishes were kept in the dark at 37°C in a humidified and containing 5% carbon dioxide atmosphere and in black paper. MTT, 3-[(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma), was dissolved in PBS, filter sterilized and stored at 4°C. The PSs were added to the wells to the final concentrations of 0.5, 1, 2.5, 5, 10, 50 and 100 uM and incubated with the cells for 3h for spontaneous redistribution throughout them. There were three special controls in both light and dark experiments: (1) without MTT (with crystal violet dye), (2) with MTT, but without PS, and (3) with PS, but without MTT.

Dishes for light experiments were then irradiated with laser light: \( \lambda_{ex} \) 633 nm (helium-neon laser) for "Photogem", and \( \lambda_{ex} \) 670 nm (SHG YAP:2 laser) for the others at the doses of 20 J/cm².

After the photoirradiation, the dishes were incubated for 39 h. MTT solution (150 ul, 0.5%) was added to each well and incubated for 4 h at 37°C to allow for MTT metabolization. The crystalls
formed were dissolved within one night by addition of 300 µl per well 25% SDS solution and vigorously pipetted to ensure homogeneity of solution prior to scanning or using a shaker overnight. Then PBS solution was added to each well (50 µl, pH 7.4). The absorbances at 530 nm were measured using a Bio-Tek Instruments Multiscan plate reader. The results were compared with the control wells and expressed with respect to the control values. The high PS concentrations of 50 and 100 µM were found to carry in a big error in the subsequent formazane absorbance measurements due to their own absorption at 530 nm, and this is why the optical density of the wells was corrected by distraction the value of optical density of control wells (with PS, but without MTT).

For every PS two experiments were done: with photoirradiation («light») and without photoirradiation («dark»), and in each pair of the dishes there was found to be an equal amount of cells. There were 118,400 cells in each well for "Photodithazine", 107,500 ones for "Photosense", and 137,600 ones - for "Photogem" as determined by an assay with dye crystal violet. Using crystal violet the number of cells was determined the following way: The cell monolayer was stained with 0.2% crystal violet in 2% ethanol. After the staining cells were washed with water and the dye was eluted with 10% acetic acid. Number of the cells was calculated from the optical density of the eluate at 550 nm, the optical density (in 1 cm cuvettes) of 0.1 corresponding to c.a. 32,500 cells.

The amount of cells was determined to introduce a correction coefficient between experiments with different PSs (different dishes, seedings, etc.). Moreover, through crystal violet it became possible to find a coefficient to determine the amount of living cells by indirect way using MTT test and to follow the quantity of living cells at MTT test, because it was shown that both in light and in dark experiments the two methods gave similar results.

**PHOTOCYTOTOXICITY ASSAY RESULTS**

The results of the photocytotoxicity assay are shown in Table 2 and Fig. 3.

<table>
<thead>
<tr>
<th>PS efficacy/ cell culture</th>
<th>EC₅₀, UM</th>
<th>Photoditazine</th>
<th>Photogem</th>
<th>Photosense</th>
</tr>
</thead>
<tbody>
<tr>
<td>pheochromocytoma</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>PC12</td>
<td>5 (EC₆₅)</td>
<td>45 (EC₆₅)</td>
<td>25 (EC₆₅)</td>
<td></td>
</tr>
</tbody>
</table>

**CYTOTOXICITY ASSAY RESULTS**

The results of the assay in respect of PC 12 test are shown in Fig 4. Cytotoxicity in this case was found only at the concentrations over 50 (UM, and it appeared to be that "Photogem" was even more cytotoxic than "Photodithazine" a in there was no cytotoxicity of "Photosense".

**Fig.3: Photocytotoxicity curves for PC12 cells**

**Fig.4: Cytotoxicity curves for PC 12 cells**
DISCUSSION

It is seen from Table 1 that "Photodithazine" and "Photogem" are close in their properties, and they are amphiphilic, however, one can hardly tell what the "Photogem" components are to which one may attribute its amphiphilic properties. Therefore, structure-activity relationships in case of "Photogem" can hardly be followed. "Photosense" is as well a complex mixture, but one may see that it is an extremely hydrophilic mixture. Thus, few are components of "Photosense" which may penetrate into (through) the plasma membrane of a cell, neither it is likely to accumulate in tumors to a somewhat considerable extent. "Photodithazine" s 1-octanol/phosphate buffer, pH 7.4 partition coefficient (Kp) has been found to be 1.4, indicating that it must be able to localize in the plasma membrane. This may be a partial explanation of its higher photodynamic efficacy in vitro, comparing to "Photosense" (Kp 0.05) and its closeness to "Photogem" (Kp 1.5).

As regards the photocytotoxicity assay, if we exclude extremely low and high concentrations in PC 12 test, and see the range of 10-50 µM, then the PSs will make up the following sequence: "Photogem" > "Photodithazine" > "Photosense" (Fig.3). It is, however, worth mentioning that "Photosense" was most photocytotoxic at the low and very high concentrations. Generally speaking, PC 12 test seemed to us quite reliable, because cell death occurring after 1.5-3 h incubation prior to photoirradiation is known to come mainly from plasmatic membrane damage, and the more lipophylic PS is, the more likely plasmatic membrane will be its primary target. So, "Photogem" and "Photodithazine" are close in their photocytotoxicity, with "Photosense" being less photocytotoxic.

At the low and very high concentrations tetapyrrol PSs are observed to possess somewhat unexpected behavior, acting as either cell proliferation promoters or specific activators of some enzymes. This observation (done by other groups, too, e.g. J.-L.Merlin et. al. showed that an increase in both light fluence and HPD extracellular concentration resulted in a decrease in cytotoxicity [28]) may partly be proved by "Photogem" dark curve (Fig.4), supposing that with photoirradiation the zone of «promoter proliferations or «enzime activation») concentrations moves to the periphery of the scale. We observed this effect with many other porphyrins, and this is, probably, due to formation of some activated species after or during the photoirradiation, that this moving occurs. This effect seems to overlap with photocytotoxicity effects, making them look so poor in the periphery of the light curves. We would rather suppose that this was for dehydrogenase enzymes activity increase, because we could see in fact the amount of ornithine crystals go up instead of increasing the amount of cells. Besides, there was no such «proliferation» effect observed in the middle of the scale (10 µM) with CaOv cells, to which DNA synthesis inhibition method was applied.

From this point of view, in PC 12 test "Photosense" was «activating» through the whole scale, "Photogem"- extremely «activating» below 50 µM, "Photodithazine" was practically characterized by none of the effects below 50 µM, with both "Photodithazine" and "Photogem" being cytotoxic in the dark at 50-100 µM concentrations.
Thus, PC 12 cell test support the prognosis of Kp determination in terms of amphiphilicity, and there is a good correlation between Kp values and photocytotoxicity of PSs in the concentration range of 5-50 µM with PC 12 cells.

CONCLUSIONS
A novel chlorin e6 drug formulation has appeared in Russia - "Photodithazine". Few were the reports about clinical applications of chlorin e6, mainly because of difficulty of its high-scale production from biological substrates. Many of the associated problems have successfully been solved by us. We have provided for longer short shell lives of the ready drug forms in solutions (1-2 years), good solubility of the drug substance in water and antibacterial filtration ability of such solutions. The elaborated production technology now allows for "Photodithazine" drug substance standard series, where contents of the main component exceed 95%, that is practically impossible for sulfonated metallocomplexes or porphyrin oligomers being non-standard mixtures with different extent or order of sulfonation or polimerization.

Photocytotoxic properties of "Photodithazine" have been evaluated, and it was supposed that its high photodynamic efficacy was connected also with its ability to penetrate into biological membranes. It did not show any toxicity on PC 12 cell culture without irradiation except the range of very high concentrations.

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