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Can Efficiency of the Photosensitizer Be Predicted by Its Photostability in Solution?[1]

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Abstract—We have investigated a possible correlation between the photostability and photodynamic efficacy for different photosensitizers; hematoporphyrin derivatives and chlorines. To perform such analysis, we combined the depth of necrosis (d_{nec}) measurement, expressed by the light threshold dose and a photodegradation parameter, measured from investigation of photosensitizer degradation in solution. The d_{nec} analysis allows us to determine the light threshold dose and compare its value with the existent results in the literature. The use of simple models to understand basic features of Photodynamic Therapy (PDT) may contribute to the solid establishment of dosimetry in PDT, enhancing its use in the clinical management of cancers and others lesions. Using hematoporphyrin derivatives and chlorines photosensitizers we investigated their properties related to the photodegradation in solution and the light threshold dose (D_{th}) in rat livers.

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I. INTRODUCTION

Photodynamic therapy (PDT) is a minimally invasive treatment with great promise in malignant and other diseases. It can be applied before, or after, chemotherapy, ionizing radiation, or surgery, without compromising these treatments or being compromised itself. Unlike radiotherapy and surgery, it can be repeated many times at the same site [1–3].

PDT is a treatment modality available for palliation or eradication of several cancers and involves the use of a photoactive drug photosensitizer, light with appropriated wavelength and oxygen [4]. The photosensitizer absorbs light energy and induces the production of reactive oxygen species, as radicals and singlet oxygen in the tumour environment, generating a cascade of events that kills the tumour cells [2, 4–8].

The first generation photosensitizer, a more purified version of HpD called Photofrin® (porfirmer sodium) (PF) a complex of porphyrin oligomers, has been approved for specific clinical applications in several countries in Europe, America, and Asia and has been under investigation for other malignant and nonmalignant diseases [8–10].

Photosan®(PS) and Photogem® (PG) are the corresponding photosensitizers produced in Germany and Russia, respectively. They are believed to present characteristics chemically, photo physically, and by diagnostic and therapeutic features identical to Photofrin® [11–13]. However, a number of problems related with the use of Photofrin®, such as extended skin photosensitivity and poor absorption of tissue-penetrating red light, have led to the development of novel photosensitizers with more favorable characteristics, especially absorption of longer wavelength light, which penetrates deeper into tissue and have faster clearance from normal tissue [6, 14].

The second-generation photosensitizers have shorter periods of photosensitization, longer activation wavelengths, higher yields of singlet oxygen, and tumor selectivity [6, 15]. Chorines and bacteriochlorines, which present absorption bands in the red and near-infrared regions allow better tissue light penetration, thus making these photosensitizers interesting candidates for photodynamic therapy [13, 15, 16].

Photodithazine® (PDZ), Radachlorin® (RAD), and Foscan® (FOS) are photosensitizers of second generation. The PDZ, a glucosamine salt of chlorine e6 is a hydro soluble photosensitizer [17, 18]. RAD presents in aqueous solution a mixture of three chlorines, including sodium chlorine e_6 (90–95%), sodium chlorine p_6 (5–7%), and a third chlorine (1–5%) which is not disclosed. These chlorine constituents (called "chlorine active substance") form 98% of the drug substance by dry weight [19]. FOS is a m-Tetra(hydroxyphenyl) chlorine (m-THPC or temoporfin), and has been approved by the European Medical Agency (EMEA) for use in head and neck squamous cell carcinomas [16, 20].

[1] The article is published in the original.

Most photosensitive drugs such as porphyrins, chlorines and phthalocyanines, which are used in the photodynamic therapy of tumors, are not photostable. In simple solutions as well as in complex environments, photosensitizers undergo light-induced modification resulting in a decrease of their initial absorption and fluorescence intensity [21–24]. This process is normally referred as photodegradation or photobleaching and is defined as the chemical transformation of the photosensitizer molecule when irradiated by light (phototransformation of the molecule) leading to the formation of new absorbing photoproducts [22, 25].

It is believed that photodegradation during irradiation of photosensitizers in tissues modifies the photodynamic efficacy. Photodynamic reaction is normally evaluated investigating the photodynamic action in cell culture or depth of necrosis (d_{nec}) studies [26, 27].

Many authors have investigated the aspects of d_{nec} in normal tissues and they have observed the occurrence of a sharp boundary between necrotic and normal tissue [26]. Such results gave rise to the concept of a threshold light dose, implying that the cell death only occurs when the absorbed energy exceeds a minimum value. High values for light threshold doses imply in low photodynamic efficacy, since many photons must be present to cause irreversible cell damage [28].

Previously, we have found in a study limited to hematoporphyrin derivatives a correlation between photostability and photodynamic efficacy [27, 29].

We have investigated a possible correlation between the photodegradation and the photodynamic efficacy of different porphyrins and chlorines based photosensitizers. To perform such analysis we combined the d_{nec} measurement, expressed by the light threshold dose; and the photostability parameter, obtained from investigation in solution for the three main used photosensitizers based in hematoporphyrin derivatives: Photofrin®, Photogem® and Photosan® and chlorines: Photodithazine®, Radachlorin®, and Foscan®.

2. MATERIALS AND METHODS

2.1. Photosensitizers

The hematoporphyrin derivative photosensitizers used were: Photogem® (PG) obtained by Photogem LLC Company in Moscow, Russia, Photofrin® (PF) obtained from Axcan Pharm, Inc., in Canada and Photosan® (PS) obtained from Seehof Laboratories, in Germany. The chlorines photosensitizers used were: Photodithazine® (PDZ) and Radachlorin® (RAD) obtained by Rada-Farma and Veta-Grand Company in Moscow, Russia and Foscan® (FOS) obtained from Biolitec Pharma Ltd. in Dublin, Ireland. The solutions were prepared in water solution in the concentration of 17 μg/ml [21]. The porphyrins in solution are aggregates/monomeric species in equilibrium, however at this concentration, the solution is quite transparent (less than 10% attenuation through a 1 cm^3 cuvette), confirming that in this range of concentration (17 μg/ml) the aggregates in solution do not interfere with the measurements [29].

2.2. Photosensitizer Degradation

Photodegradation of photosensitizers were induced at a concentration of 17 μg/ml illuminated by a light emitting diode (LED) device, composed of emitters centered at the wavelength of 630 nm with variable irradiance. The illuminating system was composed of two LED devices, planar arrays facing each other emitting at 630 nm with 350 mW/cm^2. Porphyrins and chlorines were irradiated at 630 nm. The cuvette was placed in between the two emitting plates. The absorbance spectrum was obtained using a Hitachi U-2000 spectrometer. While illuminating, the fluorescence of the solution was periodically measured. The fluorescence collection was done using a system composed by a doubled frequency Nd:YAG laser emitting at 532 nm and a spectrometer, collecting fluorescence in the range of 540–850 nm. The excitation laser was coupled to a *Y* type probe which delivers the excitation light and collects the emitted light from the solution through six fibers distributed around the central excitation one. The collected light is filtered with a band pass filter, minimizing the scattered light. The fluorescence spectrum was obtained with the probe immersed about 1 mm in the solution. The fluorescence spectrum was taken in less than 10 s, preventing long time light exposure of the solution.

2.3. PDT in Animals

Male Wistar rats weighting between 200 to 250 g, were used in this study and were maintained in accordance to the guidelines of the Committee on Care and Use of Laboratory Animals of the National Research Council and the Commission for Ethic in Research of the Hospital das Cllnicas de Ribeirao Preto, University of Sao Paulo, Ribeirao Preto, Sao Paulo, Brazil.

2.3.1. Experimental design. For liver necrosis induction, 24 male Wistar rats were used for each photosensitizer divided into two groups: hematoporphyrin derivatives (PG, PF, and PS) and chlorines (PDZ, RAD, and FOS). The photosensitizer was administered via cava vein [26] in different concentrations and its presence was confirmed using a fluorescence spectroscopy technique [30]. The time between drug administration and irradiation of liver was determined by kinetic measurements [31]. Between hematoporphyrin derivatives, the accumulation time in liver is larger for PS (12 h) than PF (2 h) and PG (30 min) and between the chlorines RAD and FOS (8 h) showed longer accumulation time than PDZ (1.5 h).

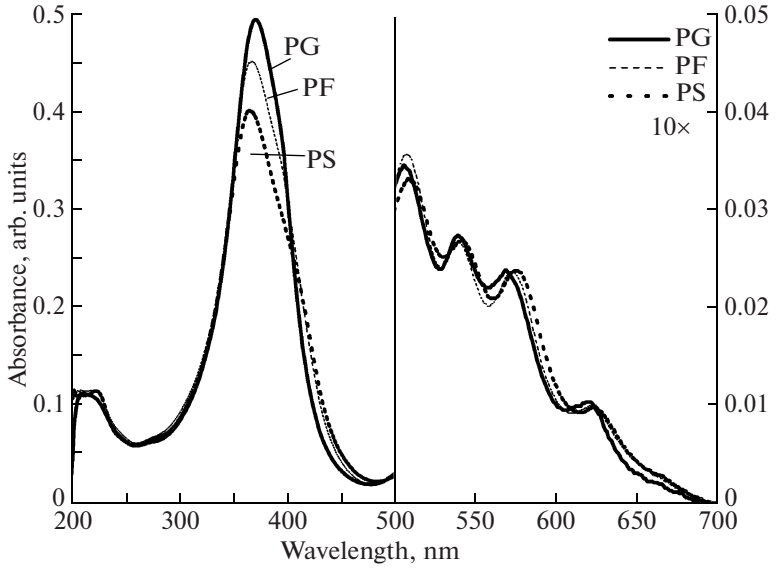

Fig. 1. Absorbance spectra for PG, PF, and PS in the concentration of 17 μg/ml with cuvette of 0.2 cm [21].

A determined 1 cm^2 area was irradiated with a diode laser at 630 nm for porphyrins and chlorines at 660 nm with 250 mW/cm^2. Each porphyrin group was irradiated with doses from 20 to 200 J/cm^2 and chlorines (PDZ and RAD) with the same doses set at 200 mW/cm^2. FOS was illuminated set at 130 mW/cm^2 with irradiation doses from 10 to 30 J/cm^2 [31]. After irradiation, the animals were sutured to recover from treatment. The animals were killed by an overdose of anesthesia 30 h after irradiation [32] and then the liver was removed. The irradiated area was macroscopically evaluated and samples were prepared for histological analysis.

Histological evaluations allow us to determine the overall aspects of the necrotic and healthy portions of the liver as well as the d_{nec} [33]. Using the d_{nec} analysis, it is possible to determine the threshold values (D_{th}) for each photosensitizer using different concentrations as a function of different light doses [33].

2.4. Fluorescence Spectroscopy System

The fluorescence collection was done using a system composed by a doubled frequency Nd:YAG laser emitting at 532 nm and a spectrometer, collecting fluorescence in the range of 540–850 nm. The excitation laser was coupled to a Y type probe which delivers the excitation light and collects the emitted fluorescence from the surface through six fibers (diameter = 100 μm) distributed around the central excitation one (diameter = 130 μm). The source emitted about 7 mW, assuring no thermal effect by the incident illumination. The backscattered light is about a thousand times more intense than the rest of the fluorescence signal collected by the fibers. To simplify the analysis we used optical filters centered at the excitation wavelength with a high wavelength band pass to reduce the intensity of the collected backscattered light producing a signal comparable to the rest of the spectrum of collected light. We have focused our attention in the fluorescence peaks related to the photosensitizer and the autofluorescence previously to injection.

2.5. Statistics

Measured fluorescence values and depth of necrosis in liver were statistically compared using an analysis of variance (ANOVA) combined with Student–Newman–Keuls test. For all tests at a p-value of less than 0.05 was considered to be statistically significant.

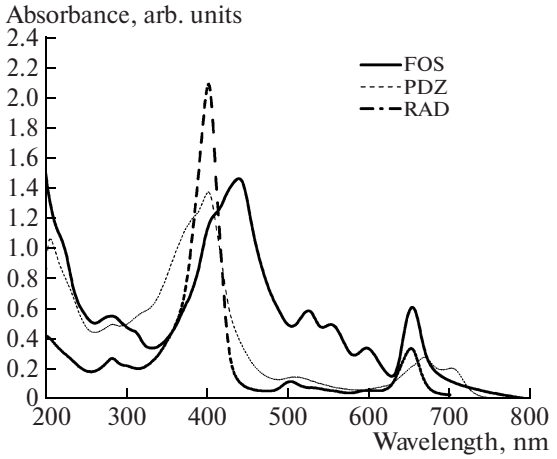

Fig. 2. Absorbance spectra measured for FOS, PDZ, and RAD in the concentration of 17 μg/ml with cuvette of 0.5 cm [24].

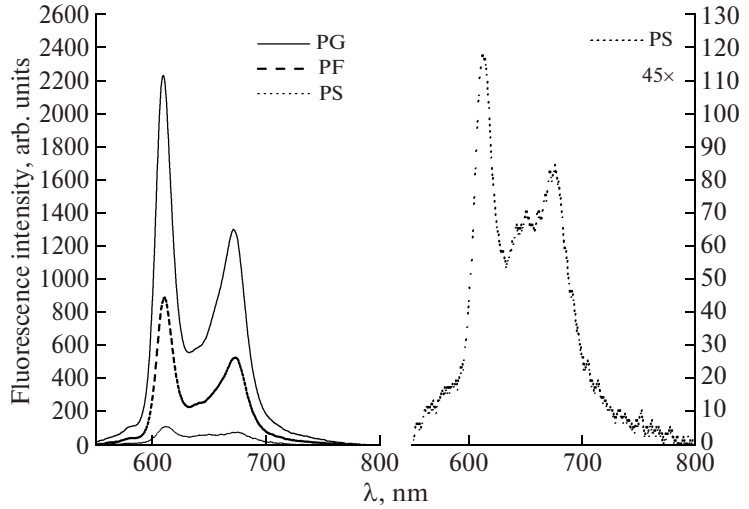

Fig. 3. Fluorescence spectra for PG, PF, and PS in the concentration of 17 μg/ml with cuvette of 1 cm, excitation at 512 nm [21].

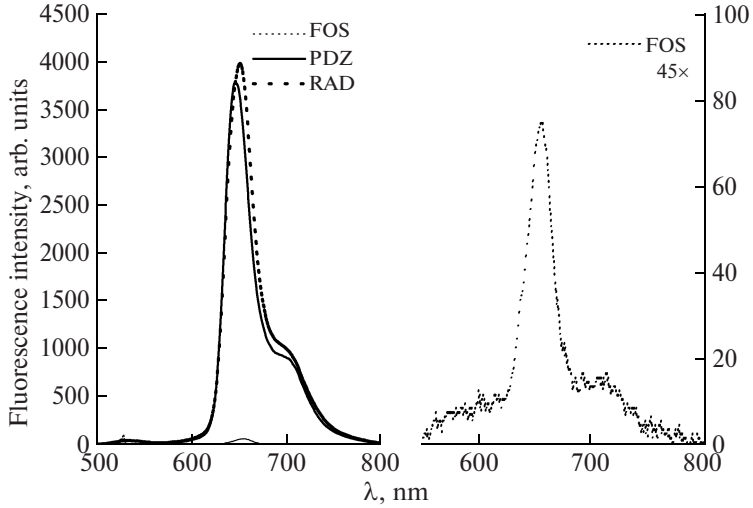

Fig. 4. Fluorescence spectra for FOS, PDZ, and RAD in the concentration of 17 μg/ml with cuvette of 1 cm, excitation at 512 nm [24].

3. RESULTS AND DISCUSSION

3.1. Photosensitizers Degradation: Photostability Parameters

The absorption spectrum for hematoporphyrin derivatives in water solution is show in Fig. 1 [21]. The largest absorption band around 370 nm corresponds to the Soret band however there are anotherm four Q bands (507, 540, 570, 620 nm). The porphyrins possess present similar absorption spectrum bands.

Figure 2 [24] shows the absorbance spectrum for chlorines with the Soret band around 400 nm and a band in the red region of higher absorption. This red shift of the Soret band for chlorines is an indicative of high proportion of monomeric species in solution [34].

The fluorescence spectrum for porphyrins is shown in Fig. 3 [21] and it is possible to visualize an additional band at 610 nm and another at 670 nm. PG presents a higher fluorescence band than PF followed by PS (20 times). In case of chlorines, Fig. 4 [24], PDZ and RAD presented similar fluorescence intensity, however FOS showed the lowest fluorescence (45 times). In agreement with Fig. 3 [21] and 4 [24], PS and FOS show more proportion of aggregate species in solution [21]. The amplitudes of the typical fluorescence peaks at the main peak for the porphyrin (610 nm) and chlorines (650 nm) molecules were monitored as a function of irradiation time at 350 mW/cm^2 during 60 min with LEDs at 630 nm.

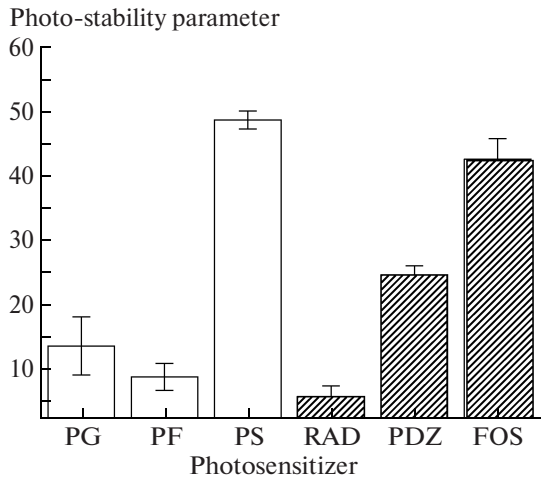

Fig. 5. Photostability parameter for hematoporphyrin derivatives (PG, PF, and PS) and chlorines (FOS, PDZ, and RAD) in the concentration of 17 μg/ml [21, 24].

In a previously paper [21], we have defined the photostability parameter (η) as:

$$\eta = \frac{(\textit{Fraction of Absorbed light})(\textit{number photons/mW})}{\alpha},$$

where α is the quantity of molecules, expressed in mass, that are transformed per unit of time, for each 1 mW/cm^2 of irradiance used to illuminate the solution.

This parameter defines the ability of the photosensitizers to undergo degradation by light. Higher values of η mean low ability to undergo degradation, i.e., higher photostability.

Figure 5 [21, 24] shows the photo stability parameter for porphyrins and chlorines. It is observed that between the porphyrins, PS is more photo stable than PF and PG and between chlorines, FOS is more photo stable than PDZ and RAD [24]. These differences can be correlated with the physical-chemical characteristics of each photosensitizer.

3.2. Depth of Necrosis and Light Threshold Dose

The D_{th} values for all photosensitizers at the conditions evaluated in this study are presented in Table 1 for hematoporphyrin derivatives and Table 2 for chlorines, respectively. The photodynamic efficacy as determined by D_{th}, is higher for PG followed by PS and finally PF and for chlorines is higher for PDZ followed by FOS and RAD. Lower values of D_{th} means higher ability to induce tissue necrosis in low light doses and, therefore, higher photodynamic efficacy [26].

3.3. Photostability Parameter and Light Threshold Dose

Figure 6 shows the relationship between the photo stability parameter (η) and light threshold dose (D_{th}) for both porphyrins (Fig. 6a) and chlorines (Fig. 6b). High values of η means higher photo stability and a low light threshold dose means high photodynamic efficacy. There is a similarity between porphyrins and chlorines but no correlation between them.

PS and FOS show high photo stability and a low light threshold dose when compared to PF and RAD. This is due to PF and RAD being unstable and requiring higher drug concentration to lower its D_{th} value [31].

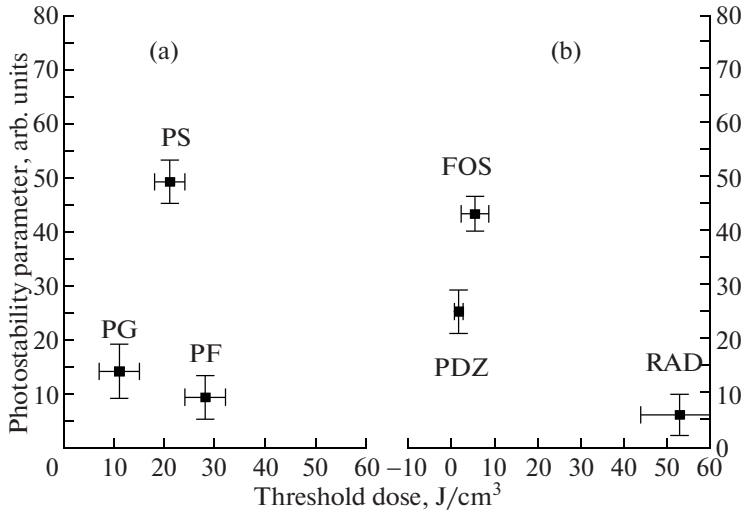

Fig. 6. Photostability parameter as a function of light threshold dose (D_{th}) for hematoporphyrin derivatives (a) and chlorines (b) in the high concentration at the conditions of this experiment (Tables 1 and 2).

Table 1. Depth of necrosis and light threshold dose for hematoporphyrin derivative photosensitizers in different concentrations and light doses (J/cm^2)

Photosensitizer	Dose, J/cm^2	PG		PF		PS	
		mg/kg					
		1.0	2.0	1.0	2.0	1.0	2.0
d_{nec}, mm	20	0.32	1.433	0	0	0	0
	50	1.59	1.795	0	0.490	0.470	1.066
	100	2.14	2.639	0.796	1.044	1.506	1.337
	200	2.79	3.023	1.000	1.966	2.014	1.925
D_{th}, J/cm^2		15 ± 2	11 ± 2	55 ± 2	28 ± 2	26 ± 4	21 ± 4

Table 2. Depth of necrosis and light threshold dose for chlorine photosensitizers in different concentrations and light doses (J/cm^2)

Photosensitizer	Dose, J/cm^2	RAD		PDZ	
		mg/kg			
		0.5	1.0	0.5	1.0
d_{nec}, mm	20	0	0	1.81	2.83
	50	0	0	2.82	3.96
	100	0	2.30	2.87	4.09
	200	2.9	3.51	2.84	4.39
D_{th}, J/cm^2		150.0 ± 2.0	52.6 ± 2.0	1.1 ± 0.5	1.5 ± 0.5

Photosensitizer	Dose, J/cm^2	FOS		
		0.1	0.2	0.3
d_{nec}, mm	10	0	0	1.32
	20	1.30	2.27	2.73
	30	2.95	2.95	3.42
D_{th}, J/cm^2		11.7 ± 2.0	9.7 ± 2.0	5.3 ± 2.0

The low photostability and high D_{th} can be related to the formation of photoproducts less photostable and less cytotoxic, leading to a more rapid elimination from the body. From the literature, we know that PDZ has more cytotoxic photoproducts, which then explain its low D_{th} value [35].

In another publication of this group [36], the influence of a detergent in the process of degradation with different hematoporphyrin derivatives was studied. The detergent mimics the effect of the cellular membrane. PG and PS have higher photodegradation than PF in presence of the detergent. This behavior may explain the PF results on this experiment, which showed lower d_{nec} values and high D_{th} values in vivo than PG and PS.

In a recently published paper [29] a correlation between photostability and D_{th} was seem for HpD, which is not observed in this study. That study however was done for a fixed 30 min DLI (Drug light interval) [37]. The difference in this study is that the DLI was optimized for the delivery of treatment and the study was extended to chlorines.

For chlorines, which are simple substances predominantly monomeric ones, the interaction with the membrane is tied to the amphiphilic compound characteristics of the molecule influencing their intracellular localization [38]. FOS is highly hydrophobic being highly phototoxic in low concentrations and light dose [39]. PDZ and RAD are more hydrophilic [38]. RAD is less photo stable than PDZ with a smaller d_{nec} and high D_{th} values. This behavior can be explained by the higher elimination of RAD due to its higher hydrophilicity [38].

4. CONCLUSIONS

The photostability of the three hematoporphyrin derivatives and three chlorines were studied in vitro. At the same time, these photosensitizers were used to treat normal rat livers and their efficacy studied in vivo by measuring d_{nec}.

According to Vollet-Filho et al. [40] there is a correlation of the in vivo degradation of PG with d_{nec} and therefore to light threshold dose. In this study, we failed to demonstrate the correlation between the photo stability parameter η and light threshold dose, probably due to the DLI optimization. We also have extended this correlation study to chlorines photosensitizers.

There are suggestions in the literature [36, 40] that a correlation might exist between this parameter and light threshold dose both determined *in vivo*.

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