Perfluorocarbon nanoemulsions containing fluorinated photosensitizer for photodynamic cancer therapy

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Abstract. The composition of the photosensitizer and fluorocarbons with high oxygen capacity allows to increase the effectiveness of photodynamic therapy, which is shown on tumor cells. To achieve the solubility of photosensitizers in fluorocarbons, a number of fluorinated porphyrins were synthesized and their spectral characteristics were obtained. Fluorocarbon emulsions without a photosensitizer are inert for tumor cells of human colon adenocarcinoma. Fluorocarbon nanoemulsion with leader fluorinated porphyrin with fluoroaliphatic substituents accumulates in tumor cells and causes photoinduced necrosis due to peroxidation of membrane lipids. Perfluorocarbon nanoemulsions in combination with a dissolved fluorine phase photosensitizer are promising compositions for the photodynamic therapy of cancer.

1. Introduction

The promising development direction of photodynamic therapy (PDT) is using of a photosensitizer (PS) with fluorocarbon nanoemulsions in combination [1]. Fluorocarbon emulsions with high oxygen capacity increase the efficiency of PDT. In hypoxia conditions they create a fundamental possibility of using PDT method to initiate the death of tumor cells. Introduction of PS into the fluorine phase of the emulsion and modification of PS to give them solubility in fluorocarbons are demanded. The additional introduction of such fragments besides the solubility, can also contribute to a higher resistance to oxidation by reactive oxygen species. Development of such emulsions requires solving the following tasks: synthesis of PS (porphyrins) soluble in fluorocarbons, investigation of spectral characteristics and cytotoxicity, developing the composition of stable fluorocarbon emulsions, and investigation of the emulsions photodynamic activity in vitro.
2. Photosensitizes soluble in fluorocarbons

2.1. Synthesis

Fluorine-containing porphyrins were obtained according to the previously report [2], from unsubstituted pyrrole and perfluoroalkyl-containing aldehydes. Perfluoroalkyl-substituted benzaldehydes were prepared from commercially available pentafluorobenzaldehyde (PFBA) and polyfluorinated alcohols with different aliphatic chain length. Nucleophilic substitution of fluorine in the para-position of PFBA was carried out using a soft base - potassium fluoride in dimethylformamide (DMF). The pyrrole-aldehyde condensation was carried out according to the Lindsey method in methylene chloride under with boron trifluoride etherate as a Lewis acid and a dehydrating agent. Then unstable porphyrinogen (without isolation) was oxidized by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (Scheme 1).

\[
\begin{align*}
\text{PFBA} & \rightarrow 1 \text{a-c} \\
2 \text{a-c} & \rightarrow \text{Scheme 1. Synthesis of porphyrins 2a-c from perfluoroalkyl-substituted benzaldehydes 1a-c.}
\end{align*}
\]

Synthetic strategy for obtaining porphyrins from aliphatic polyfluorinated aldehydes (Scheme 3) has several differences. In the first step pyrrole-aldehyde condensation proceeds without acid, the product (oxyalkylated pyrrole) was isolated by crystallization from the reaction mixture and was used further without purification. The tetramerisation of oxyalkylated pyroles 3a,b was carried out in the presence of a strong acid (para-toluenesulfonic acid) in toluene with azeotropic water distillation. The oxidation of porphyrinogen, as in the previous method, was carried out in situ by DDQ.

\[
\begin{align*}
\text{Scheme 3. Synthesis of porphyrins 4a,b from polyfluoroaliphatic aldehydes.}
\end{align*}
\]

Porphyrins obtained by these methods were purified by silica gel column chromatography. Synthesis procedures and characterization of all compounds are given in Supplementary materials.

2.2. Solubility

The introduction of polyfluoroaliphatic substituents into the porphyrin structure was necessary first of all to give them sufficient solubility in fluorocarbons, in particular in perfluorodecalin, the main component of our fluorocarbon emulsions. Saturated solutions of porphyrins were prepared by the following steps: heating to complete dissolution of porphyrin, keeping at room temperature for a day. The final concentration of porphyrins was determined by spectrophotometry using a series of dilutions to obtain more correct results. The obtained porphyrins showed good solubility in perfluorodecaline -
1 mM, and the solubility values were practically independent of the length of the perfluorocarbon fragment.

2.3. Optical characteristics
Absorption spectra of porphyrins with perfluoroaliphatic substituents were classic for this type of compounds, i.e. the spectra of all compounds consist of an intensive Soret band of about 400 nm and four Q-bands of lower intensity (Figure 1A), all data are shown in Table 2. The extinction coefficient of the fourth Q-band at 650 nm for porphyrins with aliphatic substituents in the meso-position several times higher than aromatic analogues, which is generally characteristic of non-fluorinated compounds (Figure 1B).

The fluorescence spectra of porphyrins have two maxima (Figure 1C), their intensities for aromatic derivatives are close, while for aliphatic long-wave maximums are noticeably lower. The fluorescence quantum yield for all PSs is low, which is explained by the predominant formation of an excited triplet state, rather than a singlet, which is necessary for the fluorescence emission.

2.4. Reactive oxygen species generation
Reactive oxygen species (ROS) are formed during energy transfer from the excited triplet state of a PS to an unexcited oxygen molecule. It is believed that in nonpolar media (nonpolar organic solvents, perfluorocarbons), singlet oxygen significantly prevails over other types of ROS, so they can be neglected in this case [3]. The efficiency of the singlet oxygen formation by the PS can be quantified by measuring its phosphorescence at 1275 nm. The quantum efficiency of this process is calculated relative to the well-known standard with which measurements were made under the same conditions. The values of the quantum efficiency of the singlet oxygen generation were calculated according to formula (1) relative to the literature standard meso-tetrakis(pentafluorophenyl)porphyrin [3], the obtained values are presented in Table 1.

\[
\phi = \phi_{st} \times \frac{a_{exp}}{a_{st}},
\]

where \( \phi \) is the quantum yield, \( a \) is the slope of the I-D graph (I is the emission intensity, D is the optical density).
Table 1. Optical characteristics of porphyrins 2a-c and 4a,b, the singlet oxygen generation efficiency.

| № | Absorption | Fluorescence | Singlet oxygen generation | φ |
|---|-------------|--------------|---------------------------|---|
|    | λ, нм (ε, 10^3 М^-1 см^-1) | λ, нм | Qy(1,0) | Qy(0,0) | Qx(1,0) | Qx(0,0) | Qx(0,1) |
| 2a | 407 | 503 | 533 | 582 | 653 | 660 | 715 | 0.016 |
| 2b | 407 | 502 | 532 | 582 | 653 | 660 | 715 | 0.017 |
| 2c | (137.2) | 501 | * | 583 | 654 | 660 | 715 | 0.016 | 0.83 |
| 4a | 408 (55.3) | 512 (5.1) | 546 | 595 | 649 | 655 | 720 | 0.012 | 0.70 |
| 4b | 410 | 514 | 547 | 598 | 650 | 655 | 720 | 0.011 | 0.72 |

All spectra registered in benzene at the same conditions as the selected standard. The values of the singlet oxygen generation efficiency allow us to conclude that the fluorine-containing porphyrins are promising PS for PDT. Porphyrins 2c and 4b were selected for further study. Fluorocarbon emulsions were prepared with them and were studied in vitro for photodynamic activity.

3. Fluorocarbon emulsions

Direct emulsions perfluorocarbons-in-water are stabilized using pluronics [4], phospholipids [5] and some others [6]. We used the proxanol-268 as emulsifier, which has proven itself in the composition of the “Perforan” (medical acceptable fluorocarbon emulsion). The composition of the fluorocarbon phase was also taken by analogy with Perforan - perfluorodecalin (PFD) - / perfluoromethylcyclohexylpiperidine (PMCP) = 2: 1. An ultrasonic dispersion apparatus Sonicator W-375 (Heat systemultrasonics) for emulsification was used. Our experience has shown that to obtain nanoscale emulsions, it is necessary to use a glass reactor cooled in ice-bath (sufficient heat removal and the possibility of visual control of the process). The dispersion conditions were selected by varying the power and exposure time, the particle size was controlled by dynamic light scattering, the obtained data are shown in Table 2.

Table 2. Test conditions of preparation of fluorocarbon emulsion.

| Entry | Full time, min | Ultrasound mode (time, min/power, %) | Average particle size, nm |
|-------|----------------|--------------------------------------|--------------------------|
| 1     | 0.5            | 0.5/50                               | 384±5                    |
| 2     | 2              | 1/50 + 1/50                          | 219±6                    |
| 3     | 3              | 3/80                                 | 204±10                   |
| 4     | 4              | 2/50 + 2/50                          | 205±2                    |
| 5     | 6              | 3/50 + 3/50                          | 216±16                   |
| 6     | 6              | 3/50 + 3/80                          | 212±7                    |
| 7     | 6              | 3/80 + 3/80                          | 224±11                   |
| 8     | 6              | 2/50 + 2/50 + 2/80                   | 232±16                   |
| 9     | 6              | 2/50 + 2/80 + 2/80                   | 229±8                    |

As a result of preliminary experiments, optimal conditions for preparing emulsions were found. The average particle size was about 205-220 nm, which is optimal for us (grey lines in Table 2). Subsequently, for the preparation of “single” (without PS) and loaded (with PS) fluorocarbon emulsions, we used the following dispersion conditions: two times 3 min at 50% and 80% power with and cool 2-5 min between actions. The content of PS in the fluorocarbon phase of the emulsion was chosen based on their solubility. As shown in Section 2.2., the solubility of all porphyrins in PFD was
about 1 mM. To prevent possible precipitation, because the PFD / PMCP mixture is used, we took half of this value, i.e. 0.5 mM in the fluorocarbon phase and 0.033 mM in the emulsion. Thus, the ratio of the emulsion components was taken as follows: porphyrin - 0.033 mM, a mixture of fluorocarbons - 12.2 wt. %, proxanol-268 - 2.5 wt. %, NaCl - 0.7 wt. % and water. The average particle size of the emulsion samples obtained under optimized conditions was 210-220 nm.

4. Cytotoxicity of porphyrins in emulsion on tumor cells in vitro

A photosensitizer (PS) as a potential agent for the photodynamic treatment of tumors should be low cytotoxic in the dark and cause the death of tumor cells due to its photoactivation. We investigated the dark and photoinduced cytotoxicity of fluorinated porphyrins using the MTT assay [2]. As PS, we chose compounds 2c and 4b containing fluoroaryl and fluoroalkyl substituents, respectively. Photosensitizers were used in a “free” form and in “emulsion” form. “Free PS” is a solution of PS in DMF (10 mM), diluted serially with DMEM culture medium to final concentrations when introduced into cells. Emulsions with 2c and 4b contained 0.033 mM of the corresponding PS and were introduced into the cells until the final concentrations of the PS were reached. Compounds were introduced into the cells of human colon adenocarcinoma HCT116 in the range of photosensitizer concentrations of 5-20 μM. In this experiments, the final concentration of DMF solvent did not exceed 0.5%, which allows us to neglect the toxic effect of this solvent for free PS.

Dark cytotoxicity after 72 hours of incubation of cells with 2c free, 4b free, 2c emulsion, 4b emulsion was insignificant (half maximal inhibitory concentrations IC₅₀ were not achieved in the concentration range up to 20 μM and 72h incubation). This means that dark cytotoxicity of fluoroporphyrins is very low (less than 10%, curves not shown). An emulsion without a PS (“Emulsion w/o PS”) is also non-toxic in the dark, it contributed to an increase in cell growth up to 10%.

The study of photoinduced toxicity of fluoroporphyrins 2c and 4b (free form and emulsion form) included the following steps. Porphyrins were accumulated in HCT116 cells during 24 h of incubation, laser irradiated, incubated for 24 h for death, the survival rate was determined by the MTT test (data on cell survival in relation to the number of untreated cells was taken as 100%). As is known, absorption in the red region is important for potential photosensitizers, since this light has the greatest ability to penetrate tissues. For the photoexcitation of porphyrins, a 633 nm laser was used, a dose of 33 J / cm². Upon photoexcitation of fluoroporphyrins 2c and 4b, photoinduced cell death was present for both free and emulsion forms (Figure 2).

Figure 2. Photoinduced cytotoxicity of fluorporphyrins in DMF and emulsions on HCT116 cells.

In the free form, fluoroporphyrins 2c and 4b cause small (up to 30%) cell death due to photoinduced cytotoxicity. They are photoactive, but the small cell death for the free form can be
explained by their increased hydrophobicity, since they have fluorine substituents. An increase in the efficiency of the photo-damaging effect of fluoroporphyrins was achieved due to their inclusion in the composition of fluorocarbon emulsions. The photoinduced cytotoxicity of emulsions with porphyrins is greater than in free form due to the solubility of PS and oxygen in fluorocarbons. Emulsion 4b is more cytotoxic than emulsion 2c. This correlates with spectrometry data: porphyrins with fluoroalkyl substituents in comparison with fluoroaryl analogues have a large absorption in the red region for the presented series. Thus, the formulation of fluoroporphyrins in emulsions contributes to an increase in their photoinduced cytotoxicity compared to free fluoroporphyrins.

Fluoroporphyrin 4b is characterized by a large difference between the photoinduced cytotoxicity in the emulsion compared to the free form than by fluoroporphyrin 2c. Thus, the advantage of introducing fluoroporphyrins into the composition of fluorocarbon emulsions with the aim of increasing their photoinduced cytotoxicity has been shown. The data obtained made it possible to choose emulsion 4b as a leader composition for characterization the mechanism of cell death.

5. Emulsion-accumulated cells die according to the mechanism of photoinduced necrosis
It is known that photosensitizers after photoexcitation generate reactive oxygen species, which leads to damage to cells, in particular, their lipid membranes. Along with the necrotic effect, PS can also cause other types of cell death. For fluorinated porphyrins 2c and 4b, dark cytotoxicity after 72h of incubation with cells is minimal; therefore, we characterized photoinduced necrosis as the main mechanism of cell death. The excitation of PS by such a dose of light, which is capable of causing necrosis, is interesting for initiating cell death of various tissue origin. The fast and irreversible destruction of plasma membranes, characteristic of necrosis, allows the use of the PDT method on a wide range of tumor cells, including cell subline resistant to proapoptotic chemotherapy drugs. In the case of initiation of photonecrosis, the main events of the mechanism are the accumulation of a PS in the cell (in this case, emulsions), its photoexcitation with the generation of reactive oxygen species, and lethal damage to membranes, in particular, cytoplasmic membrane.

The distribution of the leader composition in the cells was visualized after 24 h incubation HCT116 cells with 4b emulsion (final PS concentration 10 μM) by confocal laser scanning fluorescence microscopy microscopy (Figure 3). The 4b emulsion enters the cells and remains into it for 24 hours; it accumulates not only in the cytoplasmic membrane, but also in the cytoplasm. The emulsion does not entres the nucleus.

![Figure 3. Cytoplasmic distribution of the 4b emulsion. Green - Hoechst Ex405Em415-485, purple - porphyrin 4b Ex405Em600-700.](image)

Lipid peroxidation was visualized by confocal microscopy and shown on 4b emulsion. HCT116 cells were incubated with a emulsion for 24 h (final PS concentration 10 μM), after which they were stained with bodipy ceramide, a marker of lipid peroxidation. The reduced and oxidized form of the
marker has different characteristics of excitation and fluorescence, which allows us to separate the signals in microscopy (Figure 4).

Figure 4. Lipid peroxidation after photoexcitation of fluoroporphyrin 4b in an emulsion. Green - Hoechst Ex405Em415-485, blue - BODIPY581/591C11 Ex543Em560-650, red - BODIPY581/591C11 Ex488Em500-580.

Untreated control cells and cells incubated with an emulsion without a photosensitizer do not cause lipid peroxidation. Fluoroporphyrin in the emulsion causes an increase in the ratio of the oxidized form of the lipid peroxidation marker to the reduced one. This effect develops within 2 minutes after photoexcitation. This corresponds to lipid peroxidation of the cytoplasmic membrane and membrane organelles in accordance with the intracellular distribution of the PS. The oxidation of membranes, especially cytoplasmic, is lethal for the cell and is an event preceding necrotic death due to photoexcitation.

The cell membranes photodamage resulting from the excitation of a PS is visualized through the penetration of propidium iodide (PI) into the cell, and such membrane permeability is irreversible and is a marker of cell necrosis [7]. Emulsion 4b (final concentration of a 10 μM PS, 24h) accumulates in HCT116 cells and, after illumination, after illumination, lipid peroxidation leads to cell membrane damage. Propidium iodide penetrates into such damaged cells and intercalates in the nuclei - this necrotic effect is visualized in fluorescence microscopy (Figure 5). Fluoroporphyrin 2b is not detected by a fluorescence microscope, which does not interfere with the signal of PI.
Figure 5. Phase and fluorescence microscopy of HCT116 cells, 1h after photodamage (633nm, 33 J/cm²), propidium iodide staining.

PI-positive cells in which necrosis occurred due to photoexcitation 4b of the emulsion was visualized by fluorescence microscopy 60 minutes after photoexcitation. We added PI to the cell buffer to the cells that accumulated the 4b emulsion, and performed a photoexcitation of the 4b PS with a 405 nm laser and a dose of 33 J / cm² using a confocal microscope, recording the dynamics of PI entering the cell nucleus from the buffer. It was revealed that after photoexcitation the minimum time of PI entry is 50 minutes (Figure 6).

Figure 6. Nuclei staining of HCT116 cells that accumulated emulsion 4b, (final concentration of PS 10 μM) and 2-50 minutes after photoexcitation (405nm, 33 J / cm²). Green - hoechst Ex405Em415-485, Red - PI Ex488Em560-630.

Thus, photoinduced necrosis is the mechanism of action of fluoroporphyrin 4b in emulsion form. An accumulation of emulsion 4b in the cells and its cytoplasmic distribution takes place within 24 hours, peroxidation of membrane lipids during 2 minutes after photoexcitation at a dose of 33 J / cm², and after 1 hour, cell necrosis. Necrosis develops irreversibly, as shown by staining with a propidium iodide - necrosis marker.

Conclusions
We synthesized fluorine-containing photosensitizers of the porphyrin class, soluble in fluorocarbons, for the photodynamic treatment of tumors. The introduction of fluorine substituents into the structure of the porphyrin cycle made it possible to achieve the solubility of photosensitizers in fluorocarbons, gas-transporting components of emulsions. The prepared fluorocarbon emulsions containing a
fluorinated photosensitizer showed low dark cytotoxicity and submicromolar photoinduced cytotoxicity on human HCT116 adenocarcinoma cells. Emulsions with a photosensitizer accumulate in the cells within 24 hours and within 72 hours do not show a dark damaging effect. With photoexcitation of the photosensitizer in the emulsion, it was possible to achieve peroxidation of the cell membranes, including the cytoplasmic one, and to cause rapid photonecrosis, which develops within 1 h after photoexcitation. An emulsion without a photosensitizer is inert to cells for 72 hours both in the dark and after illumination. The data obtained make it possible to characterize fluorocarbon emulsions as promising compositions for placing fluorinated photosensitizers in them for photodynamic therapy of tumors.

The materials and methods used in the work are given in the Supplementary materials.

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