One More PDT Application of Chlorin E_6

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ABSTRACT

In vitro and w vivo biological evaluation of a novel drug substance "Photodithazine" has been performed. *In vitro* photocytotoxicity (EC50) was 1 μM together with some cytotoxicity. *In vivo* acute toxicity has been found to be 170 me/kg for LD10, 175 mg/kg for LD16, 197 mg/kg for LD50, 220 mg/kg for LD84 (male hybrid mice Fl {CBA x C57Bl/6t}) following 1% aqueous solution i.v. injection. Pharmacokinetics and biodistribution studies have been done using the same mice bearing innoculated under the skin of the flanks T36 embryocarcinomas injected i.v. with 40 mg/kg dose of the drug. Maximal tumor and most organs' uptake was attained 1 h p.i., however, the drug's level in the organs rapidly decreasing to zero (generally by 24 h p.i.) with the best tumor/muscle ratios over 10 by 5 h p.i. "Photodithazine" has been" found to possess rapid clearance from the organism: 94% elimination 24 h p.i., and 98% - 48 h p.i. PDT has been performed in vivo involving 21-23 g A/Snell mice with the same type 0.9-1 g tumors injected 40 mg/kg drug i.p., with 670 nm light (SHG YAP:2 laser) being delivered 5-6 h p.i. at different doses (30-210 J/cm2). The best irradiation dose has been found to be 170 J/cm^2 with a sound necrotic effect and 1-3 week remission. Thus, "Photodithazine" represents a potent photosensitizer for PDT.

Keywords: drug substance, photodynamic therapy, photosensitizer, photocytotoxicity, cytotoxicity, toxicity, chlorin E6, biodistribution, pharmacokinetics, pre-clinic studies.

INTRODUCTION

A search for an optimal compound - photosensitizer (PS) allowing for the most successful PDT practical embodiment is supposed to be one of the acute problems of this modality.

An only widely used PDT drug now is known to be «Photofrin II» that together with an ability to tumor accumulation characteristic of porphyrins and their normally low toxicity is suffering from a series of drawbacks [1, 2, 3], e.g.:

- a low therapeutic ratio, equalling to 0,8 for the skin overlaying a tumor, 1,1 for the skin surrounding the tumor, and 2 (up to 5) for muscles;
- a very long (2.5-3 months) clearance period, mainly due to big systemically introduced doses and high non-specific affinity to normal proteins and glycoproteins;
- a low PDT efficacy connected with low yields of singlet oxygen as a consequence of little 630 nm light penetration to tissues;
- a considerable affinity to epithelial tissues, resulting in the red colour of skin during and after treatment accompanying with increased skin sensitivity to the daylight;
- a need in a pause of 24-72 h between «Photofrin II» introduction to a patient and irradiation of the tumor lesion, connected both with the fact that the therapeutic ratio is increasing by more rapid clearance from normal tissues comparatively to tumor ones, and with a marked growth in cytotoxicity (reallocation effect). During this pause patients must stay in a darkened room.

These «Photofrin II» (and its analogues «Photogem», «Photosan», ets.) draw-backs have led to an intencive search for PSs, the so called "second generation PS". As the body of experimental and clinival information grew, basic requirem' an optimal PS including biological (toxic and

pharmacocinetic), photophysical, chemical and technolological criteri'i postulated [4]:

- a strong absorption in the spectral range where biological tissues are mostly penetratable for light (red and near infra-red regions);
- an optimum between fluorescence quantum yield and interconversion quantum yield, the second one determining PS ability to generate singlet oxygen (at the same time, PS ability to fluoresceate determines its diagnostic value, makes it easier to follow its biodistribution and pharmacokinetics);
- their low dark and light toxicity in therapeutic doses;
- a high selectivity of their accumulation in neoplastic tissues and a rapid PS clearance from the skin and epithelial tissues;
- a high quantum yield of singlet oxygen in vivo;
- an easy way of preparation or chemical synthesis, a homogenious chemical composition;
- a good solubility in water or permitted for the intravenous injection liquids;
- stability to photobleaching and at storage.

The main question is still the depth of light penetration to tissues. The drugs used in clinic possess absorption maxima in the region of 620-675 nm. The penetration of light to biological tissues is little in this diapason and equals to a few millimeters. Maximum light tissue transparence lies in the far red and near infrared region of 750-1500 nm and corresponds to the generation diapason of efficient, reliable and available lasers. Creation and putting into practice PSs allowing for an effective singlet oxygen generation in this field of the spectrum will broaden the sphere of PDT application. At present there is an intensive search for such PSs among chlorin, benzoporphyrin, naphthalo- and pthalocyanine derivatives. PSs possessing a capacity of a quick accumulation in tumors and high rates of catabolism are of special interest. With time, as the history of cancer chemotherapy reads, a bank of PDT drugs of targetted action will be created, with adapting them for certain nosological and hystological forms of cancer.

Here we are reporting a brief overview of what has been done during the pre-clinic studies of a novel chlorin eg drug formulation - second generation PS, chlorin E6 N-methyl-D-glucosamine complex "Photodithazine".

STUDIES *IN VITRO*

METHODS

Cytotoxic and photocytotoxic studies of "Photodithazine" have been performed *in vitro* using CaOv cell line (human ovary carcinoma) routinely used at N.N.Blokhin All-Russian Cancer Research Center for prescreening of new anti-cancer substances.

In CaOv test the cells were maintained in RPMI 1640 medium, supplemented with 10% heat inactivated fetal calf cerum, penicillin and streptomycin at the temperature of 37°C in a 5% carbon dioxide atmosphere.

To excite the PSs a wide-band light source was used - a halogene lamp with the power of 250 Watts, bands of either 350-630 nm or 650-900 nm light being cut from its integral spectrum using a thermal and pasportized light filters. Using a projective optical system with a spherical reflector to increase irradiation flow intensity, an area was formed of 8x8 cm to deliver light to glass cultural bottles. The integral intensity of the flow was measured with a pasportized optical irradiation flow meter IMO-4S and it was found to be 22 mW/cm^2 with a bell-like fall of

intensity to the periphery of the irradiation zone up to 15 mW/cm^2. The cells were irradiated in such a manner that the area of each monolayer was c.a. 4 cm^2 (simultaneously 6 bottles, 2.5 cm in diameter each), thus exposition dose being 15 J/cm^2 or c.a. 60 J per each sample.

For cytotoxic studies, PSs of interest ("Photodithazine", "Photogem" and "Photosense") were added as aliquots of aqueous stock solutions to different final concentrations (100 μM, 50 μM, 25 μM, 5 μM, 0.5 μM) to the cells in medium and incubated in the dark for 48 hrs at the cultivation conditions.

To study photocytotoxicity, the PSs were added as aliquots of aqueous stock solutions to different final concentrations (100 μM, 50 μM, 25 μM, 5 μM, 0.5 μM) to the cells in medium and incubated 1.5-2 hrs to allow for their spontaneous redistribution throughout the cells and then were exposed to 350-630 nm (for "Photogem") and 650-900 nm (for "Photodithazine" and "Photosense") light at doses of 15 J/cm^2.

The irradiation was followed by incubation in the dark for 48 hrs. One hour prior to the end of the incubation period in both cases ^{3}H-mymidine was given to the media at the final concentrations of 1 μCi/ml. After incubation, the cells were thoroughly washed with cold Hanks' solution, perchloric acid (2.5%), and then acid insoluble residues were hydrolized with perchloric acid (5%) for 20 min at the temperature of 80°C. Aliquots of the hydrolizates were transferred to scintillation liquid SL-8, and the levels of sample radiation were determined using a scintillation counter. Supression of ^{3}H -thymidine incorporation in the cellular DNA served as an indicator of PS efficiency. It was calculated as radioactivity (in cpm) of the samples incubated with PS related to the one of the samples treated by the same way but without PS addition. Basing on these data the half-efficient concentration EC_{50} (of DNA synthesis suppression) was graphically determiaed (Table 1).

Table 1.: Photocytotoxicity assay results in terms of EC_{50}

cell culture	Photoditazine, μM	Photosense, μM	Photogem, μM
ovary adenocarcinoma, CaOv	1	50	56

IN VITRO ASSAY RESULTS

In CaOv model cytotoxicity (dark toxicity) was 17.2% DNA synthesis supression for "Photodithazine" and 9.2% - for "Photosense" at concentration 10 μM, e.i. "Photodithazine" appeared to be twice as much cytotoxic compared to "Photosense", and there was no cytotoxicity at "Photogem". "Photodithazine" toxicity on CaOv cells without irradiation can be characterized as low.

The order of efficiency in the light experiment (photocytotoxicity) was as follows: "Photodithazine" >> "Photosense" = "Photogem".

STUDIES *IN VIVO*

Obtaining of promicing data on "Photodithazine" photocytotoxicity promoted further studies on its acute toxicity *in vivo*, biodistribution, pharmacokinetics and photodynamic therapy (anti-cancer specific efficiency).

STUDYING ACUTE TOXICITY

The investigation was performed with 250 male hybrid mice Fl {CBA x C57Bl/6t} weighing 20-25 g. The drug was introduced in the form of 1% aqueous solution i.v. Quantitative and qualitative parameters of its toxicity were compared. The toxic doses were calculated by the known method of Litchfield and Wilkokson. Besides, the animal behaviour was being observed, terms of their deth were estimated. Clinical intoxication picture, behaviour reactions, macroscopic view of the internal organs of the dead animals were also carefully studied.

At a single i.v. "Photodithazine" injection at the doses of 150-300 mg/kg the following clinical intoxication picture was observed: flank position in 5-10 seconds accompanying with limb convulsion and a quick surfacial breath A complete adynamia was observed after 1-2 min that continued for 1-20 min. Some animals then started to feel better, limped walked sideward, fell on the flank, and then their state normalized after 5-8 min. Other mice developed apnoe, followed with the Chain-Stock type deep breath after 1-2 min, with the full abscence of the reaction on pain-causing and mechanical stimulations. A full muscle relaxation -grade III cataleption was seen in the next 1-2 min. Bradicardia was observed Signs, of death were seen during the following 1-2 min: breathing stop and abscence of heart activity. Eyes at this were open At lancing the following picture was observed: the heart was in the systole, the lumens of brain vessels were widen, bloodful, lungs were green-black, dense, with foam-like contents of the same colour. The weght of lungs was 2.5 times more than that of the intact animals. The weght of lungs of the survived mice normalized after 1 month.

The data obtained on clinical intoxication picture showed that the cause of the death of the experimental animals was the heart-respiratory insufficiency.

"Photodithazine" acute toxicity has been found to be 170 mg/kg for LD10, 175 mg/kg for LD16, 197 mg/kg for LD50, 220 mg/kg for LD84.

BIODISTRIBUTION

Redistribution studies of "Photodithazine" and Russian photosensitizers "Photosense" and "Photogem" as reference drugs have been done using 250 male hybrid mice Fl {CBA x C57Bl/6t} of 20-25 g body weight bearing innoculated under the skin of the flanks T36 embryocarcinomas and injected i.v. with 40 mg/kg dose of the drug as 1% aqueous solution.

The differing feature of "Photodithazine" distribution was its considerable uptake by the liver - an order of magnitude higher than by the other organs. However, this was for a short period of time, and the liver concentration decreased 5.5 times during 5 hs after administration, and dropped to zero after 24 hs.

Maximum tumor and most organs' uptake was attained 1 h p.i., however, the drug's level in the organs rapidly decreasing to zero (generally by 24 h p.i.). Maximum contrast was observed 4-6 h p.i. with the maximum tumor-to-muscle ratio about 15.

PHARMACOKINETICS

Pharmacokinetics studies of "Photodithazine" and the above reference drugs have been done using 250 male hybrid mice Fl {CBA x C57Bl/6t} of 20-25 g body weight bearing innoculated under the skin of the flanks T36 embryocarcinomas injected i.v. with 40 mg/kg dose of the drug as 1% aqueous solution. Fluorimetry was used to study pharmacokinetics.

The main way of "Photodithazine" elimination from the organism was found to be intestinal, that was evidenced by fluorescence intencity data. It was also found that the kidneys played an essential role in the elimination of the compound from the organism.

The basic amount of "Photodithazine" was eliminated in the first 2 days. No more than 6% of the drug initial dose was registered after 24 hs, and only 2% after 48 hs. The only permitted by now to the clinical usage in Russia drug "Photogem" (oligomerized hematoporphyrin-IX mixture, "Phorofrin I" analogue) had maximum tumor-to-muscle ratio up to 5 at 24-30 h p.i. with retention period of more than 4 weeks. Another drug - "Photosense" (sulphonated aluminium phthalocyanine mixture) undergoing stage III clinical trials now - kept in the organism of the mice for up to 3 months.

Thus, "Photodithazine" was found to possess a rapid clearance from the organism: 94% elimination 24 h p.i., and 98% - 48 h p.i. Pharmacokinetic properties of "Photodithazine" have been found to be considerably better than references' ("Photosense" and "Photogem").

OTHER STUDIES

Recently standard series of drug form "Photodithazine" have been estimated as regards their pirogenity, sterility, histamine-like impurities, acute toxicity with two kinds of animals (mice and rats), chronic toxicity, local irritating action, allergenity and immunogenity, the preliminary results unambiguously indicating to the possibility of prospective broad clinical testing.

By now, it has been shown that the drug is pyrogenic (with rabbits), this pyrogenicity being proved to be connected rather with the chemical structure of the photosensitizing part of the molecule, than with that of the aminosugar or impurities. The pyrogenity was registered in some cases already in the doses of 1/15 from the therapeutic dose 1 mg/kg.

The histamine-like action was studied on cats, and the studies resulted in the negative answer: the drug did not develop the likewise action even in doses hundred times exceeding therapeutic.

LD50 values in the experiments on acute toxicity with two kinds of animals (mice and rats) did not differ much (160 and 140 mg/kg, correspondingly), correlating well with the data received earlier with male hybrid mice and reported in part 3.1.

PHOTODYNAMIC THERAPY

Chlorin E6 as sodium salt has already been used by other groups as PS able to accumulate in tumors and causing their necrosis. Depending on a model, tumor-to-muscle ratio at the maximum concentration in tumor was reported to be 3 [5], 6-8.8 [6], 8.9 [7].

PDT was performed involving 21-23 g A/Snell mice with innoculated under the skin of the flanks T36 embryocarcinomas injected 40-50 mg/kg drug i.p. (under etheral narcosis), with 670 nm light (SHG YAP:2 laser) being delivered 5-6 h p.i. at different doses (30-210 J/cm^2) the ninth-tenth day after tumor innoculation. Depilation was perfomed prior to the irradiation. The weght of tumors in the control and experimental groups was 0.9-1 g.

PDT was given the following way. Five mice having tumors of the same weight and size made up a group Three of them were irradiated, the other two served as a control. Totally there were 4 PDT sessions, differing by laser irradiation flow intencity and time of the treatment (Table 2).

Table 2. Photodynamic therapy in mice.

Mean laser irradiation flow intencities, mW/cm	Time of treatment, min	Mean light exposure doses, J/cm^2
100	5	30
260	5	80
280	10	170
290	12	210

Each animal, except those from the control group, once irradiated, was observed for 1 month. The area of necrosis and general physiological parameters were estimated.

During 1st week after PDT there was a tumor surfacial necrosis in the central part of the irradiation zone with a maximum area of 50-70 mm^2. Comparing to the control group, some retarded tumor grouth was observed in the central part for 8-10 days after the light delivery, with the resumption of the grouth the next two weeks in the periphery and scab formation with no progression in the necrotic zone By the end of the 4th week the weight of the tumors in the experimental group was 3.0±0.3 g, whereas in the control group it varied from 3.8 to 4.2 g accompanying with an obvious cachexia.

Besides, the obtained results evidenced about the direct correlation between light doses and observed biological effects. For example, in the group of the animals irradiated with 30 J/cm2 the surfacial necrosis was observed on 7-8[11] day after treatment that was approx. 3 times later than usual. For the mice irradiated with 80 J/cm^2 some biological changes were registered on the 5 day whereupon necrotic processes were expressed weakly. At the irradiation with 210 J/cm^2 a sound necrotic effect was seen as a dark-brown spot in the middle of the tumor the 2nd day after PDT. However, 1 of the 3 mice from this group died after 3 days. The best irradiation dose was found to be 170 J/cm^2 with a strong necrotic effect and 1-3 week remission.

The data received led us to the conclusion about an expressed photodynamic efficiency of "Photodithazine" *in vivo* experiments with mice.

CONCLUSIONS

A novel chlorin e6 drug formulation has appeared in Russia - "Photodithazine".

- "Photodithazine" possesses an intensive absorption band in the long-wave red field of the spectrum (Mr 983, λ_{max} 663 nm, ε=38,200 M^{-1} $cm^{"1}$, $E^{1\%\ lcm}$ = 390, borate buffer, pH 9.2).
- It is fluorescent between 660 and 680 nm (half-width of the band) and, at the same time, it has a high interconversion quantum yield of 75%. Though, regarding exciting the molecule in 663 nm band, the resolution is poor.
- It has been found to be characteristic of a low dark acute toxicity. For comparison, synthetic phthalocyanine metallocomplex "Photosense" is an even better photosensitizing and fluorescent drug, but its general toxicity after i.v. administration has appeared to be considerably stronger than "Photodithazine"'s. The insufficiency of "Photosense" as regards its acute and chronic toxicity has become even more obvious since its clinical trials in Russia started.
- Maximum contrast with "Photodithazine" was observed 4-6 h p.i. with the maximum tumor-to-muscle ratio about 15 and the full clearance period of 24-36 h p.i. Optimally, light was to be delivered 0.5-7 h p.i. "Photosense" displayed no tumor selectivity and clearance period for up to 3 months. "Photogem" therapeutic index was less than 5 after 24-30 hs p.i., the

- Maximum contrast with "Photodithazine" was observed 4-6 h p.i. with the maximum tumor-to-muscle ratio about 15 and the full clearance period of 24-36 h p.i. Optimally, light was to be delivered 0.5-7 h p.i. "Photosense" displayed no tumor selectivity and clearance period for up to 3 months. "Photogem" therapeutic index was less than 5 after 24-30 hs p.i., the drug being preserved in the organs and tissues for more than 4 months.
- As a drawback of "Photodithazine", its pyrogenicity can be mentioned.
- "Photodithazine" favours an expressed specific PDT activity. However, PDT protocol needs to be further specified. The proposed therapeutic doses for the humans are between 1 and 2 mg/kg.
- "Photodithazine" is easily obtained from *Spirulina Platemis* cyanobacteria or some other source of plant origine and has a homogenious chemical composition;

Thus, "Photodithazine" represents a promising second generation PS for the clinical testing.