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# Previous Illumination of a Water Soluble Chlorine Photosensitizer Increases Its Cytotoxicity[1]

**J. C. Correa[a], V. S. Bagnato[b], H. Imasato[a], and J. R. Perussi[a, *]**

[a] *Instituto de Química de São Carlos, Universidade de São Paulo—Av. Trabalhador saocarlense, 400–13566-590, São Carlos, SP, Brazil*

[b] *Instituto de Física de São Carlos, Universidade de São Paulo—Av. Trabalhador saocarlense, 400–13566-590, São Carlos, SP, Brazil*

*e-mail: janice@iqsc.usp.br

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**Abstract**—Photodithazine (PDZ) is an N-methyl-D-glucosamine derivative of chlorine e6 that is water soluble and has an intense absorption in the range of 650–680 nm. PDZ photobleaching and photoproduct formation were induced by illumination with laser at two wavelengths: 514 nm (ion argon laser) as well as in 630 nm (dye laser). The time constants of PDZ photobleaching were: 18 min for 630 nm irradiation and 50 min for 514 nm irradiation, suggesting that degradation after irradiation with red light is faster than with green light. Photoproducts formation was evidenced by the appearance of a new absorption band at 668 nm with slight broaden of the Soret band, suggesting that there was no break of the macrocycle. The cytotoxicity of the photodegradated PDZ was investigated and showed to be lower in the dark and higher than non irradiated PDZ. These results may have important clinical implications for PDT such as the possibility to use the previously irradiated PDZ just before clinical application in order to get increased efficiency.

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## 1. INTRODUCTION

Photodynamic therapy (PDT) is a valuable technology for the treatment of benign and malignant diseases using a photosensitizer (PS), light and oxygen. For cancer treatment it can be applied before, after or in conjunction with chemotherapy, radiotherapy or surgery and can be repeated many times at the same site [1]. PDT is based on the administration of a PS which is retained longer in tumor tissue and it is then activated by visible light in a specific wavelength compatible with absorption of the PS producing singlet oxygen, which kills the tumor. Regulatory approvals for the clinical use of several photosensitizers for the treatment of non-malignant and malignant diseases already exists in many countries and several clinical trials has been performed with malignant diseases previously thought to be unsuitable for PDT. This methodology has also been employed to treat skin diseases such as psoriasis and vitiligo as well as presenting remarkable medical and aesthetic results [2].

The first generation of PSs was based on hematoporphyrin derivatives and had several inconveniences as being a complex of porphyrin oligomers, contamination with impurities, relatively low absorbance at 630 nm and a long biochemical lifetime, resulting in photosensitization that persists up to 6–8 weeks after the clinical treatment. Photofrin® has approval for specific clinical applications in several countries of Europe, America, and Asia such as for treatment of late endobronchial lesions, Barrett's esophageal obstructing lesions, bladder cancer and lung cancer, brain tumors, breast carcinomas as well as head and neck neoplasms [3] and is under investigation for other diseases besides cancer. The analogous sensitizer used in Brazil is called Photogem®.

These negative aspects lead to the search for new second generation photosensitizers with valuable physicochemical properties such as chemical purity and higher molar absorptivity in the red region or phototherapeutic window (650–850 nm), resulting in increased therapeutic answers even using lower doses of photosensitizers [4]. In this class are the chlorines, phtalocyanines, benzoporphyrins, bacteriochlorines, porphycenes, etc. The called third generation of PS consists of the second generation of PS bound to carriers in order to obtain selective accumulation in the tumor [5]. It has been described having rapid tissue accumulation and clearance from the organism decreasing risks of high skin photosensitivity [6, 7].

Photodithazine® (PDZ) is a PS synthesized in Russia that has been obtained from *Spirulina Platensis* cyanobacteria as a noncovalent complex of N-methyl-D-glucosamine chlorine $e_6$ salt on basis of chlorophyll A derivatives (Fig. 1). Photodithazine belongs to the second generation of PS and is under clinical evaluation. Photodithazine has long shelf life; it is water soluble and has a powerful absorption band in 655 nm where biological tissues [6] are more transparent to light than in 630 nm. In comparison to hematoporphyrin derivatives used in clinical practice, PDZ has a

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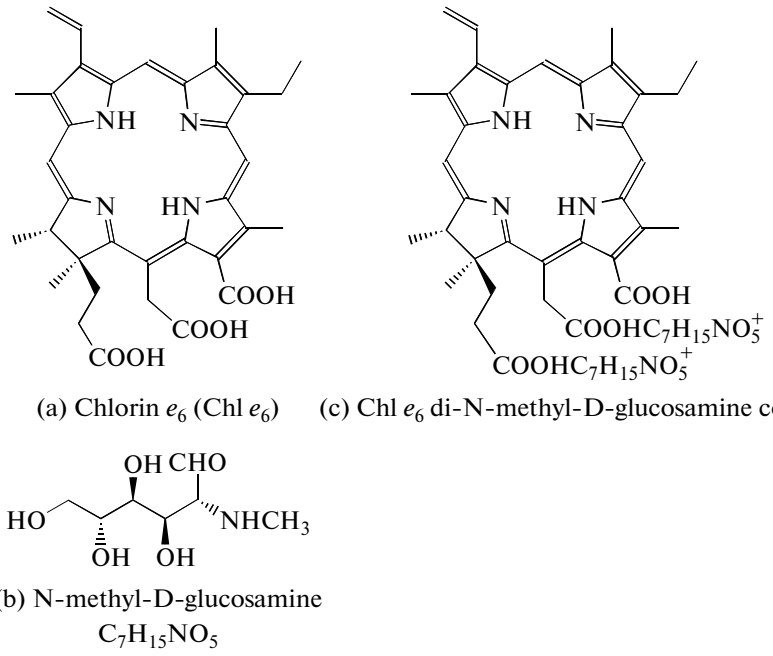


**Fig. 1.** Chemical structure of Photodithazine by VETA-GRAND Co., Moscow, Russia. A complex of di-N-methyl-D-glucosamine of Chl e6.

number of special properties, being its rapid tissue accumulation and clearance from the organism the most significant [6, 7]. As a consequence, there is no complication associated with possible high skin photosensitivity [7]. Besides that, PDZ possesses a higher photodynamic activity [8] and high tumor to normal tissue ratio of accumulation (10:1) [9]. The octanol/phosphate buffer partition coefficient ($K_p$) of Photodithazine has been found to be 1.6 [9], indicating that it must be able to localize in the cell membranes, corroborating with the $\log K_p$ found in our group of 0.2 [8]. This may be a partial explanation for its higher photodynamic efficacy in vitro, comparing to the hematoporphyrin derivatives Photofrin and Photogem with $\log K_p$ of 0.15 and 0.11, respectively [8].

Photosensitizers used in PDT are liable to degradation by light (photobleaching) in simple solutions as well as in complex environments, which can be visualized by spectral changes as well as by the decreasing of their initial absorption and fluorescence intensities [1, 10–16]. This phenomenon should be taken into account when developing the appropriate protocol to treatment since it is very important for dosimetry. In this study the photobleaching of PDZ was induced in vitro by previous illumination with laser at two wavelengths. The spectral changes and the kinetics of the appearance of possible photoproducts were followed as well as the cytotoxicity of PDZ before and after photobleaching.

## 2. MATERIALS AND METHODS

### *2.1. Photosensitizer*

Photodithazine® was obtained from Veta-Grand, Russia, at a concentration of 0.55%. Photogem® was obtained from Photogem LLC Company, Moscow—Russia. The 5 mg $mL^{-1}$ stock solutions of the photosensitizers were prepared in 20 mM phosphate buffered saline (PBS) pH 7.4 and stored in the dark at 4°C.

### *2.2. Photodegradation*

Solutions of 20 μg $mL^{-1}$ PDZ in PBS were irradiated at 514 nm (argon ion laser-Coherent Innova 100) as well as at 630 nm (Rhodamine 101 laser pumped with argon ion laser (Coherent Innova)), both with intensity of 25 mW $cm^{-2}$. The photodegradation was performed during 10–180 min and followed by absorption and fluorescence spectroscopy.

### *2.3. Constant Constrain Analysis*

The set of absorption spectra of PDZ as a function of irradiation time was analyzed by the Convex Constraint Algorithm (CCA) [17, 18] (a software program kindly supplied by Dr. G.D. Fasman) which allows the decomposition of all the experimental spectra into pure spectra which correspond to different forms of the chromophores in the solution during the induced photodegradation.

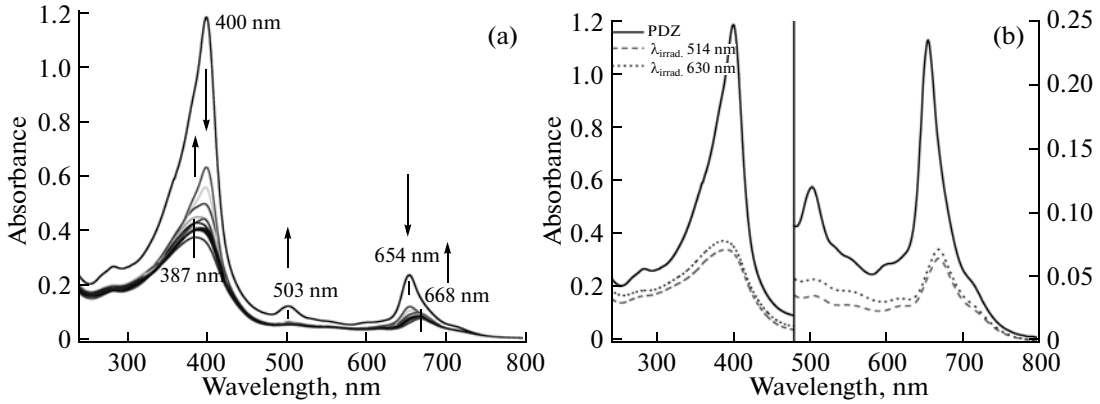


**Fig. 2.** (a) Absorption spectra changes of a 20 μg/mL PDZ solution in PBS irradiated at 630 nm as a function of irradiation time. Arrows indicate the direction of variation of intensity. (b) Spectra of a 20 μg $mL^{-1}$ solution of PDZ in PBS (solid-line) after irradiation at 514 nm (dashed line) and 655 nm (dotted line), irradiation time 180 min, 25 mW $cm^{-2}$.

### *2.4. Cell Cultures*

A tumor cell line originated from human larynx carcinoma (HEp-2, ATCC-CCL-23) and a non-tumor cell line (VERO, ATCC-CCL-81) originated from the kidney of an adult African green monkey were cultivated in monolayers in 25 $cm^2$ flasks with Eagle and Dulbecco's modified Eagle medium (DMEM) medium, respectively, supplemented with 10% fetal bovine serum (FBS), ampicillin and streptomycin at 37°C under a humidified atmosphere consisting of 5% $CO_2$ and 95% air [14].

### *2.5. Cytotoxicity of PDZ and Its Photoproducts*

Aliquots of 200 μL suspension containing 1 × $10^5$ viable cells/mL were plated in each of the 96 wells of the test plates in culture medium supplemented with 10% FBS. After 24 h, the cells were incubated for 2 h at 37°C with PDZ (not irradiated and previously irradiated) in the concentration range of 0 to 25 μg $mL^{-1}$ (based on untreated dye concentration) in the appropriated culture medium with 3% FBS. All samples were made in triplicate. After the incubation time the photosensitizer-containing medium was removed and replaced by fresh medium. The samples for phototoxicity study were irradiated with a LED 630 nm and the cells for dark toxicity were not irradiated. Then, the cells were incubated in ordinary medium by an additional 48 h in order to evaluate the number of surviving cells. After this recovering period, the cells were treated for 3 h with 3-(4,5-dimethyl) thiazol-2-il-2,5-diphenil bromide tetrazolium (MTT). This method of cell counting is based on the reduction of MTT to formazan blue (absorbance at 570 nm) by the mitochondrial dehydrogenases present only in living cells [19]. The absorbances were measured using a well plate reading unit (Benchmark BIO-RAD). The median inhibitory concentration ($IC_{50}$) was determined using the Calcusyn program [20, 21] and the cell survival (%) was assessed as a function of PDZ concentration for each irradiation time.

## 3. RESULTS AND DISCUSSION

### *3.1. Photodegradation*

In order to assure that PDZ was present as a monomer in the solution used to induce photodegradation, control experiments were performed and suggested that PDZ does not aggregate up to 120 μg $mL^{-1}$, concentration very superior to the one used in this study, i.e., 20 μg $mL^{-1}$ in PBS. The Fig. 2a presents the absorption spectral changes of PDZ solution as a function of irradiation time with laser at 630 nm. The results indicate the photodegradation of PDZ and the formation of another compound (668 nm). According to Rotomskis, the photoproducts that absorb at red spectral region are significant since these photoproducts may have activity in PDT [22]. Similar profiles were observed with 514 nm irradiation (Fig. 2b).

In a previous study we have found that the induced photodegradation of Photogem [12] leads to a decrease of the Soret (369 nm) as well as at Q bands (507, 540, 570, and 620 nm) and the appearance of a new band at 640 nm, suggesting that the photoproduct is a chlorine-like [10].

The chlorines present the Soret band around 400 nm and this red shift compared with the porphyrins is an indicative of high proportion of monomeric

**Table 1.** Decay time constant of the spectral bands of PDZ after irradiation at 514 and 630 nm

| Irradiation wavelength, nm | τ (401 nm), min | τ (655 nm), min |
|---|---|---|
| 630 | 14 | 13 |
| 514 | 51 | 65 |

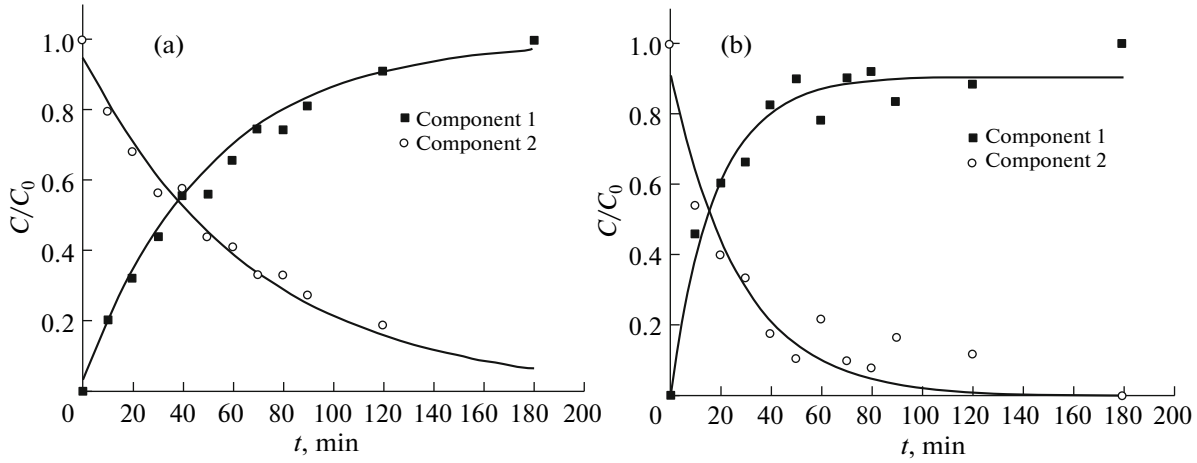


**Fig. 3.** Analysis obtained by the CCA method of the spectral changes due to the photodegradation of PDZ 20 μg $mL^{-1}$ in PBS using an argon ion laser (a) at 514 nm and (b) 630 nm as a function of time.

species in solution [23]. When PDZ was irradiated at 514 and 630 nm, a decrease in intensity at the Soret band and of the Q bands occurred, followed by the appearance of a new band at 668 nm, suggesting that chemical transformations leads to photoproducts formation. The analyses of the spectra of Photogem under photobleaching (results not showed) suggested that there are two distinct species, one that is degradated as a function of time (photosensitizer) and another (photoproduct) that is formed (red shifted $Q_1$ band) as a function of irradiation time at both wavelengths. The results indicate that both processes occur simultaneously and that the photobleaching and photoproducts formation reach saturation at the same irradiation time, although different for each wavelength of irradiation. It was also found that the photodegradation of the hematoporphyrin derivative is slower than that of chlorine suggesting that PDZ is less photostable than Photogem. In a comparative study it was obtained the order of susceptibility of chlorine photosensitizers through the photodegradation rate: Radachlorin < Photodithazine < Foscan. This difference in the photodegradation rate for Foscan was explained by the high proportion of aggregates in solution of this photosensitizer that inhibit the photo-oxidative process, holding up the singlet oxygen formation [1].

The decay time constant for the Soret band (400 nm) as well as at 655 nm for PDZ photodegradation are presented in Table 1. It can be seen that these values are in the same order of magnitude in both bands and that the photodegradation is faster at 630 nm than at 514 nm. This behavior can be attributed to the fact that PDZ presents a high value of absorptivity in 655 nm, close to the wavelength of irradiation at 630 nm.

**Table 2.** Decay time constant obtained using the results of the CCA method for the Soret band of PDZ after irradiation at 514 and 630 nm

| Wavelength of irradiation, nm | Photobleaching τ, min |
|---|---|
| 630 | 18 |
| 514 | 50 |

The obtained optical absorption spectra during the induced photodegradation of PDZ were analyzed also by the CCA method and deconvoluted into two components representing the integer and the photooxidized PDZ base spectrum, respectively. In Fig. 3 are presented the linear combination factors variation as a function of irradiation time. Based on the trends of the values of factors, it is possible to assign the component 1 to PDZ and component 2 to oxidized PDZ.

In order to compare the decay time constants obtained by absorption decay in Table 1 with the ones obtained through Fig. 3 using the CCA method, these later values are presented in Table 2. It can be seen that these constants are similar and suggest that both methods of analysis are correct.

As a result, the average values for the degradation time constant obtained by both methods (Tables 1 and 2) are 16.0 and 50.5 min for irradiation at 630 and 514 nm, respectively. It is worth to mention that although these degradation rates are not the same, the photoproducts absorption spectra at the end of the 180 min irradiation are similar (Fig. 2), suggesting that the induced photobleaching at both wavelengths lead to the same compounds.

During the induced photodegradation of PDZ, the changes in the emission spectra were also followed by fluorescence (results not showed). An expressive decrease at the fluorescence band of PDZ was detected with no shift of the bands. Table 3 presents the decay time constant obtained by the changes in the fluorescence intensity during the induced photodegra-

dation. Again, the irradiation at 630 nm has resulted in a faster process, corroborating with the absorption spectroscopy.

The photobleaching of the hematoporphyrin derivative Photogem was also performed in this study and irradiation at the same conditions at 630 nm resulted in a decay time constant of 32 min. This result suggest that PDZ photodegradates faster (2.3 fold) than Photogem so PDZ is less photostable than Photogem [12].

Studies described in the literature comparing the photodegradation of several photosensitizers as hematoporphyrin derivatives, chlorins and phthalocyanines show that chlorin $e_6$ is the less photostable of them. It has been suggested that one of the possible mechanisms of photo-oxidation of chlorin $e_6$ involves the attack of the oxygen in the diene unity. Chlorin $e_6$ contain a vinyl substituent which can be susceptible to photoxidation. The authors have also studied the efficiency of red absorbing photoproducts formation and did not observe the same results for chlorin $e_6$. Therefore, the results obtained in this study with PDZ are in accordance with Rotomskis [24].

According to Rotomskis et al. [22] and Bonnett and Martinez [10], spectral changes may occur due to photoinduced modification of porphyrins with or without rupture of the macrocycle. It has been described that the break of the macrocycle implies in the appearance of a band in the UV region after the irradiation and that the absence of breaking of the macrocycle leads to a decrease in the absorbance of the Soret and Q bands, associated to the appearing of a new Q band [10, 11, 25, 26]. In the present study it was not observed a new band in the UV region after irradiation in both wavelengths. Thus, the obtained results suggest that the irradiation of PDZ with light at 514 and 630 nm did not lead to the opening of the ring, but only to the formation of photoproducts of the bacteriochlorines type (absorption at 660 nm). For Photogem, the induced photodegradation causes a decrease of the Soret absorption band (369 nm) and of the Q bands (507, 540, 570, and 620 nm). Also, the appearance of a new band in 640 nm suggests that the photoproduct can be a chlorine which absorbs at lower wavelength relative to bacteriochlorines [10, 12, 22].

### *3.2. Comparative Cytotoxicity of Photodithazine and Photogem*

Concerning the accumulation of hematoporphyrin derivatives, a study of Atif et al. [3] with Photofrin in HEp-2 cells have shown that this photosensitizer starts to accumulate in those cells just after 25 h.

Table 4 shows the $IC_{50}$ values of Photodithazine and Photogem in the dark and under illumination for HEp-2 cells. Comparing the $IC_{50}$ of PDZ in the dark ($32 \pm 4$ μg mL$^{-1}$) with the one for Photogem ($8.8 \pm 0.2$ μg mL$^{-1}$) in HEp-2 tumor cells, PDZ is 3.6 fold less cytotoxic in the absence of light than Photogem. This result indicates that this chlorine photosensitizer is much safer than porphyrin derivatives. Under illumination PDZ presents $IC_{50}$ just 20% lower than Photogem. The potentiation of PDZ with light is about 14 fold and just 3 fold for Photogem what means that PDZ is (about 4.5 fold) more potentialized by the light than Photogem.

Ribeiro et al. have investigated the phototoxic and cytotoxic effects on normal cell lines using Photogem irradiated for 66 min with red laser and evaluated by scanning electron microscopy (SEM) 24 h after PDT concluding that the morphological alterations were maintained, demonstrating the irreversible damage caused by this therapy with this photosensitizer [27].

Cavalcante et al. have used a way to make a fast previous evaluation of photosensitizers efficacy by a combination of techniques: (a) use of bovine serum albumin and uric acid as chemical dosimeters; (b) photohemolysis of red blood cells used as a cell membrane interaction model, and (c) octanol/phosphate buffer partition to assess the relative lipophilicity of the compounds. They have found that PDZ presents a higher

**Table 3.** Values of decay time constant of the spectral emission band of PDZ after its irradiation at 514 and 630 nm

| Wavelength of irradiation, nm | $\tau_{F656}$, min |
|---|---|
| 630 | 18 |
| 514 | 36 |

**Table 4.** Cytotoxicity in the dark (no light) and light (under illumination, dose = 31.5 J cm$^{-2}$) with LED at 630 nm for PDZ and PG in HEp-2 cell line. The cells were incubated by 2 h at 37°C and 5% $CO_2$

| Photosensitizer | Dark | Light |
|---|---|---|
| Photodithazine | $32 \pm 4$ | $2.3 \pm 0.7$ |
| Photogem | $8.8 \pm 0.2$ | $2.9 \pm 0.9$ |

**Table 5.** Dark cytotoxicity of PDZ before and after photodegradation with irradiation at 514 and 630 nm in tumor cells HEp-2, 2 h incubation time at 37°C and 5% $CO_2$

| Irradiation time, min | $IC_{50} \pm$ sd, μg mL$^{-1}$ ($n_{exp}$) | |
|---|---|---|
| | Irr. 514 nm | Irr. 630 nm |
| 0 | $32 \pm 4(12)$ | |
| 10 | $69 \pm 1(5)$ | $64 \pm 1(5)$ |
| 20 | $70 \pm 1(5)$ | $65 \pm 1(5)$ |
| 40 | $73 \pm 1(5)$ | $71 \pm 1(5)$ |
| 60 | $75 \pm 1(5)$ | $80 \pm 1(5)$ |

Note: sd—standard deviation; $n_{exp.}$—number of replicas; Irr.—irradiation wavelength.

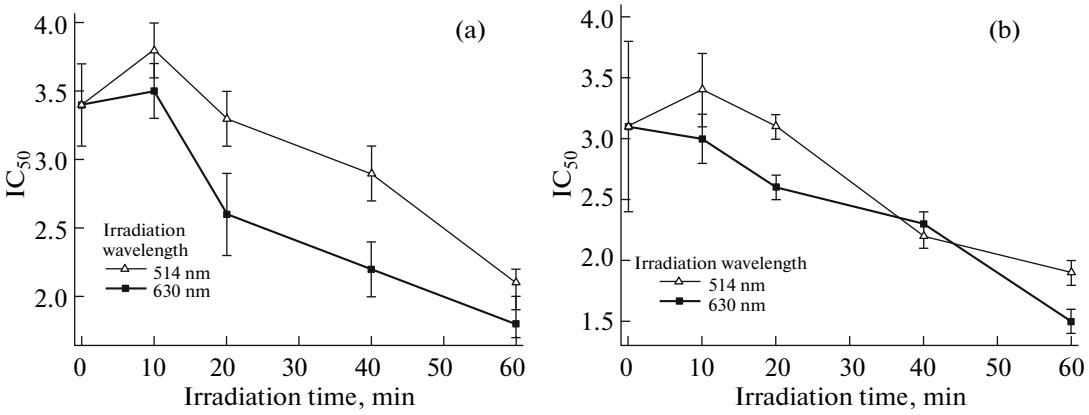


**Fig. 4.** Phototoxicity of PDZ photoproducts ($IC_{50}$). VERO (a) and Hep-2 (b) cells were incubated for 2 h using standard procedure with photoproducts obtained by variable irradiation time (maximum 60 min). Phototoxicity assays: 630 nm LED, 25 mW$cm^{-2}$, and 14 min of irradiation adding up to a dose of 21 J $cm^{-2}$.

photodynamic efficiency than Photogem and these results are in agreement with the cytotoxicity of these photosensitizers in normal and tumor cells [8]. So, the present results also agree with that study.

A study performed by Banfi et al. [28] with uterus colon tumor cells have shown that Foscan® (a chlorine derivate) is significantly more cytotoxic under irradiation than Photofrin® (an hematoporphyrin derivative), since they obtained an $IC_{50}$ for Foscan® around 3 times lower than for Photofrin®. In this way, the obtained results for PDZ and Photogem® in the present study agree with the ones described in the literature.

### *3.3. Dark Cytotoxicity of Photodithazine Non Irradiated and Previously Irradiated*

Table 5 presents the values of $IC_{50}$ in VERO tumor cells for non irradiated PDZ and PDZ after irradiation with laser at 514 and 630 nm as a function of the illumination time.

**Table 6.** Dark cytotoxicity of the PDZ before and after photodegradation with irradiation at 514 and 630 nm in VERO cells, 2 h incubation time at 37°C and 5% $CO_2$

| Irradiation time, min | $IC_{50} \pm$ sd, μg $mL^{-1}$ ($n_{exp}$) | |
|---|---|---|
| | Irr. 514 nm | Irr. 630 nm |
| 0 | 62 ± 3(12) | |
| 10 | 73 ± 1(5) | 65 ± 1(5) |
| 20 | 75 ± 1(5) | 72 ± 2(5) |
| 40 | 80 ± 1(5) | 75 ± 1(5) |
| 60 | 83 ± 1(5) | 86 ± 2(5) |

Note: sd—standard deviation; $n_{exp.}$—number of replicas; Irr.—irradiation wavelength.

It can be observed in Table 5 an increase in $IC_{50}$ of PDZ as a function of the photodegradation time which means a decrease in cytotoxicity of PDZ in the dark in VERO cells with Photodithazine previously irradiated. These results suggest that, in the dark, the photoproducts of PDZ are less cytotoxic than PDZ. Similar behavior was found in VERO cells since a progressive decrease of toxicity occurs due to PDZ irradiation (Table 6). Same findings were observed in our group for photodegradation of Photogem [29].

Interestingly a study of the phototoxicity of Quinifuryl (nitroheterociclic compound with anticancer activity) in rat leukemic cells (P388) showed that the final products of the photodecomposition of Quinifuryl present lower cytotoxicity in the dark than Quinifuryl itself, causing the death of less than 10% of cells with 100 min of incubation [30].

### *3.4. Phototoxicity of Photodithazine Non Irradiated and Previously Irradiated*

On the other hand, the photoproducts of PDZ showed an enhanced phototoxicity. Figure 4 presents the $IC_{50}$ values of the photoproducts of PDZ obtained by previous irradiation in 514 and 630 nm for time intervals from 0 to 60 min in VERO and HEp-2 cells. It can be observed that with the increase of the previous illumination time of the PDZ solution, the phototoxicity increases pointed by the $IC_{50}$ values decrease (~50%). Comparing the previous irradiation of PDZ in both wavelengths, it can be concluded that the irradiation with red light causes a more significant decrease in $IC_{50}$ than green light irradiation. In the spectroscopic studies it was observed that the photodegradation and consequently the photoproducts formation are more pronounced at 630 nm than at 514 nm. This way, it can be concluded that under the illumination conditions in which photoproducts formation is favored, the phototoxicity is increased.

These results suggest that the photoproducts of PDZ are more phototoxic than the starting material. It is important to point out that the photoproducts of Photodithazine are about 15–20 times more cytotoxic under light than the original PDZ. Besides, Belitchenko et al. [31] have studied the cytotoxicity in the presence of light of chlorine m-THPC (Foscan®) non irradiated and previously irradiated in uterus colon tumor cells and have found a higher cytotoxicity for non irradiated dye and this cytotoxicity decrease as a function of the pre-irradiation of the dye.

Contrasting to PDZ photoproduct toxicity, previous studies concerning on the photobleaching of Photogem and Photofrin described that the photoproducts of porphyrins derivatives are less cytotoxic than the original photosensitizers [12, 32]. The values of $IC_{50}$ increase when Photogem is irradiated in 488, 514, and 630 nm [12]. It was concluded that the cytotoxicity as well as the phototoxicity can be diminished through photodegradation of Photogem.

Thus, the opposite behavior observed under photodegradation of chlorine sensitizers and the hematoporphyrin derivatives, as well as the evidences that the photoproducts of chlorins became more photoactive than the photosensitizer not irradiated may have a relevant implication in dosimetry and clinical PDT.

## 4. CONCLUSIONS

Photodithazine presents some advantages over hematoporphyrin derivatives like Photogem, for example. Some of these are higher absorbance in red shifted wavelength where the skin has enhanced transmittance; no aggregation in a wide range of concentration and fast clearance from the organism, which decrease the undesirable effect of skin photosensitivity. Concerning to cytotoxicity, this study showed that PDZ has two important advantages over Photogem: it is less cytotoxic in the dark and more cytotoxic under illumination. The photoproducts of PDZ are less cytotoxic in the dark than non irradiated PDZ and around twice more phototoxic after previous illumination. Therefore, the present results for the N-methyl-D-glucosamine chlorine e6 derivative showing the increased phototoxicity of the photoproduct may have important clinical applications and must be taken in consideration in dosimetry. In conclusion, the presented data and considerations can be an evidence of a promising approach of PDT based on fractionated application of light dose taken advantage of in situ sensitizer modification to more active photoproducts.

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