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# A threshold dose distribution approach for the study of PDT resistance development
# A threshold distribution approach for the study of PDT resistance

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ABSTRACT

Photodynamic therapy (PDT) is a technique with well-established principles that often demands repeated applications for sequential elimination of tumor cells. An important question concerns the way surviving cells from a treatment behave in the subsequent one. Threshold dose is a core concept in PDT dosimetry, as the minimum amount of energy to be delivered for cell destruction via PDT. Concepts of threshold distribution have shown to be an important tool for PDT results analysis in vitro. In this study, we used some of these concepts for demonstrating subsequent treatments with partial elimination of cells modify the distribution, which represents an increased resistance of the cells to the photodynamic action. HepG2 and HepaRG were used as models of tumor and normal liver cells and a protocol to induce resistance, consisted of repeated PDT sessions using Photogem® as a photosensitizer, was applied to the tumor ones. The response of these cells to PDT was assessed using a standard viability assay and the dose response curves were used for deriving the threshold distributions. The changes in the distribution revealed that the resistance protocol effectively eliminated the most sensitive cells. Nevertheless, HepaRG cell line was the most resistant one among the cells analyzed, which indicates a specificity in clinical applications that enables the use of high doses and drug concentrations with minimal damage to the surrounding normal tissue.

## 1. Introduction

Photodynamic Therapy (PDT) is an anticancer technique based on the creation of oxidative species in the tumor through the excitation of a compound by light [1]. For a successful PDT treatment, damage must be caused beyond a resistance threshold of the cells for guaranteeing their death [2].

Some types of lesions require a fractionated protocol, consisting in repeated sessions of PDT, to allow the recovery of surrounding normal tissues and tumor re-oxygenation [3]. In clinical trials of PDT for non-melanoma skin cancer treatment, several protocols were based on two sessions (days or months apart of each other) [4,5]. However, lesions may present different tolerances to damage and respond differently to PDT. The recurrence rates of the treatment may be related to the fact that no study is performed to investigate how the tumor reacted to the first session prior to the following ones.

At the occurrence of damage, the primary cell response is to repair itself, adjusting its metabolism and machinery in order to survive. When that damage is not sufficient to cause death in all the irradiated cells, the remaining ones may repopulate the tumor and form a more resistant lesion to PDT. In chemotherapy, it is possible to find studies that observed a 2500-fold increase in drug resistance [6]. Due to its significantly different mechanisms of action compared to chemotherapy, PDT was once believed not to have the ability to induce resistance even by natural selective mechanisms, as it is based on oxidative stress. Nevertheless, further studies have shown that, despite presenting distinctly lower increasing rates, it is possible to observe resistance resulted from repeated sessions of PDT [7–12]. The proposed mechanisms to explain resistance are: altered photosensitizer uptake and release, changes in intracellular PS distribution, insufficient light or oxygen, and increased inactivation pathways, such as photobleaching and increased intracellular scavengers levels [13].

In this study, we evaluated the development of PDT resistance using a threshold dose distribution model. This concept was described, as well as the parameters used to characterize the distributions. It was shown how these parameters correlate to resistance, and why the threshold

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dose distributions are a suitable model to investigate it.

## 2. The Threshold Distribution Model

A threshold dose distribution concept was introduced by Sabino et al. [14] and further explored in a previous study from which its advantages were shown over analyzing only the dose-response curve [15]. While information in fact is contained into the dose-response curves, it is not usually explored, thus the importance of threshold dose distributions in elucidating them. Through this tool, it is possible to obtain information regarding variability and sensitivity of the population.

The sigmoid behavior of cytotoxic dose responses is the evidence of the existence of a distribution of tolerances within a population, since this curve would be a step one if all the cell presented the same sensitivity. This variability has been observed in a series of studies that showed the differences in response of a genetically identical population of tumor cells in response to a cytotoxic stimulus [16,17].This distribution can be expressed as $g(D)dD$ and it represents the probability of a cell to have a threshold between the doses $D$ and $D + dD$. Given an energy of $D_0$, the cell fraction $f(D_0)$ with a threshold value lower or equal to $D_0$ will respond. Mathematically, this can be described by:

$$f(D_0) = \int_0^{D_0} g(D)dD. \quad (1)$$

Therefore, the threshold dose distribution can be obtained by differentiating the experimental dose-response curve:

$$g(D0) = \frac{df}{dD}\Big|_{D_0} \quad (2)$$

The $g(D)$ curve depends strongly on the PDT parameters, such as photosensitizer (PS) used and its concentration and incubation time, wavelength of the light source and the cell type. Concentration and incubation time of the PS determine the amount of reactive oxygen species generated upon light excitation and its subcellular localization, which impacts the damage caused by PDT and triggers mechanisms of repair. Additionally, the absorption of photons by the PS molecule depends on the wavelength of excitation, which also affects the penetration of light in the tissues, including the tumor. Last, the uptake of PS and response to PDT damage are very dependent on the type of cells treated.

The distribution is mainly characterized by its width ($\Delta D$), measured at half-maximum amplitude, and its peak center ($D_p$), which express the dose corresponding to the maximum amplitude. The parameter $D_p$ represents the most frequent threshold dose in the population, which quantifies its characteristic resistance, while $\Delta D$ is a measurement of how heterogeneous the population is regarding the threshold dose. The variability is also related to resistance, since it is more likely for resistant individuals to be isolated from a very heterogeneous population than from a homogeneous one.

In our previous study, it was observed that $\Delta D$ value is numerically close to the doses applied. That means that the probability of a significant number of cells with high threshold doses to survive the treatment is quite large. Fig. 1 shows the threshold distributions of PDT experiments using the prostate carcinoma R3327-AT cell line, obtained from the dose-response curve presented in the study of Moore et al. [18]. Photofrin was the photosensitizer used, and light doses from 0.5 to 3 J/cm$^2$ were delivered. It is possible to observe that the widths of the distributions increase as the PS concentration decreases and, consequently, they become closer to the maximum applied doses. Therefore, in a clinical situation, the light dose must be significantly higher than the values of $D_p$ and $\Delta D$ to avoid the induction of resistant cells.

As the parameters $D_p$ and $\Delta D$ are closely related to resistance, the threshold dose distribution is an adequate tool to study the subject in an in vitro setup. It could allow proposing optimized PDT protocols in order to reduce tumor recurrence in clinical treatments.

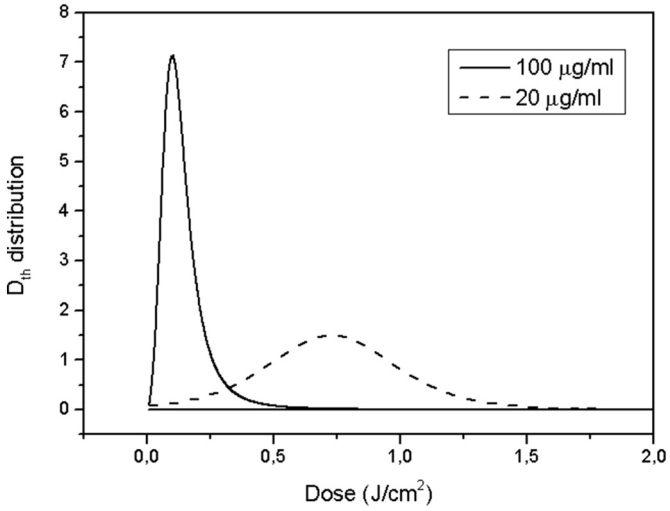


**Fig. 1.** Threshold distributions from the experiments of Moore et al. [18].

## 3. Material and Methods

### 3.1. Cell Line and Cell Culture

HepG2 and HepaRG cells were obtained from ATCC (American Type Culture Collection, HB-8065 and HTB-22) and Gibco (HPRGC10), respectively. The cells were cultured in DMEM (Dulbecco's Modified Eagle's medium – Cultilab, Campinas, Sao Paulo, Brazil) supplemented with phenol red and 10% Fetal Bovine Serum (FBS, Cultilab, Campinas, Sao Paulo, Brazil), and maintained in an incubator at 37 °C in a humidified atmosphere (95% air and 5% $CO_2$, Sanyo, MCO 19-AIC UV). All experiments were performed at a passage lower than 30 and the cells were not tested during the period of experiments. Cell morphology was assessed by inverted microscopy (Zeiss Observer Z1 Microscope, Oberkochen, Germany).

### 3.2. Light Source

The laser source used for all PDT experiments was composed by 24 LEDs predominantly emitting at 630 nm (prototype “Biotable®”, developed by the Technological Support Laboratory, Sao Carlos Institute of Physics, University of Sao Paulo, São Carlos-SP, Brazil), with an output power of 45.0 mW/cm$^2$. The emission was chosen due to its proximity to the 620 nm Q-band of Photogem®. The device irradiates plates from the bottom, so the light source-to-plate distance is fixed.

### 3.3. Photosensitizer

Photogem® (High Chemical Technology, Moscow, Russia) was chosen as the photosensitizer for this study, mainly due to its previous wide clinical application for PDT in Brazil, as it has been the first PS to be approved by the Brazilian National Health Surveillance Agency (ANVISA). All procedures involving PS manipulation were performed under low light to avoid undesired photobleaching. Stock solutions of 5 mg/ml were prepared by dissolving the powder in sterile saline solution (PBS) and then maintained at 4 °C in the dark.

### 3.4. Resistance Development

Resistance was induced in the cell lineHepG2. Tumor cells were subjected to ten sessions of PDT, with Photogem® concentration of 5 μg/ml and a light dose of 5 J/cm$^2$. Cells were cultured in 25 cm$^2$ flasks and, at confluence, incubated with PS for 4 h in phenol-free medium, supplemented with 5% of FBS. Then, prior to the illumination, cells were washed twice with PBS and fresh medium was added. After PDT,