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Modifying excitation light dose of novel photosensitizer PVP-Hypericin for photodynamic diagnosis and therapy

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ABSTRACT

Conventional photodynamic diagnosis (PDD) and therapy (PDT) makes use of photosensitizers that are excited by continuous light irradiation of specific wavelengths. In the case of PDT, the overdose of continuous excitation may lead to an expansion of necrosis in cancer cells or morbidity in healthy surroundings. The present study involves 5-h fluorescence imaging of living human lung epithelial carcinoma cells (A549) in the presence of a novel photosensitizer, PVP-Hypericin (PVP: polyvinylpyrrolidone) to optimize the excitation light doses for PDD and PDT. A number of time-lapse imaging experiments were performed using a low-power blue LED operating in either continuous or pulsed mode. The irradiances I^* were 1.59, 6.34 and 14.27 mW/cm^2, the pulse lengths L being 0.127, 1.29, 13, 54.5, 131 and 60,000 ms. Then, the relation between irradiance, various exposure times, photobleaching and phototoxicity of PVP-Hyperycin was investigated. Results showed a nonlinear relationship between the amounts of excitation dose, cell viability and toxicity. For all experimental I^*, minimal phototoxicity and photobleaching was detected when cells were exposed to brief pulses of light ($L \leqslant 13$ ms). On the other hand, pulsed excitation with $I^* = 14.27$ mW/cm^2 and $L = 131$ ms induced high percentages of apoptosis comparable to the long exposures of $L = 60{,}000$ ms and the continuous excitation. Thus, replacement of continuous excitation by a pulsed method seems applicable for PDT.

1. Introduction

Photodynamic diagnostic imaging (PDD) and photodynamic therapy (PDT) have received much attention in recent years due to their use to detect and treat proliferative disorders, including cancer [1–4]. Conventional PDD and PDT involve the administration of a photosensitizer, which preferentially accumulates in diseased cells, followed by a continuous light excitation of specific wavelengths. Most photosensitizers are dyes that generate fluorescence and reactive oxygen species (ROS) upon light irradiation [2,3].

Fluorescence photobleaching of photosensitizers and generation of ROS increase with increasing excitation light dose, but these are less pronounced or absent at medium to low excitation [5,6]. It has been reported by Stephens and Allan [6] as well as Goldman and Spector [7] that cells may have intrinsic enzymatic mechanisms for converting ROS to less harmful compounds, so as long as these systems are not saturated, cells tolerate generated ROS. Immoderate generation of ROS leads to oxidative stress which can cause apoptosis, necrosis or autophagy-associated cell death [7–9]. Therefore, according to the above explanation, the opportunity for prolonged detection and diagnostic imaging of cancer cells needs low photobleaching and thus a low level of excitation [5,10–12]. However, for therapy applications, appropriate and sufficient excitation is necessary to ensure complete treatment and to allow for consistent and reproducible patient outcomes [4,13]. The overdose of the excitation, which may lead to an expansion of necrosis in cancer cells (compared with the more preferable apoptosis mechanism of cell death) or morbidity in healthy surroundings, should also be avoided (see Fig. 1) [13–15]. The usefulness of a particular excitation light dose for PDD and PDT depends on the photosensitizer and cancer cells or tissues being investigated [7,16]. There is no general guideline or model to optimize the excitation for PDD and PDT; it has to be assessed empirically for each photosensitizer and cell type [13,16–18]. As a consequence, choosing an appropriate excitation dose for PDD and PDT is almost always a challenging problem.

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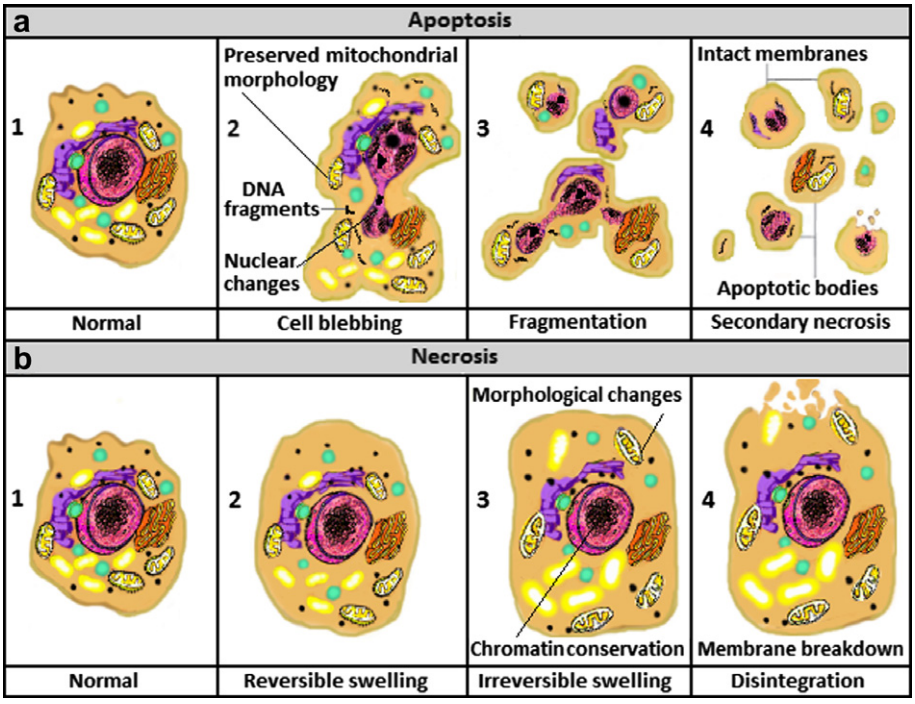

Fig. 1. Two main types of cell death modified from Ref. [15]. (a) Apoptosis is explained by characteristic changes in the morphology of the nucleus, blebbing of the plasma membrane, overall cell shrinkage, and formation of apoptotic bodies. As the apoptotic bodies are surrounded by an intact plasma membrane, apoptosis usually occurs without any leakage of cellular contents and thus without provoking an inflammatory response. The apoptotic bodies are recognized and eaten by professional phagocytes or neighboring cells. (b) Necrosis (negative death) induces chromatin condensation, swelling and vacuolation of cytoplasm, dilation of organelles such as mitochondria, endoplasmic reticulum and Golgi apparatus. It also causes the rupture of the plasma membrane and the proinflammatory leakage of the intracellular content. Swelling and bursting of cancer cells due to necrosis causes the spilling of the cell contents over their non-cancer neighbors and elicits an inflammatory response. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We have done a series of experiments to modify the excitation light dose of a strong photosensitizer called PVP-Hypericin for PDD and PDT of A549 cells in vitro studies. PVP-Hypericin is a novel formulation based on a water-soluble complex formed between Hypericin and PVP with fluorescence emission mostly within the red spectral region [4,19]. Therefore, to evaluate the potential of PVP-Hypericin as a photodiagnostic and phototherapeutic agent, a real need for optimization of the excitation dose and minimizing the side-effects exists [4,19,20]. Based on our experiments, we have previously reported that excitation of PVP-Hypericin with brief pulses of blue LED (I^* = 9.83 mW/cm^2 and L = 1.29 ms) minimizes the phototoxicity and photobleaching, and thus provides long-term PDD of A549 cancer cells [5]. In this paper, we aim to introduce a method to modify the excitation light dose of PVP-Hypericin and to find the optimum irradiation dose that can replace the conventional continuous excitation (with side-effects) in PDT of the A549 cells. The distinctive strategy of this paper is based on the dynamic imaging of cell viability and toxicity due to the different dosages of irradiation (three value of I^* and various exposure times) before and after the addition of PVP-Hypericin. In order to facilitate the study of cell viability, an additional fluorescent marker called carboxyfluorescein-diacetate-succinimidyl-ester (CFSE) in a non-toxic concentration was used, which improves the fluorescence signals in time lapse images [21,22]. Then, to evaluate the efficacy of PVP-Hypericin for PDT, a model is developed that shows a nonlinear relationship between the percentages of cell death and various exposure times at each of the experimental I^*. Based on this model, the thresholds of irradiation time the cells can tolerate as well as the optimum I^* and L showing the best overlap with PDT are presented.

2. Materials and methods

2.1. Preparation of cell culture and marking of A549 cells with CFSE and PVP-Hypericin

A549 cells cultured in RPMI 1640 medium with 10% fetal calf serum (FCS) (all from the Cancer Research Institute, Medical University of Vienna, Austria) were marked with CFSE fluorescent dye by using a method adapted from Quah et al. [21]. CFSE was diluted to 5 mM in dimethyl sulfoxide and then to 1 μM in phosphate-buffered saline. The dye solution was added to the cells to give a final concentration of 1 μM, which is non-toxic for the cells [21,22]. After 10 min, staining was stopped by washing twice with ice-cold RPMI. Finally, the cells were cultured in RPMI with 10% FCS in 35 mm Petri dishes (WillCo-dish™, GWSt-3522, Amsterdam) with a density of 6.6 × 10^4 cells/dish in a standard incubator (CO2CELL, MMM Group) at 5% CO_2 and 95% humidity. After 24 h, when the CFSE was distributed among parent and daughter cells, the proliferation of these marked cells, the so-called reference cells, were monitored upon different dosages of excitation by a fluorescence microscope for 5 h.

The solution of PVP-Hypericin was prepared by the formulation of Hypericin with PVP40, in a ratio of 1:100, using the procedure described by Kubin et al. [19]. PVP-Hypericin was dissolved in RPMI with 10% FCS at 1 mM Hypericin for the stock solution. Then it was freshly added to the cells marked with CFSE to give a final concentration of 50 μM Hypericin [19,23]. Then, the phototherapeutic efficacy of PVP-Hypericin in these dual-marked cells was monitored for 5 h. Each experiment – characterized by a specific excitation dose- was independently carried out three times for statistical reliability and reproducibility.