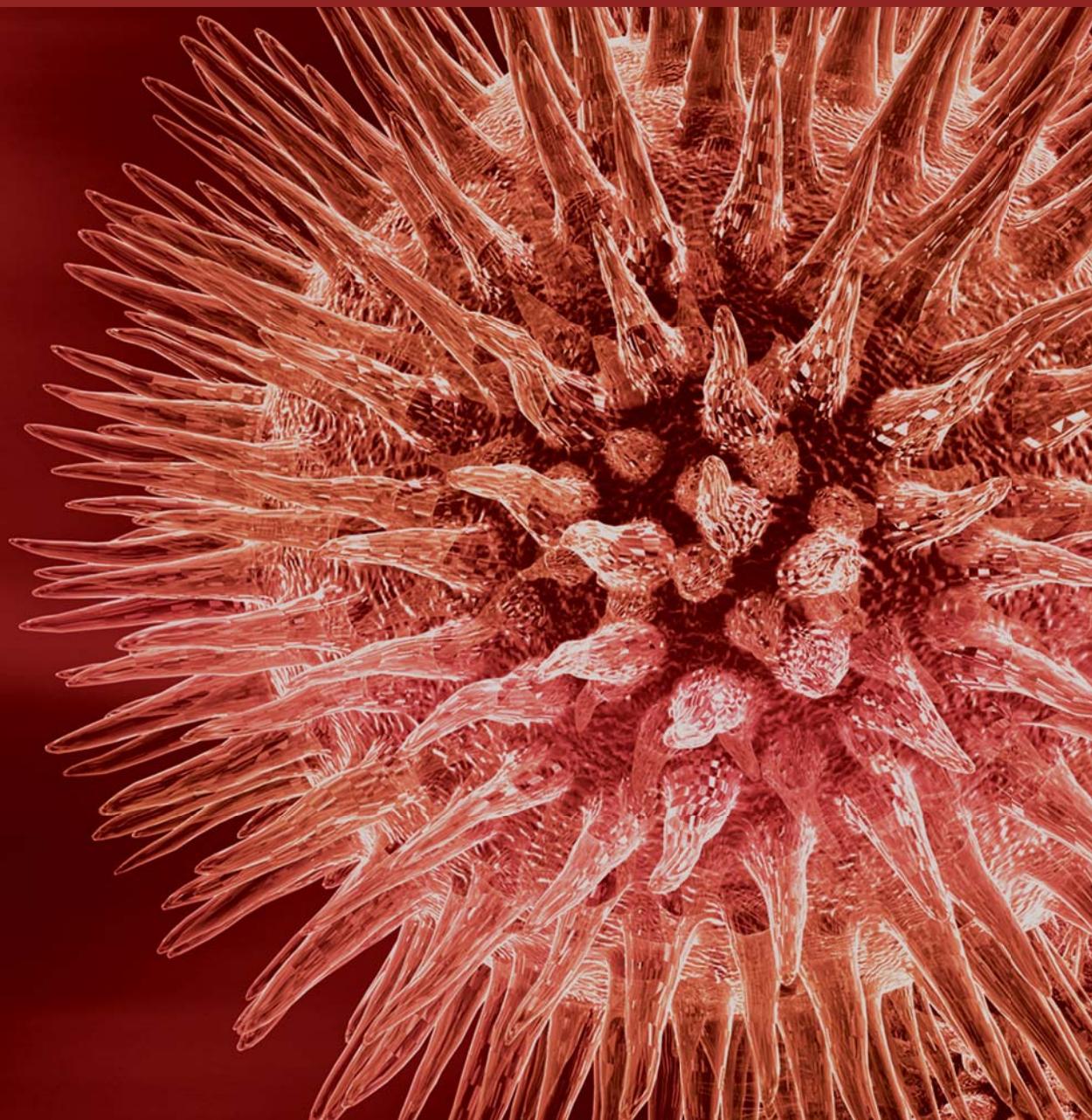


# New Applications of Photodynamic Therapy in Biomedicine and Biotechnology

Guest Editors: Kristjan Plaetzer, Mark Berneburg, Tobias Kiesslich, and Tim Maisch



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## Editorial

# New Applications of Photodynamic Therapy in Biomedicine and Biotechnology

Kristjan Plaetzer,<sup>1</sup> Mark Berneburg,<sup>2</sup> Tobias Kiesslich,<sup>3,4</sup> and Tim Maisch<sup>5</sup>

<sup>1</sup> Laboratory of Photodynamic Inactivation of Microorganisms, Department of Materials Science and Physics, University of Salzburg, Hellbrunnerstraße 34, 5020 Salzburg, Austria

<sup>2</sup> Department of Dermatology, Eberhard Karls University, Liebermeisterstraße 25, 72076 Tuebingen, Germany

<sup>3</sup> Department of Internal Medicine I, Paracelsus Medical University and Salzburger Landeskliniken (SALK), Müllner Hauptstrasse 48, 5020 Salzburg, Austria

<sup>4</sup> Institute of Physiology and Pathophysiology, Paracelsus Medical University, Strubergasse 21, 5020 Salzburg, Austria

<sup>5</sup> Department of Dermatology, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

Correspondence should be addressed to Kristjan Plaetzer; kristjan.plaetzer@sbg.ac.at

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Photodynamic procedures are based on the light-induced, photosensitizer-mediated overproduction of reactive oxygen species for removal of harmful or unwanted cells/pathogens. With approvals for various applications by health agencies in most industrial countries, Photodynamic Therapy (PDT) represents the method of choice for treatment of age-related macular degeneration and is appreciated as minimally invasive therapeutic procedure to treat skin, esophageal, head and neck, lung, and bladder cancers with high cure rates, low side effects, and excellent cosmetic outcome. Being motivated by the success of PDT in management of various human diseases, new and very promising applications of this procedure are being identified and explored by the PDT research community. So, for example, Photodynamic Inactivation of microorganisms (PDI) has the potential to avert the severe threat of increasing antimicrobial resistance. This special issue presents a collection of papers dealing with novel photosensitizers with improved photochemical and photophysical properties, alternatives to (single) photon excitation of the photoactive drug, new clinical targets of photodynamic procedures, and innovative reviews on crucial aspects, which substantiate the success of Photodynamic Therapy and Photodynamic Inactivation.

In a research paper entitled “*Hydrogen bond acceptors and additional cationic charges in methylene blue derivatives:*

*photophysics and antimicrobial efficiency*,” A. Felgenträger et al. report on the synthesis and photodynamic efficiency of six novel methylene blue derivatives with highly polar and/or hydrophilic groups. Based on a systematic analysis of singlet oxygen formation in combination with the absorbed light energy and on investigations of the phototoxicity towards *Staphylococcus aureus* and *Escherichia coli*, a structure-activity relationship has been drawn from a chemical point of view. The data presented in this study demonstrate that hydrogen acceptor bond moieties and additional tertiary charges in the substituent have a positive influence on the overall antimicrobial efficacy of methylene blue derivatives, resulting in a convincing inactivation of viable Gram (+) or Gram (−) bacteria up to 7 log units.

Conjugates of nanoparticles and photosensitizers represent a very promising new generation of photoactive substances. The correlation of the size of purpurin-18-*N*-methyl-*D*-glucamine gold nanoparticles and the respective photodynamic activity is described by B. Lkhagvadulam et al.’s “*Size-dependent photodynamic activity of gold nanoparticles conjugate of water soluble purpurin-18-*N*-methyl-*D*-glucamine*”. The authors demonstrate that the PDT efficiency of photosensitizer-nanoparticle conjugates against A549 lung cancer cells is higher when compared to the unbound photosensitizer because of the increased internalization of the PS

into the target cells using the size effect. The highest photodynamic activity is reported to be associated with rather bigger conjugates with a diameter of about 60 nm.

Y.-S. Chou et al.'s "Photo-induced antitumor effect of 3,6-Bis(1-methyl-4-vinylpyridinium) carbazole diiodide" evaluated the photo-induced antitumor effect of 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC) in a lung tumor cell line (TC-1) and the corresponding *in vivo* murine model (TC-1 cell tumors in C57BL/6 mice). BMVC is able to induce a phototoxic effect in TC-1 cells at light fluences greater than 40 J/cm<sup>2</sup> and significantly reduced the tumor growth rate *in vivo* following fine-needle interstitial light illumination of the photosensitizer. Immunohistochemistry studies showed that the microvascular density was lower not only in the PDT group but also in animals, which received light only (without photosensitizer). Therefore, the antivascular effect might be partially attributed to mild hyperthermia induced by the laser illumination.

The study of V. Ziegler et al. entitled "Photosensitizer adhered to cell culture microplates induces phototoxicity in carcinoma cells" discusses the phototoxic effect of adherence of photosensitizers on the surfaces of cell culture microplates. Using two rather lipophilic and two hydrophilic substances currently employed in PDT, the authors show that lipophilic photosensitizer molecules adhered to microplates are able to induce cytotoxicity in A431 human epidermoid carcinoma cells upon illumination, independently of whether the photoactive molecule relocates from the surface into cells or not. This effect is negligible for hydrophilic photosensitizers. The ability of plastic materials to (reversibly) store photosensitizers might, according to V. Ziegler et al. represent a new approach for photosensitizer delivery or development of antimicrobial coatings.

Inactivation of microorganisms within skin or tissue based on photosensitizers activated by illumination may be complicated by the relatively low penetration depth of visible light. As an alternative to light illumination, F. Nakonechny et al.'s "Sonodynamic excitation of rose bengal for eradication of Gram-positive and Gram-negative bacteria" excited the photosensitizer Rose Bengal with 28 kHz ultrasound. The results presented in the study demonstrate for the first time that the novel "sonodynamic" activation of Rose Bengal is able to reduce the number of viable *Staphylococcus aureus* and *Escherichia coli* by up to 4 log units. Sonodynamic treatment represents a promising approach for curing internal infections and sterilization.

In a comprehensive review article entitled "Innovative strategies to overcome biofilm resistance" by A. Taraszkiewicz et al. strategies to combat bacterial biofilms are discussed. If organized in biofilms (e.g., in or on humans), bacteria are much more resistant to antimicrobial therapies, including Photodynamic Inactivation. The authors suggest two innovative approaches to increase the bactericidal effect of PDI, namely, the employment of enzymes that affect the biofilm or a combination of PDI with antibiotics, plant extracts, or other biofilm disrupting substances. Application of PDI together with enzymes specific for microbial structures might furthermore increase the selectivity of phototoxicity

and/or allow for the usage of lower photosensitizer concentrations.

Patients suffering from the genetically induced skin disorder epidermolysis bullosa (EB) are exposed to an increased skin tumor risk. P. Larisch et al.'s "In vitro analysis of photosensitizer accumulation for assessment of applicability of fluorescence diagnosis of squamous cell carcinoma of epidermolysis bullosa patients" investigated the applicability of fluorescence diagnosis of hypericin and endogenous protoporphyrin IX using an *in vitro* model system, consisting of different (EB) skin cell lines exposed to normal and proinflammatory conditions. A proinflammatory milieu simulated by addition of TNF-alpha appeared not to influence photosensitizer accumulation in skin cells. Carcinoma cells of recessive dystrophic EB showed lower hypericin or PpIX-induced fluorescence than nonmalignant recessive dystrophic EB cells. Further experiments will be necessary to evaluate the possible application of fluorescence diagnosis for early cancer detection of EB patients.

Two-photon absorption is a nonlinear optical process, by which two photons are absorbed simultaneously, thus allowing for doubling the wavelength of excitation of a given photosensitizer in PDT, thereby increasing the penetration depth of the PDT illumination light in tissue and allowing for treatment of thicker malignancies. In this issue, K. Ogawa and Y. Kobuke through their paper "Two-photon photodynamic therapy by water-soluble self-assembled conjugated porphyrins" review a set of studies on two-photon PDT with water-soluble and self-assembling conjugated porphyrins. Based on data of two-photon cross section values obtained by open-aperture Z-scan measurements, singlet oxygen production and phototoxicity towards cancer cells *in vitro*, the conjugated porphyrins are suggested as candidates for two-photon PDT agents.

In the paper entitled "Killing effect of Ad5/F35-APE1 siRNA recombinant adenovirus in combination with hematoporphyrin derivative-mediated photodynamic therapy on human nonsmall cell lung cancer," L. Xia and coworkers report on the PDT efficiency and the molecular mechanism of cytotoxicity of the Ad5/F35-APE1 siRNA recombinant adenovirus in combination with hematoporphyrin derivative (HpD) in A549 human lung adenocarcinoma cells. Infection of cancer cells with the Ad5/F35-APE1 siRNA recombinant adenovirus significantly reduced the HpD-PDT-induced expression of the APE1 protein and could therefore enhance the photokilling of HpD-mediated PDT.

Three papers discuss the interconnection between PDT and the host's immune response. The methodology report entitled "Effective combination of photodynamic therapy and imiquimod 5% cream in the treatment of actinic keratoses: three cases" of L. Held et al. evaluate the therapeutic effect of combining PDT using 5-aminolevulinic-acid-(ALA-) induced PPIX as photosensitizer with imiquimod 5% cream for the clinical treatment of actinic keratoses. Imiquimod is known to regulate the level of cytokines and thereby to influence the immune response and was applied to the treatment area two weeks after PDT. Although a rather small population of patients was part of the pilot study, the results are promising and should be studied in subsequent larger clinical trials:

PDT followed by imiquimod treatment was well tolerated and combination of both improved the reduction of actinic keratoses, as demonstrated by the authors.

Photodynamic treatment of actinic keratoses and non-melanoma skin cancer in solid organ transplant recipients as well as alternatives to PDT are reviewed by C. Wlodek et al.'s "*Use of photodynamic therapy for treatment of actinic keratoses in organ transplant recipients*". It is well known that long-term immunosuppressive medication performed on organ transplant recipients increases the risk to develop skin cancers and/or their precursors. The authors present PDT as efficient, safe, and well-tolerated treatment option for these malignancies in immunosuppressed patients but point out that early and numerous treatments on a regular basis are necessary to obtain an optimal response and to prevent development of new actinic keratoses.

E. Panzarini et al. in their paper "*Immunogenic cell death: can it be exploited in photodynamic therapy for cancer?*" review the undoubted key role of the immune response triggered by PDT for the control of cancer cells revival after an antitumor treatment. By elucidating the role of damage associated molecular patterns (DAMPs) at the crossroad between cancer cell death and immunogenicity in PDT, the convincing advantage of PDT over several other cancer treatment options, which is to potentially elicit a specific antitumor immunity, is discussed.

The process of scientific development of new applications of PDT is still highly active and viable. The finalizing contribution of this special issue is therefore an invitation to other researchers to join the successful photodynamic community. T. Kiesslich and coworkers in the paper entitled "*A comprehensive tutorial on in vitro characterization of new photosensitizers for photodynamic antitumor therapy and photodynamic inactivation of microorganisms*" present a comprehensive tutorial on the initial experimental procedures *in vitro*, which help to identify possible applications of newly synthesized photosensitizers in the frame of PDT/PDI. The article covers a detailed description of the experimental approach to the characterization of the photochemical and photophysical properties of a photosensitizer, its uptake kinetics into cells, intracellular localization, and photodynamic action in both, tumor cells and microorganisms. The authors hope that the special issue on PDT in general and the tutorial article in particular will help to stimulate the efforts to expand the convincing benefits of photodynamic procedures within both established and new fields of applications.

## Acknowledgments

The guest editors are grateful to all referees who devoted considerable time and effort to maintain the high scientific standard of this special issue by their excellent and constructive comments on the papers.

*Kristjan Plaetzer  
Mark Berneburg  
Tobias Kiesslich  
Tim Maisch*

## Review Article

# A Comprehensive Tutorial on *In Vitro* Characterization of New Photosensitizers for Photodynamic Antitumor Therapy and Photodynamic Inactivation of Microorganisms

Tobias Kiesslich,<sup>1,2</sup> Anita Gollmer,<sup>3</sup> Tim Maisch,<sup>3</sup> Mark Berneburg,<sup>4</sup> and Kristjan Plaetzer<sup>5</sup>

<sup>1</sup> Department of Internal Medicine I, Paracelsus Medical University/Salzburger Landeskliniken (SALK), Muellner Hauptstrasse 48, 5020 Salzburg, Austria

<sup>2</sup> Institute of Physiology and Pathophysiology, Paracelsus Medical University, Strubergasse 21, 5020 Salzburg, Austria

<sup>3</sup> Department of Dermatology, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

<sup>4</sup> Department of Dermatology, Eberhard Karls University, Liebermeisterstraße 25, 72076 Tuebingen, Germany

<sup>5</sup> Laboratory of Photodynamic Inactivation of Microorganisms, Department of Materials Science and Physics, University of Salzburg, Hellbrunnerstraße 34, 5020 Salzburg, Austria

Correspondence should be addressed to Kristjan Plaetzer; kristjan.plaetzer@sbg.ac.at

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*In vitro* research performed on eukaryotic or prokaryotic cell cultures usually represents the initial step for characterization of a novel photosensitizer (PS) intended for application in photodynamic therapy (PDT) of cancer or photodynamic inactivation (PDI) of microorganisms. Although many experimental steps of PS testing make use of the wide spectrum of methods readily employed in cell biology, special aspects of working with photoactive substances, such as the autofluorescence of the PS molecule or the requirement of light protection, need to be considered when performing *in vitro* experiments in PDT/PDI. This tutorial represents a comprehensive collection of operative instructions, by which, based on photochemical and photophysical properties of a PS, its uptake into cells, the intracellular localization and photodynamic action in both tumor cells and microorganisms novel photoactive molecules may be characterized for their suitability for PDT/PDI. Furthermore, it shall stimulate the efforts to expand the convincing benefits of photodynamic therapy and photodynamic inactivation within both established and new fields of applications and motivate scientists of all disciplines to get involved in photodynamic research.

## 1. Introduction

Photodynamic procedures combine three per se harmless components, namely, a light-sensitive molecule (the photosensitizer, PS), non-UV light corresponding to an absorption peak of the PS, and molecular oxygen to remove harmful pathogens, cells, or tissue(s). Within the last decades, the most prominent application of this approach, photodynamic therapy (PDT), has become a valuable alternative to classical treatment of localized malignant diseases since clinical studies prove its effectiveness, particularly in early stage tumors. The success of PDT is based on three mechanisms by which, either alone or in combination, the light-induced and PS-catalyzed overproduction of reactive oxygen species

(ROS) destroys tumors: direct tumor cell death, damage set to the vasculature, and induction of a local inflammation with a subsequent immune response. For some indications (e.g., skin precancers and cancers) PDT represents a valuable therapeutic option also due to the excellent cosmetic outcome. Based on the design of new PS (with near infrared absorption), PS formulations, or PS/nanoparticle conjugates as well as technical improvements, still new applications of PDT are regularly identified [1].

Motivated by the achievements of recent PDT research and justified by the severe health threat that antimicrobial resistance poses to humans' health photodynamic inactivation of microorganisms (PDI) has been (re)introduced as revolutionary approach to kill bacteria, viruses, yeasts,

and parasites. The lack of new antibiotics classes combined with the propagation of multidrug resistant bacteria/fungi and the economic and regulatory challenges thereof have boosted the research on PDI [2–4]. As the properties of ideal PS for PDT and PDI differ, numerous new substances especially synthesized for this promising approach are being developed in the photodynamic research community and expand the field of applications (e.g., to water-borne diseases) [5, 6].

Photodynamic therapy and PDI require, as multidisciplinary approaches, the tight cooperation of chemists (e.g., for synthesis of new PS), biologists (for testing new substances), physicists (e.g., for light dosimetry), and clinicians (for the transfer from the lab bench to the clinical application). The development of novel PDT/PDI applications in biomedicine and biotechnology is mainly driven by chemists and biologists. The former design new PS with promising properties, sometimes without the claim to intend the new substance for a specific target, and the latter test these new dyes *in vitro* on eukaryotic or prokaryotic cell cultures in order to estimate the possible field of application of a PS. Although many experimental steps of PS testing make use of the wide spectrum of methods readily employed in cell biology, some special aspects (e.g., fluorescence of the PS molecule or the requirement of subdued light conditions) have need of being considered when doing *in vitro* experiments in PDT/PDI. Up to date, the scientific photodynamic community did neither suggest a standard strategy for PS testing, nor does a tutorial on PS testing exist.

The aim of this paper is therefore to provide a comprehensive overview of experimental strategies and methods—without extensive referencing in order to maintain readability—by which novel photoactive drugs can be tested *in vitro* for their employment in photodynamic procedures. It shall represent a suggestion of operative instructions by which novel photoactive molecules may be identified as suitable for PDT and PDI, based on photochemical and photophysical properties of a PS, its uptake into cells, the intracellular localization, and photodynamic action in both tumor cells and microorganisms. This tutorial shall stimulate the efforts to expand the convincing benefits of photodynamic procedures within both established and new fields of applications and motivate (young) scientists to contribute to the photodynamic research.

## 2. Characterization of the Photochemical and Photophysical Properties

**2.1. Recording of Absorption/Fluorescence Spectra in Various Solvents/Cells.** The absorption/excitation wavelength(s) of a given PS represents a key selection criterion for its application in PDT or PDI. As a common agreement, UV activation of photoactive drugs is inadmissible to exclude damage set to the cells' genetic information. Also, light wavelengths which are absorbed by the major tissue or cell chromophores should be avoided for excitation of the PS. For PDT on solid tumors, the effective penetration depth of the excitation light highly depends on the interference with the absorption spectra

of the major tissue chromophores, namely (oxy-/deoxy-) hemoglobin and melanin, as well as water. The absorption spectra of these molecules define the optical window for PDT in tissue which covers the wavelength range of 600–850 nm [7]. The upper limit of this window is set by the minimal quantum energy, which is required for an efficient production of singlet oxygen, considering thermal loss of energy combined with the shifts of the electrons during the photophysical processes [8]. Due to a different composition of chromophores in microorganisms, the lower wavelength limit of the optical window defined for PDT applications does not necessarily apply for photosensitizers employed in PDI.

As the photophysical processes in a fluorescing molecule are dependent on the solvent, excitation and emission spectra of a PS should be read in aqueous solutions (buffers) or biocompatible solvents such as dimethyl sulphoxide (DMSO) or ethanol. In order to increase the water solubility of rather lipophilic PS, fetal calf/bovine serum (FC/BS) or other solubility enhancers such as polyvinylpyrrolidone [9, 10] may be added for recording of the spectra. In very rare cases (e.g., for photosensitizers showing absorption peaks with a narrow spectral half-width in combination with laser light illumination) the recording of spectra of the photosensitizer inside the cells might be necessary to assure a wavelength overlap of the light used for excitation with the absorption of the dye. Here, cells are incubated with the photosensitizer for a sufficient period of time, detached from the surface of the cell culture receptacle (e.g., by trypsinization) and washed with buffer to remove PS not internalized into cells. Fluorescence spectrometers allowing for stirring the solution of cells inside the cuvettes might be necessary and the spectra have to be corrected by samples with cells and without PS [11, 12].

Additionally, the experimenter has to take into account that not only the primary PS itself but also secondary molecules with different absorption resulting from photo-modification of the primary PS may contribute to the overall photodynamic efficiency [11, 13].

The preparation of stock solutions of the photosensitizing agents might prove useful, as they might, depending on the chemical stability of the photosensitizer and the storage conditions, allow for a good experiment-to-experiment reproducibility of the PS concentration in PDT experiments. If stock solutions are prepared, the final concentration of solvents other than physiological buffers in the *in vitro* application should be as low as possible (for DMSO and ethanol <1% v/v), and the possible cytotoxic effect of the solvent should be tested by means of a viability assay (see the following chapter) under conditions identical to those used for incubation of the PS with the target cells. Storage of the PS should be performed according to the manufacturer's recommendations or may require individual optimization. The stock solutions of some PS may be kept frozen (exception: e.g., liposomal formulations). Independent of the storage conditions (as solid powder or in solution) the control measurements of the PS spectral properties on a regular basis are highly recommended to rule out PS decomposition.

**2.2. Monitoring of Singlet Oxygen Production.** Depending on the chemical structure of a photosensitizer there are two alternative kinds of pathways to generate reactive oxygen species (ROS) after light activation. A type I process involves the direct interaction of an excited photosensitizer with surrounding substrates to generate radicals or radical ions like hydroxyl radicals ( $\text{HO}^\bullet$ ) and superoxide anions ( $\text{O}_2^{\bullet-}$ ) via charge transfer. Whereas in a type II mechanism the generation of singlet oxygen ( ${}^1\text{O}_2$ ) usually takes place by direct energy transfer from the excited triplet state of the PS to molecular oxygen (see Figure 1). In both cases, the initially generated reactive oxygen species initiate further oxidized intermediates at the cell wall, cell membrane, on peptides, and lipids depending on the localization of the photosensitizer.

The detection of reactive oxygen species is usually shown indirectly by deactivation of ROS using quenchers like sodium azide (type II), histidine, mannitol (type I), betacarotene, and superoxide dismutase (type I). In biological systems, the lifetime of ROS can be very short (e.g., few  $\mu\text{s}$  for singlet oxygen) [14]. Thus, the quencher/detection agents must be located directly at the site of ROS generation with a sufficient high concentration, which can be difficult and a source of ambiguous results. However, in microorganisms the transport of such quenchers is rather complicated and the quenchers may not reach the site of ROS generation.

Increasingly sophisticated optical techniques have been developed and employed over the last years to both create and monitor ROS such as  ${}^1\text{O}_2$  in samples that range from liquid solutions to single living cells [15].  ${}^1\text{O}_2$  is believed to play the major role in PDT/PDI. Therefore, the following text will focus on frequently used detection techniques for this type of ROS. Wu et al. have recently summarized methods of  ${}^1\text{O}_2$  detection [16], with focus on the recent technical advances. To investigate the chemistry of  ${}^1\text{O}_2$ , several analytical tools are available to obtain information about the concentration, the spatial distribution, and the temporal behavior of  ${}^1\text{O}_2$  formation, including spectrophotometry, fluorimetry, and (chemi)luminometry.

With respect to spectrophotometry, over the years, the design of appropriate probes for  ${}^1\text{O}_2$  has developed significantly. Up until the late 1990s many chemical  ${}^1\text{O}_2$  traps had been reported [17–21]. Of the trapping molecules used, 1,3-diphenylisobenzofuran (DPBF) has certainly been one of the favorites [22].  ${}^1\text{O}_2$  rapidly and irreversibly reacts with DPBF to initially yield an endoperoxide which, in turn, evolves into other products that do not fluoresce and have absorption spectra different from that of DPBF. The development and use of DPBF-dependent techniques have recently played a key role in yielding useful information about  ${}^1\text{O}_2$  in a wide variety of systems [23–26]. 9,10-diphenylanthracene (DPA) was also a widely used chemical trap for  ${}^1\text{O}_2$  [27, 28]. In both cases, as well as for many other molecules, the fluorophore itself acts as a reactive moiety and changes its photophysical properties upon reaction with  ${}^1\text{O}_2$ . The decrease in absorbance, due to the loss of conjugation, has been used for anthracene derivatives as a quantitative measure of the formation of the endoperoxide. However,

because the detection of  ${}^1\text{O}_2$  was based on the measurement of absorbance changes, these probes are not highly sensitive.

In 1999 Umezawa and coworkers designed and synthesized novel fluorometric probes for  ${}^1\text{O}_2$  based on fluorescence changes in order to improve the sensitivity [29]. They developed linked two-component systems called DPAXs, consisting of a fluorophore reporter portion and a  ${}^1\text{O}_2$ -reactive anthracene moiety. The fluorophore part is based on fluorescein, which is widely used in cell biology for labeling and sensing [30]. In 2001, Tanaka et al. reported a rational design strategy for an optimal fluorescent probe for  ${}^1\text{O}_2$  also based on a fluorescein-anthracene combination called 9-[2-(3-carboxy-9,10-dimethyl)anthryl]-6-hydroxy-3H-xanthen-3-one (DMAX) [30]. The mechanism these probes are based on is photoinduced electron transfer (PeT). The experimental approach has been to devise a two-component system comprised of a trapping moiety coupled to a light emitting chromophore. Prior to the reaction with  ${}^1\text{O}_2$ , emission from the chromophore is quenched by electron transfer from the adjacent trapping moiety. Upon reaction with  ${}^1\text{O}_2$ , however, the resultant oxygen adduct is no longer an efficient intramolecular electron donor, and light emission readily occurs from the fluorescent moiety. Commercial vendors such as molecular probes also used this fluorescein-anthracene combination to develop singlet oxygen sensor green (SOSG, see Figure 2) [31].

Fluorescent probes are sensitive and can afford high spatial resolution via microscopic imaging [30]. The fluorescent properties of fluorescence probes such as fluorescence intensity, wavelength, quantum yield, or fluorescence lifetime can change upon reaction with  ${}^1\text{O}_2$ . Two clear advantages of this indirect method to detect  ${}^1\text{O}_2$  exist: (1) the luminescence quantum efficiency of the optical probe is comparatively large ( $\Phi_{\text{fl}} \sim 10^6$ ,  $\Phi_{\text{ph}}({}^1\text{O}_2) \approx 1$ ), and (2) emission occurs in the visible region of the spectrum where optical detectors are very efficient. However, for a number of reasons, caution must be exercised when using fluorescent probes such as SOSG. First, it was shown by Ragàs et al. that SOSG is able to generate  ${}^1\text{O}_2$  [32] and Gollmer et al. have shown that the immediate product of the reaction between SOSG and  ${}^1\text{O}_2$  is, itself, an efficient  ${}^1\text{O}_2$  photosensitizer [33]. Second, SOSG appears to efficiently bind to proteins which, in turn, can influence uptake by a cell as well as behavior in the cell [33]. Third, some fluorescent probes are not selective enough for one particular ROS. Recently, Nakamura et al. showed that SOSG reacted with other ROS such as  $\text{O}_2^{\bullet-}$ , hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and  $\text{HO}^\bullet$  resulting in a small increase in the fluorescence response [34]. As such, incorrect use of fluorescent dyes can yield misleading data on yields of photosensitized  ${}^1\text{O}_2$  production and can also lead to photooxygenation-dependent adverse effects on the system being investigated.

Therefore, direct measurements are highly recommended to detect ROS like  ${}^1\text{O}_2$  via its luminescence at 1270 nm. In this case, photons that are emitted by the excited molecule itself are detected (Figure 1, right side). An advantage is that there is no need for any additional substances, which might be either toxic or too large for transportation in tissue. The time-resolved detection technique has been used for (1) the

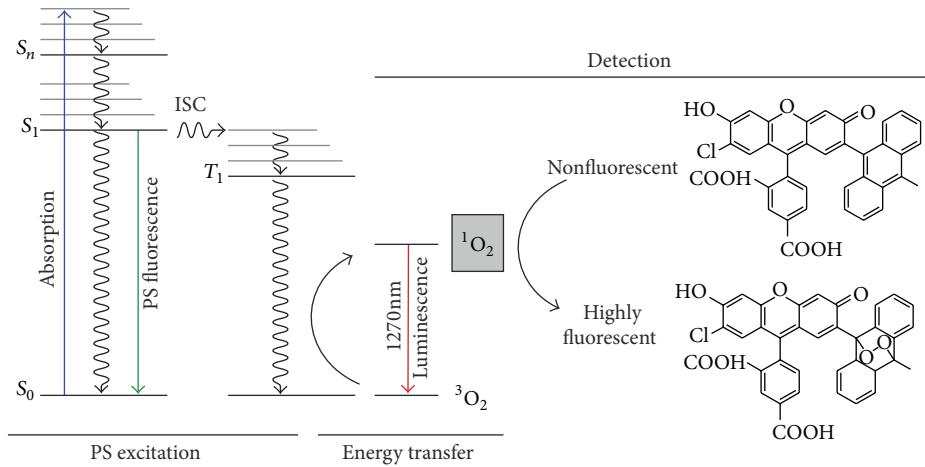


FIGURE 1: Jablonski diagram for the photosensitized production of  ${}^1\text{O}_2$  (left side) and its detection either by direct measurement of the singlet oxygen photons at 1270 nm (singlet oxygen luminescence) or indirectly using a fluorescent probe (right side).

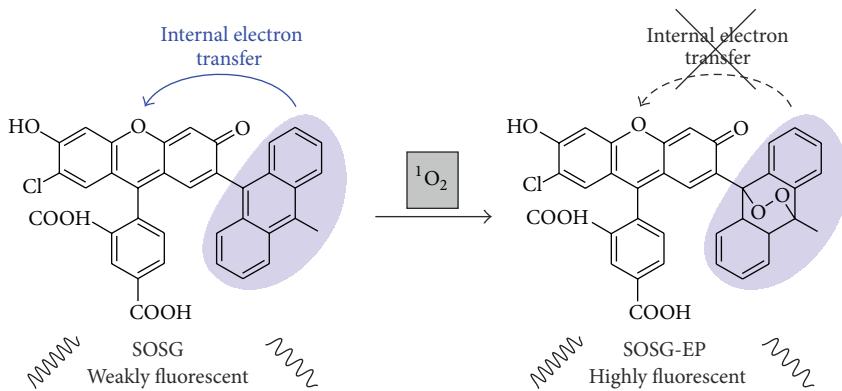


FIGURE 2: Formation of the endoperoxide of SOSG (singlet oxygen sensor green) upon reaction of SOSG with  ${}^1\text{O}_2$  as an indirect method for detecting  ${}^1\text{O}_2$ . Prior to the reaction with  ${}^1\text{O}_2$ , internal electron transfer (ET) quenches the fluorescence from the light-emitting chromophore. Upon reaction with  ${}^1\text{O}_2$  and the formation of the endoperoxide, electron transfer is precluded, and fluorescence is observed.

identification of  ${}^1\text{O}_2$ , (2) measurement of quantum yields of  ${}^1\text{O}_2$  production in photosensitized processes,  $\Phi_\Delta$ , and (3) the determination of rate constants for the interaction of  ${}^1\text{O}_2$  with substrates.

The  ${}^1\text{O}_2$  signal depends on many parameters that are expressed in the following formula:

$$[{}^1\text{O}_2](t) = \frac{[T_1]_0 k_{T_1\Delta} [{}^3\text{O}_2]}{k_T - k_d} (e^{-k_d t} - e^{-k_T t}), \quad (1)$$

where  $[T_1]_0$  is the concentration of the photosensitizer molecules in the excited  $T_1$  state,  $k_{T_1\Delta}$  is the rate constant for deactivation of the  $T_1$  state by  ${}^1\text{O}_2$ ,  $[{}^3\text{O}_2]$  is the concentration of molecular oxygen in its ground state,  $k_T$  is the rate constant for all channels of  $T_1$  deactivation, and  $k_d$  is the rate constant for all channels of  ${}^1\text{O}_2$  deactivation.

The time dependence of the  ${}^1\text{O}_2$  signal is used to better understand the interaction of  ${}^1\text{O}_2$  with its environment. It tells one about the kinetics of the production and the decay of  ${}^1\text{O}_2$  in different environments ranging from polymer systems

to single living cells. These direct measurements can reflect the complex and dynamic morphology of a cell [35–39].

The  ${}^1\text{O}_2$  luminescence quantum efficiency ( $\Phi_{\text{Ph}}$ ) is given by (2):

$$\Phi_{\text{Ph}} = \Phi_\Delta k_r \tau_\Delta, \quad (2)$$

where  $\Phi_\Delta$  represents the quantum yield of  ${}^1\text{O}_2$  formation,  $k_r$  the radiative rate constant for the transition  ${}^1\text{O}_2 \rightarrow {}^3\text{O}_2$ , and  $\tau_\Delta$  the lifetime of  ${}^1\text{O}_2$ .

However, despite the wide spread use of the direct 1270 nm detection, it has limitations because of the low  ${}^1\text{O}_2$  luminescence (the quantum efficiency is about  $10^{-5}$ – $10^{-7}$  depending on its environment) [40] and low signal-to-noise ratios, which makes the measurement of the  ${}^1\text{O}_2$  signal a nontrivial task and limits the effectiveness of this technique for many applications especially in the biological field. In the presence of molecules that can physically quench or chemically react with  ${}^1\text{O}_2$ , such as water or proteins, for example, in

a biological cell,  $\tau_{\Delta}$  will decrease and  $\Phi_{\text{Ph}}$  will also decrease, resulting in a low signal-to-noise ratio. However, for the measurement of the luminescence signal a very sensitive detection system is available. With respect to  $^1\text{O}_2$  detection, the use of the 1270 nm  $^1\text{O}_2 \rightarrow ^3\text{O}_2$  luminescence in both time and spatially resolved experiments has, without doubt, been the most beneficial and informative tool. Several research groups were able to detect singlet oxygen luminescence in lipids and even in living cells/microorganisms after incubation with an exogenous photosensitizer and an optical excitation at different wavelengths [41–44].

**2.3. Assessment of Radical Formation.** In the type I mechanism of the photodynamic principle hydrogen-atom abstraction or direct electron-transfer reactions occur between the light excited state of the photosensitizer and a substrate that can be either a biological structure (e.g., lipids, proteins, amino acids, or DNA), a solvent, or an inanimate surface to yield free radicals or radical ions like superoxide radicals, hydroxyl radical, or peroxy radical [45]. The main methods for detection of type I-generated radicals include scavenging molecules such mannitol, histidine, and N,N-dimethyl-p-phenylenediamine (DMPD), as well as the total radical-trapping antioxidant parameter method (TRAP) or the oxygen-radical absorbance capacity (ORAC) method. At present, there is a need for more specific assays due to the lower specificity of these scavenging methods. Overall, type I reactions become more important at low oxygen concentrations like inside of biofilms or in more polar environments [46].

**2.4. Determination of the Photostability/Bleaching.** Due to light exposure of a photosensitizer, the absorption and fluorescence properties of the photosensitizer itself can change, which indicates that photodegradation and/or photoproduct formation appeared which results in a decreased photostability [47, 48]. Such changes can be evidenced by the appearance of new absorption bands within the specific absorption spectra of the photosensitizer [49]. Degradation can be monitored by the decrease of maxima absorption peak of the photosensitizer. Changes of the characteristic absorption spectra of a given photosensitizer depend on the wavelength of the illumination light: shorter wavelengths are more effective than longer wavelengths [50]. If the photosensitizer is bleached too rapidly, either successful inactivation of microorganisms or tumor destruction will not be completed once the minimal inhibitory concentration of the nondegraded photosensitizer in the infected/tumor tissue is exceeded upon illumination [51]. On the other hand, photobleaching can be an advantage regarding avoiding an overall skin photosensitivity which is one of the main side effects in patients treated with PDT [1, 52]. Furthermore, it is unalterable to assure the nontoxicity of photodegraded products of the photosensitizer. Overall knowledge of photodegradation as well as effects of photoproducts generated upon illumination is important to develop an appropriate dosimetry for each photosensitizer in antimicrobial photodynamic inactivation or in antitumor PDT [53].

**2.5. Positive Charge and Molecular Weight of a Given Photosensitizer.** A must-have of a successful PS for PDI is a positive charge, because bacteria are charged negatively due to their cell wall composition and *meso*-substituted, but negatively charged, porphyrins have not shown toxicity against Gram(–) bacteria [54, 55]. Furthermore, hydrophilic compounds (less than 600–700 Da, e.g., for *E. coli*) can diffuse only through the outer membrane via porins which act as a very effective permeability barrier, making Gram(–) bacteria less susceptible as Gram(+) [56]. Therefore, porphyrin-based photosensitzers like TMPyP with a molecular weight higher than 500 Da cannot diffuse through these porin channels. As a consequence, ROS can be generated only at the cell wall area of Gram(–) bacteria. Another important observation that has been made about positive-charged cationic antimicrobial photosensitzers concerns their selectivity for microbial cells compared to host mammalian cells [57]. It is thought that cationic molecules are only slowly taken up by host cells by the process of endocytosis, while their uptake into bacteria is relatively rapid. If illumination is performed within short intervals after PS application (minutes) the PDT-mediated damage to host tissue will be minimized.

### 3. PS Uptake Kinetics, Dark Cytotoxicity, and Intracellular Localization in Tumor Cells

This section describes experimental approaches for initial tumor cell-based characterization of new PS including cellular pharmacodynamics, cytotoxic effects of the PS in the absence of light, and the intracellular localization of the PS. Finally, the assessment of the penetration depth in an *ex vivo* porcine skin model is described.

**3.1. PS Uptake/Release Kinetics.** Measurement of the uptake of a new PS drug into cancer cells provides information on the (kinetics of) interaction and membrane transport characteristics of the drug and enables a first rough estimation of the drug-to-light interval—as a basis for verification in the subsequent preclinical validation of the PS. The methodological approaches described here make use of the inherent fluorescence properties of the PS and allow for either absolute quantification of the drug amount bound respective taken up by cells or a relative quantification mainly, for example, for estimation of time-dependent course of PS uptake.

By the nature of the assay format, experiments using microplates with 96 wells as the most commonly employed format allow for time-efficient and multiparametric testing of several variables possibly influencing the uptake characteristics. A first approach involves incubation of cancer cells cultured in 96-well microplates, followed by a fixed incubation period (e.g., 10–24 hrs) with a dilution series of the PS in the appropriate cell culture medium and subsequent determination of the PS-related fluorescence signal. The simplest procedure involves washing the cell cultures after the incubation and lysis of the cells by detergents such as SDS (sodium dodecyl sulphate) followed by fluorescence measurement in a microplate fluorimeter. Control experiments should be performed to exclude altered fluorescence

characteristics of the PS in the presence of such cell lysis reagents. Besides the plasma membrane permeability of the given PS, several additional factors may influence the cellular uptake including interaction of the PS with (i) constituents of fetal calf/bovine serum (FCS/FBS; [58, 59]) and with (ii) the cell culture plastic material [60, 61]. The first parameter can be assessed by using appropriate concentrations of FCS ranging from zero to the standard concentration used for routine cultivation of the respective cell type (e.g., 5%–15% v/v FCS for numerous cancer cell lines). Such data are important as similar serum constituents are present in human blood plasma which may influence the tissue distribution of PS drugs after systemic administration. The second parameter may significantly influence the results obtained from the described simple incubation-lysis-measurement approach as especially lipophilic PSs may attach to a considerable amount to the cell culture plastic surface [60]. As we have demonstrated in a recent study ([61], this issue), the surface-adhered PS in microplates without cells can even exert considerable phototoxic effects after addition of PS-free cells in a range similar to the usual protocol where cells are seeded first and the PS is added subsequently. Therefore, appropriate control experiments and controls samples need to be included to estimate the amount of PS which is bound by the microplate plastic and which might significantly contribute to the PS fluorescence signal measured after PS incubation and direct lysis of the cells. In case of a rather lipophilic PS, such false-positive fluorescence signals can be avoided by detaching the cells from the culture receptacle (e.g., trypsinization or EDTA treatment), transfer to new tubes/wells followed by cell lysis and fluorescence detection. Regardless of whether cell-bound PS fluorescence is measured directly in the microplate wells or after detachment and transfer, this approach can be designed to allow for absolute quantification of the amount of cell-bound PS if appropriate cell-free PS dilutions series are simultaneously measured.

An alternative approach is based on fluorescence-activated cell sorting (FACS) analyses based on single-cell analysis of the cellular fluorescence in a flow cytometer. As such protocols involve detachment of cells prior to analysis, no interference with the measured signal from PS molecules adhered to the plastic material is expected. On the other hand, this approach is only suitable for relative quantification and each sample usually requires manual work (e.g., for cell detachment, transfer, washing steps) implicating a reduced number of processable samples compared to the microplate format.

Both experimental approaches can be used to characterize the dose-dependent PS uptake by cancer cells by identification of the range of PS concentrations which result in a measurable PS fluorescence—that is, the minimum concentration as well as, a concentration threshold above which a saturation of PS bound/taken up by the cancer cells occurs. With both methods, the influence of the presence of serum constituents as well as for example, the influence of the chemical PS formulation can be assessed. Subsequently, the time-dependent uptake studies can be performed to investigate the kinetics of PS uptake into cells. As mentioned previously these data may give first information on the

appropriate drug-to-light interval and allow for definition of an incubation period for subsequent *in vivo* experimentation. Particularly for time-dependent experiments involving incubation times in the range of several hours up to days it is important to correct for the cell number (biomass) present at the individual time points. This can be achieved, for example, by measurement of the total protein content (e.g., colorimetric assays such as Bradford, bicinchoninic acid assays) of the cell sample as a surrogate parameter for the actual cell number. Clearly, in highly proliferating cell types, the cell number may influence the amount of PS bound/taken up by the individual cell—especially at low PS concentrations.

The release of PS by cells—that is, transport of the PS molecules out of the cell—is an important parameter as it partly determines the period of photosensitivity in the clinical application; that is, a PS drug which is rather rapidly exported from cells is likely to bring about a reduced time period necessary for the patient to readapt to normal daylight conditions. PS release experiments can be performed using both of the previously mentioned approaches and additional parameters such as the presence of FCS in the culture medium can be tested for their influence on the release kinetics. In our hands, the FACS-based approach seems more reliable for this purpose, as its single-cell measurement may allow for more accurate determination of the residual cell-bound PS (resp. the amount of PS released from cells) than if it is summarily measured in lysed cells. After the PS incubation period, PS release experiments involve careful washing of the cell cultures to remove unbound PS molecules and subsequent incubation in PS-free cell culture medium. In general, time periods ranging from, for example, 10 to 24/48 hours should serve as valid starting points for analysis of the PS release.

**3.2. PS Dark Toxicity.** The general requirements for an optimal photosensitizing agent include low dark toxicity, that is, negligible cytotoxicity in the absence of light. This ensures the validity of the dual-specificity ideal of antitumor PDT, namely that both via tumor cell (semi)selective enrichment of the PS and confinement of the illuminated area by appropriate design of the light source, the cytotoxic action of PDT is limited to the cancerous tissue while sparing adjacent healthy cells [62]. Experimental assessment of the dark toxicity involves incubation of the cells with a PS dilution series initially according to the incubation parameters established in the PS uptake experiments described in Section 3.1. Probably, the establishment of optimal parameters (e.g., incubation time, PS concentration, media composition, and cell density) may require that experiments on PS uptake characteristics and dark toxicity are performed in parallel. In general, a large array of appropriate assays is available for viability analyses in *in vitro* cell culture samples. These assays make use of either measurement of (i) metabolic parameters (activity of metabolic enzyme) as a surrogate readout for the cell's viability, (ii) biochemical and morphological changes during apoptosis, (iii) proliferation rates of cells, and/or (iv) viable cell number employing specific membrane-impermeable dyes to exclude dead (leaky) cells.

TABLE 1: Tests for cell viability and cell death modes.

Assay type	Test	Measured parameter	Signal			Instrument	Microplate <sup>a</sup>	References <sup>b</sup>
			ABS	FI	Lumi			
Metabolic enzyme(s)	MTT, XTT, WST	Mitochondrial enzymes	X			Microplate reader	Yes	[77–79]
	Resazurin	Cellular dehydrogenase enzymes and cytochromes		X		Microplate reader	Yes	[80]
Metabolites	ATP	Intracellular ATP		X		Microplate reader	Yes	[81]
	Caspase activation	Apoptosis-specific proteases	X	X	X	Western blot Microplate reader	No Yes	[77] [75]
	Nuclear fragmentation	Chromatin condensation and fragmentation		X		Fluorescence microscope Flow cytometer	Yes No	[82] [75]
Apoptotic changes	DNA ladder	DNA cleavage resulting in multiples of 180 Bp		X		Gel electrophoresis	No	[83]
	Membrane blebbing	Characteristic apoptotic bodies	X			Phase contrast microscope	Yes	[67]
	PARP cleavage	poly-ADP ribose polymerase cleavage		X	X	Western blot	No	[77]
	Annexin V	Membrane externalization of Annexin V		X		Flow cytometer Fluorescence microscope	No Yes	[77] [77]
	Cyt-c release	Mitochondrial cyt-c release	X	X		Western blot	No	[77]
	$\Delta\Psi$	Mitochondrial membrane potential breakdown		X		Flow cytometer Fluorescence microscope	No Yes	[67, 84] [85]
Cell proliferation	$^3\text{H}$ thymidine	DNA incorporation of		X		Scintillation counter	Yes	[86]
	BrdU	$^3\text{H}$ thymidine/BrdU				Microplate reader	Yes	[87, 88]
Cell number		Direct cell number		X		Flow cytometer	No	

ABS: absorbance; ATP: adenosine-5'-triphosphate; BrdU: 5-bromo-2'-deoxyuridine; caspase: cysteine-dependent aspartate-directed proteases; FI: fluorescence intensity; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Lumi: luminescence; PARP: poly(ADP-ribose) polymerase; WST: water-soluble tetrazolium salt; XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

<sup>a</sup> Assay suitable for use with microplates (yes/no).

<sup>b</sup> Methodological references or exemplary studies using the respective test in the context of *in vitro* PDT.

For determination of dark toxicity of a PS in a given cell type, usually only the overall effect on viability or proliferation is interesting. More detailed analysis of the modes of cell death is rather important for the investigation of the light-induced effects of the PS (see Section 4). Anticipatorily, these tests are listed here in Table 1 including a superficial appraisal of their various strengths.

For the particular assessment of dark toxicity, classical viability tests based on measurement of the activity of metabolic enzymes may be most efficient since these tests can be performed in microplates implicating the possibility of multiparametric testing including technical replicates for each sample.

**3.3. Intracellular PS Localization.** Following establishment of the overall PS uptake characteristics and the PS's dark toxicity, a subsequent experimental step involves the determination of

the intracellular localization and enrichment of the photosensitizing drug. Again, for this purpose, the inherent fluorescent properties of the PS are used.

Provided the microscope setup is equipped with the appropriate filter sets, first superficial information on the intracellular distribution can be obtained from conventional fluorescence microscopy. With this approach, general statements such as preferential localization in the cytoplasm, plasma membrane, or the perinuclear region can be obtained. For more detailed analysis, the use of specific organelle-localizing dyes ("organelle trackers") is recommended. Table 2 provides a list (not complete) of fluorescence dyes that might be used for analysis of colocalization with the PS investigated. The choice of a particular dye depends on its fluorescence spectrum which should not overlap with the emission wavelength of the PS. Particularly, if the localization of the PS is not confined to one clearly identifiable structure,

TABLE 2: Fluorescent probes for cellular organelle counterstaining [89].

Organelle/cell structure	Fluorescent dye(s)
Mitochondria	TMRM, TMRE, rhodamine 123, tetramethylrosamine, mitotracker, nonyl acridine orange, carbocyanines, dual-emission dyes (JC-1, JC-9)
Endoplasmic Reticulum	3,3-dihexyloxacarbocyanine iodide [DiOC6(3)], ER-Tracker
Nucleus	DAPI; Hoechst-33342, propidium iodide, SYTO dyes
Cytoplasm	Calcein AM
Golgi apparatus	Fluorescent labeled lectins
Lysosomes	LysoTracker
Cell membrane	CellTracker

DAPI: 4',6-diamidino-2-phenylindole; TMRE: tetramethylrhodamine ethyl ester; TMRM: tetramethylrhodamine methyl ester.

the use of confocal fluorescence microscopy which provides increased spatial resolution may be helpful. An alternative approach for quantification of the PS's intracellular localization could involve organelle-specific fractionation, for example, by centrifugation techniques and analysis of the organelle-bound PS fluorescence—this approach is more time-consuming and may require more extensive optimization for the particular cell type.

**3.4. Assessment of the Penetration Depth of the PS in Porcine Skin.** After a first positive prescreening of new developed photosensitzers with photodynamic activity against microorganisms in suspension *in vitro*, the next challenge in antimicrobial photodynamic inactivation is to find appropriate parameters (e.g., light dose and incubation time) to inactivate relevant key pathogens without harming surrounding tissue *in vivo/ex vivo*. Therefore penetration and localization of the given photosensitzers must be investigated using an *ex vivo* skin model. Recently it could be shown that an *ex vivo* porcine skin model can be used, because it is proposed as a good test model for human skin based on many similarities regarding physiological, histological, and permeability properties [63]. Restriction of a photosensitizer to the stratum corneum without accumulation in deeper parts of the epidermis or dermis might be useful regarding a successful decolonization of pathogens on intact skin [64]. Recently it could be shown that localization of the photosensitizer TMPyP in a water-ethanol formulation was restricted to the skin surface only [64]. However agents with a molecular weight of >500 Da exhibit a low permeability through the stratum corneum. The molecular weight of TMPyP is 682.2 g·mol<sup>-1</sup> (without counterions). Therefore the molecular weight of drugs which are used in transdermal drug-delivery systems is well below <500 Da [65]. To enhance penetration through the stratum corneum various formulations are available that contain supplements (DMSO, alcohol, pyrrolidones) which exhibit penetration enhancing activities [66]. Overall the main targets are superficial and localized infections. These areas are readily accessible for the topical application of PS and light, neither harming the surrounding tissue nor disturbing the resident microbial flora.

#### 4. Photodynamic Action in Tumor Cells

This section describes experimental approaches for characterization of the cytotoxic action induced by light including analysis of overall viability, IC<sub>50</sub> values, and, specifically, the discrimination of the cell death modes induced by PDT.

**4.1. Analysis of Tumor Cell Viability Changes after PDT.** After having optimized the incubation parameters (concentration, incubation time, and media composition) as described in Sections 3.1 and 3.2, one can proceed in analysis of the photoinduced cytotoxic effects of a new PS. Using adherent cancer cells (cell lines) the fastest approach is to use a microplate assay based on the activity of metabolic enzymes such as MTT or the resazurin assay, both of which are quick, cheap in terms of reagents, and easily established (see Table 1 for an overview). Similar to such assays, determination of intracellular ATP gives a reliable estimation of the amount of viable cells after a cytotoxic treatment as the intracellular concentration of this metabolite (as is the activity of core metabolic enzymes) is assumed to be held in a tight (millimolar) range in viable cells. Therefore, the overall amount of ATP or the enzyme activity in a population of cells is supposed to represent the overall viable cell number. Important to keep in mind is the fact that cells undergoing apoptosis (active cell death) maintain considerable levels of metabolic activity respective ATP in order to perform the energy-requiring steps during the apoptotic cascade [67–72]. Therefore, for all of the mentioned assays, the time point to perform the test should be chosen in a way so that apoptotic cells do no longer contribute to the assay readout. This might include establishment for each individual cell line in order to determine the time point after treatment where apoptotic cells have finished the cell death program and have undergone secondary necrosis. This time period may be in the range of 24–48 hrs after treatment for most cancer cell lines.

Besides PDT-treated cell samples, each particular experiment on overall cell viability should include the following control samples—again most easily to be realized in the (96-well) microplate format: untreated control (UTC), dark control (DC), light-only control (LOC), the treated samples,

and appropriate blank wells required for the assay's blank subtraction. In our experience, at least triplicate wells should be included for each of the listed sample types. Treated samples are incubated with the PS (as established in Section 3.1 to result in a measurable cellular enrichment of the PS). As illustrated in Figure 3, subsequent illumination conditions can be designed in two ways to get an overall impression on the viability changes following PDT treatment: (i) a constant PS concentration is employed for all treated samples accompanied by illumination with different light fluences ( $\text{J}\cdot\text{cm}^{-2}$ ) or (ii) incubation with different concentrations of the PS (i.e., a dilution series) followed by illumination with a constant light fluence. Particularly when different light fluences are applied to individual rows of the microplate wells (as in Figure 3; approach (i)) microplates with clear well bottoms and black walls should be employed to avoid activation light crosstalk between the rows of wells during illumination.

Similar to determination of the dark cytotoxicity of the PS, comprehensive characterization of the PDT *in vitro* model system should address—or exclude, in most cases—possible cytotoxic effects of the illumination itself. For this purpose, cells are seeded and incubated in the same medium as usually used for PS incubation but without the PS, followed by illumination with different light fluences. As mentioned, for most applications such a control experiment is to rule out possible effects of the illumination itself on the viability or proliferation rate of the cells.

All the mentioned experimental approaches should be accompanied prior to the particular assay by routine control observation in a conventional light microscope. In most cell lines, cytotoxic effects can be readily identified at this level by observation of rounding of cells (apoptosis, probably including classical apoptotic bodies) and detachment of cells (apoptosis and/or necrosis at later time points). Such visual control may help interpretation of the results gained from assays measuring metabolic enzymes or metabolites as a surrogate parameter of cell viability and help rule out false-positively or -negatively high/low signals.

**4.2. Calculation of the  $IC_{50}$  Values.** The mentioned analyses of dark toxicity and light-induced cytotoxicity (using different PS concentrations and a constant light dose) can be used to calculate a modified  $IC_{50}$  value. This parameter is usually referred to as the half-maximal inhibitory concentration of an inhibitor in, for example, enzyme inhibition experiments. In the context of PDT, the  $IC_{50}$  value is calculated by division of the concentration required for 50% cell killing in the dark (lethal dose ( $LD_{50,\text{dark}}$ ))) and the concentration required for 50% cell killing following illumination of PS-incubated cell samples ( $LD_{50,\text{PDT}}$ ) [74]. The  $IC_{50}$  value thus measures the relation between the cytotoxic effects of the PS in the dark and following photoactivation; a higher  $IC_{50}$  is indicative of a low dark toxicity or a particular high cytotoxic efficiency after illumination [75]. By its nature, use of this parameter makes only sense for direct comparison between two or more PSs in the same cell model and under comparable illumination conditions [75, 76].

**4.3. Analysis of the Cell Death Mode.** Further in-depth analysis of the cytotoxic action of a PS following illumination involves the discrimination between essentially three modes of cytotoxicity, that is, inhibition of proliferation, induction of apoptosis, and, third, induction of necrotic cell death. Very low PDT doses may also cause increased cell proliferation resulting in increased cell viability signals and/or cell numbers. As discussed recently, the mode of action of PDT can usually cause all the mentioned effects—in a dose-dependent manner as illustrated in Figure 4. This is in contrast to chemotherapeutic agents or radiation which preferentially causes apoptosis as the underlying cytotoxic effect [1, 79, 90].

For rapid and initial analysis of the cell's response in terms of proliferation, apoptosis, and necrosis, our group has developed a simple and versatile assay based on microplate assays analyzing metabolic enzymes [67, 70]. This procedure makes use of the fact that cells undergoing apoptosis (i.e., active cell death) require functioning energy supply in terms of intracellular ATP accompanied by approximately normal activity of (catabolic) pathways whose enzymes are those measured in the MTT test, for example. As shown in Figure 5, this approach employs standard viability tests based on metabolic surrogate parameters (e.g., MTT, ATP, resazurin) and involves measurement at two different time points following PDT treatment: a first measurement is taken at an “early” time point where cells undergoing apoptosis still retain their metabolic activity. A second reading is taken at a “late” time point where apoptosis has been completed and these cells have converted to secondary necrosis due to the absence of phagocytizing cells in the cell culture setting. In contrast to apoptotic cells which maintain their metabolic activity until the late steps of the apoptotic program, necrotic cells are characterized by a rapid breakdown of the plasma membrane integrity, metabolic hemodynamics, and a leakage of intracellular material in the extracellular space [91].

As shown in Figure 5, the different signals between early and late readings can be used for a first discrimination between induction of proliferation, apoptosis, or necrosis in the *in vitro* setting. This approach clearly works with sum signals; therefore, mixed populations of, for example, apoptotic and necrotic cells cannot be quantified in absolute terms. However, the 96-well microplate format—on the other hand—allows for rapid testing of, for example, ten different treatment conditions. This assay variant has been successfully used in previous publications with either the MTT assay [67, 70] or the fluorescent resazurin assay [82].

Another simple test for discrimination of whether a reduced viability signal is caused by direct cytotoxicity (apoptosis or necrosis) or by inhibition of proliferation also employs metabolic viability tests such as the MTT assay. For this purpose multiple readings at different time points following illumination are performed. The viability signals obtained at each time point are related to the initial ( $t = 0$  hrs) value and the resulting temporal dynamics of the signal for each treatment condition can be evaluated as follows: a decrease below the initial value can be interpreted as a direct cytotoxic effect as the absolute viability signal

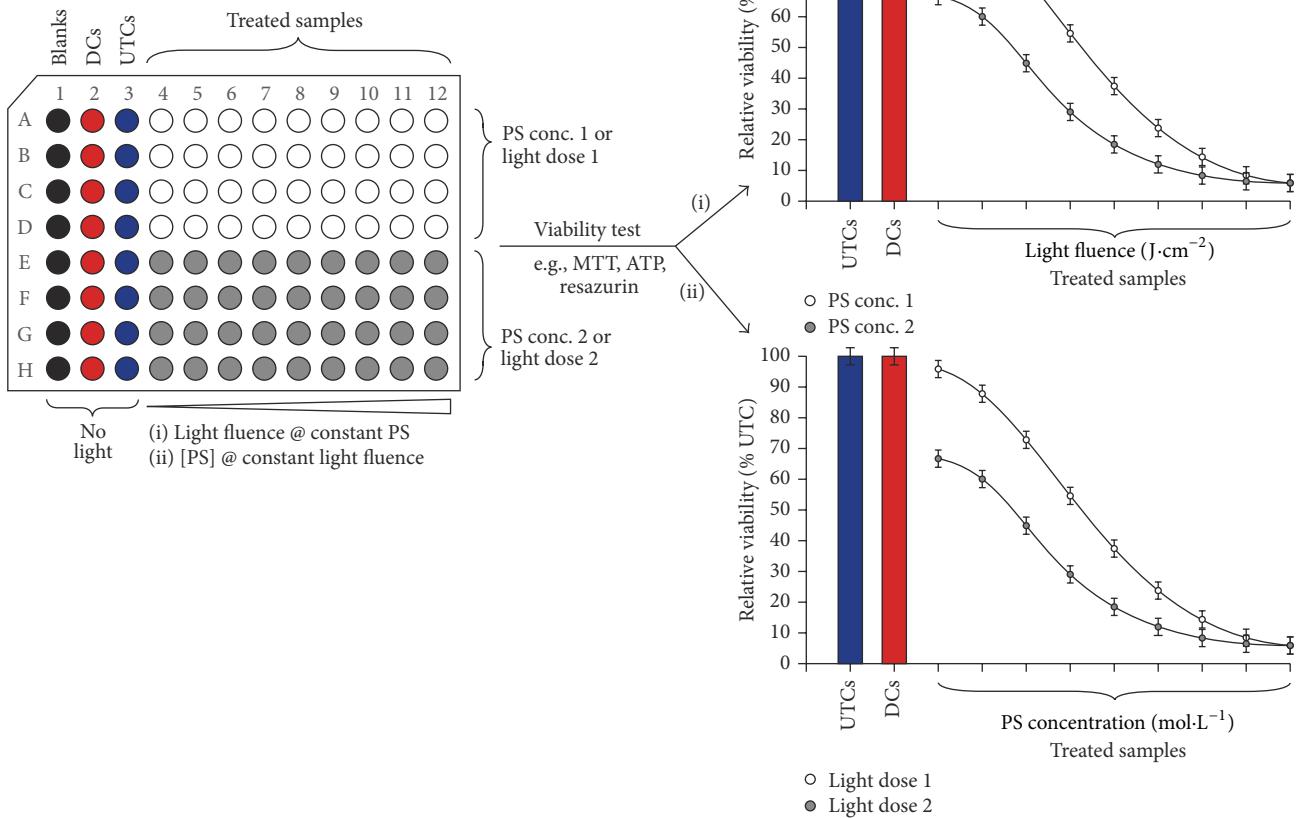


FIGURE 3: Experimental variants for analysis of overall tumor cell viability following PDT. Under constant PS conditions or constant light fluence, the light fluence or the PS concentration can be varied to obtain initial information on the phototoxic effects of a particular PS. DC: dark controls (PS without light); PS: photosensitizer; UTC: untreated control (no PS, no light).

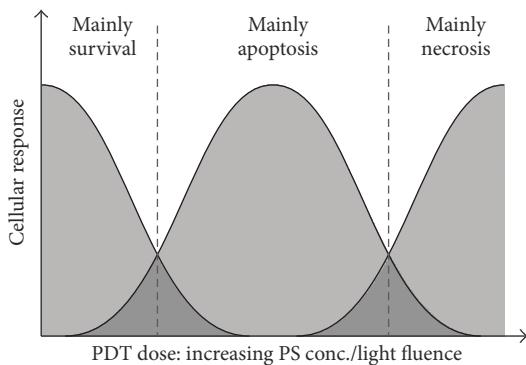


FIGURE 4: Dose-dependent transition between cellular responses following PDT. Abbreviations: PS, photosensitizer. Modified from [73].

decreases. A constant viability signal (in the range of the initial value) indicates inhibition of proliferation, whereas a signal increasing relative to the initial value (similar to untreated controls in most cases) indicates proliferation.

Clearly, as a sum measurement this test design cannot discriminate between the modes of cytotoxicity in absolute terms (i.e., on the single-cell level). However, it may assist in the interpretation in a situation where the endpoint viability measurement (e.g., 24 hrs p.i.) indicates a viability signal smaller than the untreated controls since this reduction could be solely attributed to growth inhibition without any apoptosis/necrosis induction, that is, direct cytotoxicity. Assays directly measuring the proliferation rate are classically based on DNA incorporation of nucleotide analogues such as  $^3\text{H}$ -thymidine or bromodeoxyuridine (BrdU). Incorporation of the first can be measured via scintigraphy whereas the latter is detected by BrdU-specific immunostaining. Both methods allow direct assessment of the proliferation rate of cells but should be accompanied by the mentioned viability tests for unequivocal interpretation.

After having superficially determined how the cell population responds to a photodynamic treatment (survival/proliferation, direct cytotoxicity/reduced viability), one may proceed to in-depth characterization of the specific mode of cell death in case of a cytotoxic PDT regimen. This might be relevant to elucidate the detailed mechanism

TABLE 3: Cell-based assays for discrimination and quantification of cell death modes.

Cellular/biochemical event	Method <sup>a</sup>	Assay platform	Comment <sup>b</sup>
DNA degradation	(i) Detection of “DNA ladders,” that is, multiples of 185 bp	Gel electrophoresis	Semiquantitative
	(ii) TUNEL	FM, FACS	Semi-quantitative
	(iii) COMET	Single cell gel electrophoresis	Semi-quantitative
	(iv) SubG <sub>1</sub> (cell cycle analysis)	FACS	Quantitative
Nuclear fragmentation	For example, DAPI, Hoechst-33342 DNA-stained nuclei	FM	Quantitative
Membrane blebbing	Morphological changes	Phase contrast LM	Semi-quantitative
Caspase activation	Fluorometric/luminometric detection of cleavage of artificial caspase substrates	Microplate reader, FM, FACS	Quantitative, single-cell analysis via FACS
PSer exposure	Antibody staining	FM, FACS	Quantitative, single-cell analysis via FACS
Mitochondrial cyt-c release	Subcellular fractionation and immunodetection	Western blotting	Semi-quantitative
Mitochondrial ΔΨ breakdown	Fluorochrome-based assessment of mitochondrial ΔΨ	FM, FACS	Semi-quantitative (within cells), quantitative for comparison between cell populations
Membrane integrity, release of intracellular material <sup>c</sup>	(i) Detection of necrosis-associated plasma membrane breakdown via PI staining	FM, FACS	Quantitative, single-cell analysis via FACS
	(ii) Biochemical assay for LDH enzyme release from necrotic cells	Microplate reader	Quantitative

Cyt-c: cytochrome c; DAPI: 4',6-diamidino-2-phenylindole; ΔΨ: mitochondrial membrane potential; FACS: fluorescence-activated cell sorter; FM: fluorescence microscopy; LM: light microscopy; PI: propidium iodide; PSer: phosphatidylserine; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

<sup>a</sup>Selection of methods is focused on *in vitro* experimentation (cell culture).

<sup>b</sup>Based on the author's experience.

<sup>c</sup>These methods address specific necrosis-associated cellular changes.

of a particular PS or PDT regimen and, on the other hand, might have implications for overall therapeutic effect by induction of diverse immune system-related responses [92, 93]. In this brief discussion we focus on the classical ways of cell demise—excluding autophagy which is also considered in recent reports to contribute to PDT-induced cytotoxicity [94] (for an methodological overview see [95]). Table 3 lists the most common methods and assays to address whether cells and cell populations undergo apoptosis (active/“programmed” cell death) and/or necrosis (passive cell death) (further reading [96, 97]). As commented in Table 3, the variety of methods differs with respect to their quantitative or semiquantitative results, that is, whether a percentage of cells undergoing apoptosis can be determined using the particular method. Furthermore, the assays differ with respect to price as well as prerequisites regarding instrumentation and time required. Furthermore, some of the listed approaches may depend on whether the cell (or cell line) studied shows the morphological/biochemical feature addressed by the particular test. A comprehensive weighting of the various methods is beyond the scope of

this section—useful advice in our opinion includes the following aspects: (i) appropriate—probably non-PDT-treated-control samples (i.e., 100% apoptotic/necrotic cells) help to validate the method and make the results obtained for PDT-treated samples more reliable and expressive, (ii) whenever possible, a population-based method should be accompanied by single-cell analysis(es) to gain information about the cell portion affected, (iii) all indirect (non-microscopy-based) assays should be accompanied by simple (phase contrast) light microscopy to allow comparison with the sometimes quite obvious overall cellular responses, (iv) the timing when to use individual methods may need optimization for each cell model (and treatment protocol) as some of the cellular/biochemical events listed in Table 3 occur early versus rather late following the PDT treatment, and (v) PDT treatments may cause mixed population consisting of both apoptotic and necrotic subpopulation of cells in a given sample ([73], see also Figure 4). The methods listed in Table 3 comprise assays specifically focusing on *in vitro* experimentation using cells (cell lines) in culture; for specific methods to investigate the occurrence and extent of apoptosis/necrosis

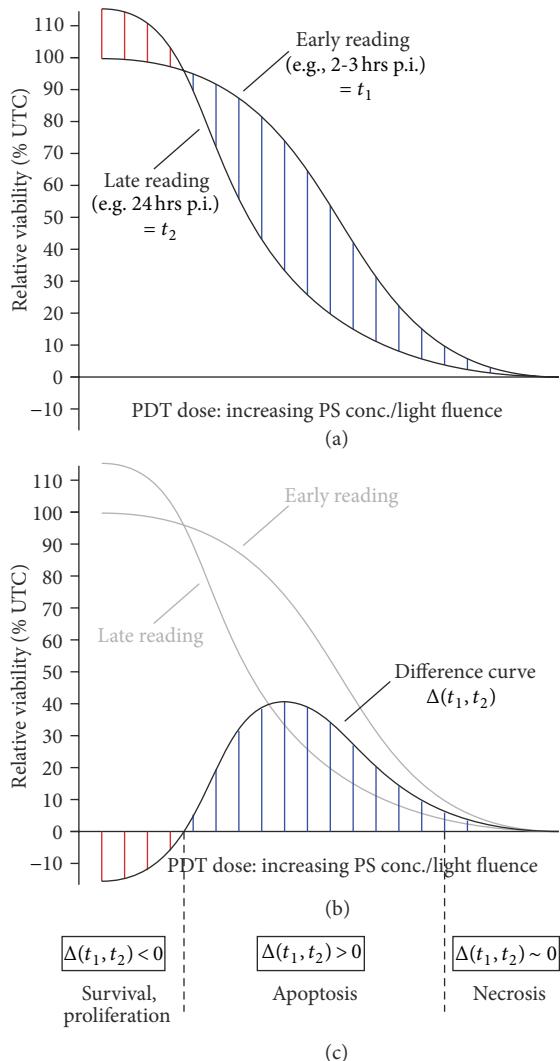


FIGURE 5: Discrimination of the cellular responses towards PDT. Measurement of the overall viability at early versus late time points following illumination (a) allows for calculation of a difference curve (b) and estimation of the dose ranges which predominantly induce cellular survival, apoptosis, and necrosis (c). PS: photosensitizer. For details on interpretation see text.

*in situ* (tissue sections) the reader is kindly referred to recent methodological overviews [98, 99].

## 5. Characterization for Photodynamic Inactivation of Eukaryotic and Prokaryotic Microorganisms

Ideally, a wide spectrum of antimicrobial action on bacteria, fungi (yeasts), and protozoa should be achieved with a given PS/PDI protocol. The primary readouts should focus first on photodynamic efficacy in suspensions, followed by biofilm inactivation (monospecies and polyspecies) *in vitro*. Later on *ex vivo* and animal studies should be considered to demonstrate a photodynamic killing efficacy of  $\geq 3 \log_{10}$  steps ( $\geq 99.9\%$  reduction of viable microorganisms). Such

a reduction of viable microorganisms must be achieved to state that an antimicrobial effect is possible. Furthermore, the efficacy should be independent of the antibiotic/antifungal resistance pattern of the investigated microbial strains. From this point of view a selection of photodynamic-resistant microorganisms should be absent after multiple sublethal treatments conditions. Due to the regulatory affairs to get approval by the FDA or the European Health Authorities, mutagenicity must be excluded. Appropriate formulations must be developed allowing an easy and specific delivery of the given photosensitizer to the infected area.

**5.1. Assessment of Cytotoxicity in the Dark and Phototoxicity Based on CFU Counting.** The American Society of Microbiology has decreed that for any technique to be called “antibacterial” or “antimicrobial” at the very least  $3 \log_{10}$  of CFU (99.9%) need to be killed. Furthermore based on the guideline for hand hygiene in health-care settings a minimum of  $5 \log_{10}$  reduction of viable counts of microorganisms must be achieved for a successful disinfection [100]. Survival of viable bacteria must be determined by the colony-forming assay. After overnight incubation, colonies are counted and viable pathogen concentration is expressed as CFU/mL using a logarithmic scale. Furthermore, no cytotoxic effects in the dark of both the given photosensitizer itself and of the possible photoproducts formed after illumination should be demonstrated either against the pathogen itself or the eukaryotic cells.

**5.2. Addition of Cell Wall-Permeabilizing Agents.** In case that the given photosensitizer is not efficient enough (less than  $3 \log_{10}$  steps of CFU/mL reduction) to kill relevant pathogens upon illumination addition of cell wall-permeabilizing agents might be useful to enhance the photodynamic efficacy. From a clinical point of view metal chelators like EDTA might be useful to cause a disorganization of lipid structures increasing the permeability of the outer membrane of Gram(–) bacteria [54, 101]. EDTA solutions might be useful, because it is well established in dentistry as it has been commonly used as a detergent for the removal of smear layers. Another permeabilizing agent is polymyxin B nonapeptide which has demonstrated a porphyrin-based photodynamic enhancement [55].

**5.3. Determination of the Efficiency towards Bacterial Biofilms.** The natural behavior of microorganisms is to grow as a biofilm rather than as free-floating cells. It is generally accepted that biofilms represent the leading cause of microbial infections. One of the main consequences of the biofilm mode of growth is the increased resistance to antimicrobial therapy, resulting in recurrent or persistent infections leading to treatment failure. Therefore, biofilms are up to 100-fold more resistant against any antimicrobial treatment modality as compared to their planktonic counterpart [102]. From this point of view it is necessary to evaluate additionally the photodynamic efficacy against biofilm growing pathogens of any positive preselected photosensitizer which has demonstrated

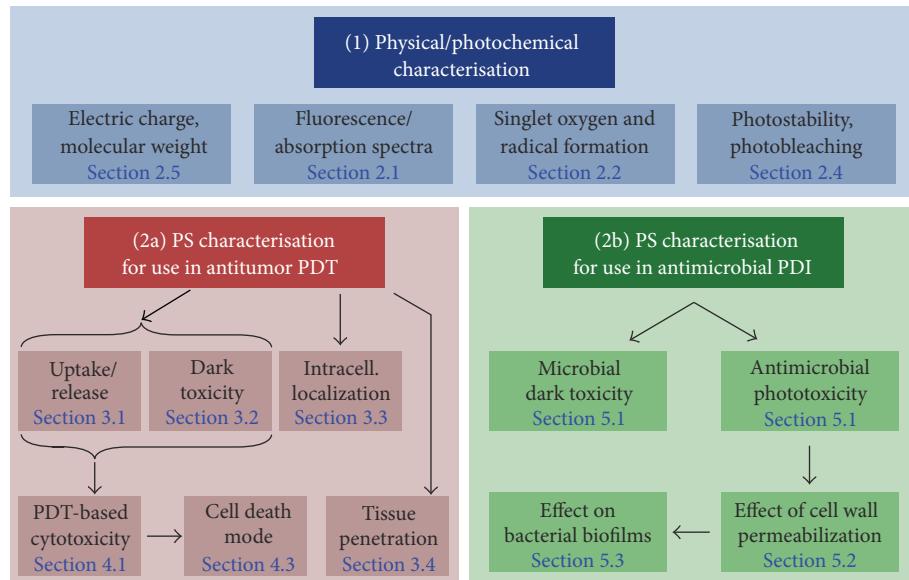


FIGURE 6: Flow chart for basic characterization of novel photosensitizers for PDT and/or PDI applications. This diagram provides a suggested stepwise procedure for basic and *in vitro* characterization of novel PS molecules involving the most important physical, photochemical and cellular characteristics. The respective sections within this paper are provided.

photodynamic killing efficacy against microorganisms in suspension.

## 6. Conclusion

Taken together, the previously mentioned experimental approaches are suited to provide comprehensive information on the potential use of a particular—and probably newly developed—PS agent in the frame of PDT or PDI. As several aspects determine the possibilities of use in the two major branches of photodynamic applications (see the aforementioned for details), we suggest a stepwise characterization of the most important physical and photochemical/dynamic features of a new PS in order to determine the possible fields of therapeutic applications. Such a step-by-step approach is depicted in Figure 6 (with reference to the chapters within this paper) providing an interdisciplinary straight forward strategy for comprehensive characterization of photosensitizing compounds. Early and unequivocal identification of the various strengths and weaknesses of an individual agent may help deciding for which particular clinical application the particular drug is worth further establishing.

*In vitro* research represents the initial step in the biological characterization of a new PS for its application in PDT or PDI. Conclusions drawn from cell culture experiments are always difficult to directly transfer to the *in vivo* situation and may not allow for a very precise prediction of clinical applications of a given substance. However, these experiments may suggest possible targets and provide first evidence on practicable PDT/PDI treatment protocols. Up to date, no standard strategy for the basic *in vitro* investigations of PS existed. Therefore, this tutorial, which is based on the authors' experience in PDT and PDI, can serve as a

guide for researchers who are involved in preclinical PS testing or plan to contribute to such research efforts. Special aspects of the experimental categorization of a novel PS, such as, for example, the possible interference of the PS with fluorochromes employed in cytological assays or the light sensitivity of the PS, have to be considered by an experimenter when performing a spectral analysis, the determination of levels of the phototoxic agents (ROS) including singlet oxygen, drug and light dose finding, intracellular localization, or assessment of the mode of cell death predominant at a given PDT protocol and are discussed in this paper. As outlined before, PDI research using microorganisms as model systems has to take into account the special nature of these pathogens. For example, the high growth rate of bacteria and yeast reasons the requirement of assays which allow for viability tests covering more than three orders of magnitude, which excludes classical colorimetric assays. Furthermore, as discussed in the previous chapters, the presence of cell wall composition and the ability to form biofilms require alternative PS and therapeutic procedures for successful photokilling when compared to cancer cells.

Until now localized infections of the skin, wounds, infections of the oral cavity, infections related to periodontitis, and endodontitis as well as infection of the middle ear are especially suitable for PDI treatment, because they are relatively accessible for PS application and illumination [103]. Overall, PDI might either be substitute standard antimicrobial therapy or act as an additional approach in the future.

Concluding, we hope that this tutorial will motivate researchers of all disciplines to get involved in photodynamic therapy and photodynamic inactivation and thereby help to further expand the convincing benefits of photodynamic procedures to new fields of applications.

## Abbreviations

$O_2^{\cdot-}$ :	Superoxide anion
ABS:	Absorbance
ATP:	Adenosine 5'-triphosphate
BrdU:	5-bromo-2'-deoxyuridine
Caspase:	Cysteine-dependentaspartate-specificprotease
CFU:	Colony-forming unit
cyt-c:	Cytochrome c
DAPI:	4',6-diamidino-2-phenylindole
DC:	Dark control
$\Delta\Psi$ :	Mitochondrial membrane potential
DMAX:	9-[2-(3-carboxy-9,10-dimethyl)anthryl]-6-hydroxy-3H-xanthen-3-one
DMPD:	N,N-dimethyl-p-phenylenediamine
DMSO:	Dimethyl sulphoxide
DPA:	9,10-diphenylanthracene
DPBF:	1,3-diphenylisobenzofuran
EDTA:	Ethylenediaminetetraacetic acid
ET:	Electron transfer
FACS:	Fluorescence-activated cell sorting
FB/CS:	Fetal calf/bovine serum
FI:	Fluorescence intensity
FM:	Fluorescence microscopy
HO $^{\cdot}$ :	Hydroxyl radicals
LM:	Light microscopy
LOC:	Light-only control
MRSA:	Methicillin-resistant <i>Staphylococcus aureus</i>
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ORAC:	Oxygen-radical absorbance capacity
PARP:	Poly(ADP-ribose) polymerase
PDI(B):	Photodynamic inactivation (of bacteria)
PDT:	Photodynamic therapy
PeT:	Photo-induced electron transfer
PI:	Propidium iodide
PS:	Photosensitizer
PSer:	Phosphatidylserine
ROS:	Reactive oxygen species
SOSG:	Singlet Oxygen Sensor Green
TMRE:	Tetramethylrhodamine ethyl ester
TMRM:	Tetramethylrhodamine methyl ester
TRAP:	Radical-trapping antioxidant parameter method
TUNEL:	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
UTC:	Untreated control
WST:	Water-soluble tetrazolium salt
XTT:	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

## Conflict of Interests

The authors declare that there is no conflict of interests.

## Authors' Contribution

A. Gollmer and T. Maisch have contributed equally to this paper.

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## Review Article

# Immunogenic Cell Death: Can It Be Exploited in PhotoDynamic Therapy for Cancer?

Elisa Panzarini, Valentina Inguscio, and Luciana Dini

Department of Biological and Environmental Science and Technology (Di.S.Te.B.A.), University of Salento,  
Via per Monteroni, 73100 Lecce, Italy

Correspondence should be addressed to Luciana Dini; luciana.dini@unisalento.it

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Immunogenic Cell Death (ICD) could represent the keystone in cancer management since tumor cell death induction is crucial as well as the control of cancer cells revival after neoplastic treatment. In this context, the immune system plays a fundamental role. The concept of Damage-Associated Molecular Patterns (DAMPs) has been proposed to explain the immunogenic potential of stressed or dying/dead cells. ICD relies on DAMPs released by or exposed on dying cells. Once released, DAMPs are sensed by immune cells, in particular Dendritic Cells (DCs), acting as activators of Antigen-Presenting Cells (APCs), that in turn stimulate both innate and adaptive immunity. On the other hand, by exposing DAMPs, dying cancer cells change their surface composition, recently indicated as vital for the stimulation of the host immune system and the control of residual ill cells. It is well established that PhotoDynamic Therapy (PDT) for cancer treatment ignites the immune system to elicit a specific antitumor immunity, probably linked to its ability in inducing exposure/release of certain DAMPs, as recently suggested. In the present paper, we discuss the DAMPs associated with PDT and their role in the crossroad between cancer cell death and immunogenicity in PDT.

## 1. Introduction

The plain success of cancer therapies crucially depends on the synergic interaction between immune cells and dying/dead cancer cells. The ideal cancer treatment should merge the direct cytotoxic action on tumor cells with potent immunostimulatory effects based on the recognition of molecular immunogenic determinants on dying cells by immune cells. Indeed, anticancer immune responses may contribute to the control of the neoplastic disease after cancer modalities since they help to eliminate residual cancer cells or maintain micrometastases in a stage of dormancy. The capability of a cancer treatment to elicit Immunogenic Cell Death is clinically relevant since it is associated with an anticancer immune response that reinforces the therapeutic effect of the therapy. The immunogenicity of the dying cancer cells involves subtle changes in their surface proteome and the secretion of soluble molecules known as Damage-Associated Molecular Patterns (DAMPs) allowing their immunogenic recognition by immune effectors.

In recent times, more and more efforts are addressed to associate particular DAMPs with a specific cell death pathway or with particular stress agents able to induce Immunogenic Cell Death (ICD) in cancer cells. One such therapeutic modality certainly associated with DAMPs is PhotoDynamic Therapy (PDT). In the present paper, we collect data regarding DAMPs related to PDT, primarily focusing on the ability of these molecules to function as ICD effectors in PDT.

## 2. Emerging Hallmarks of Cancer

During their evolution to the malignant state, tumor cells progressively evolve multiple ploys to carry out their intrinsic fateful program. Particularly, cancer cells acquire six distinctive and complementary biological capabilities allowing tumor growth and metastatic dissemination. These include self-sufficiency in growth signals, insensitivity to growth suppressors, circumventing cell death mechanisms, limitless replicative potential, sustained angiogenesis, and tissue

invasion and metastasis [1]. Cancer cells do not need stimulation from external growth factors to grow and divide since they can generate their own growth signals sustaining chronic proliferation. Unlike normal cells whose growth is kept under control by inhibitors in the surrounding environment, in the extracellular matrix and on the surface of neighboring cells, tumor cells are generally resistant to growth-preventing signals becoming masters of their own destinies. They are able to bypass apoptosis, the preferential form of Programmed Cell Death (PCD) induced by conventional cancer therapies, by the loss of Tumor Protein 53 (TP53) tumor suppressor function, the upregulation of antiapoptotic regulators (Bcl-2, Bcl-xL) or of survival signals (Igf1/2), the downregulation of pro-apoptotic factors (Bax, Bim, Puma), or the short-circuiting of the extrinsic ligand-induced death pathway. Normal cells undergo a limited number of successive cell growth-and-division cycles, since their proliferation is subjected to two distinct barriers: senescence, a viable state characterized by an irreversible arrest in proliferation limiting the lifespan of mammalian cells, and crisis, which involves cell death. On the other hand, cancer cells escape these barriers and they are capable of indefinite growth and division (immortality). In fact, the immortal cells present damaged telomeres, the regions of repetitive nucleotide sequences at each end of a chromosome, that are centrally involved in this unlimited proliferation capability [2]. In order to progress, cancer cells must turn on a blood supply, generated by the process of angiogenesis, ensuring a continual provision of oxygen and other nutrients. Angiogenesis is balanced by inducers, such as vascular endothelial growth factor (VEGF) and acidic and basic fibroblast growth factor (FGF 1/2), and inhibitors, including thrombospondin-1. Thrombospondin-1 is regulated by p53, therefore the loss of p53 can allow angiogenesis. Tumor cells can migrate from their origin site to invade surrounding tissue and metastasize to distant body areas through a multistep process, referred to as invasion-metastasis cascade [3], characterized by a succession of cell-biologic changes. These include (1) local invasion, then (2) intravasation by cancer cells into nearby blood and lymphatic vessels, (3) transit of cancer cells through the lymphatic and hematogenous systems, followed by (4) escape of cancer cells from the *lumina* of such vessels into the parenchyma of distant tissues (extravasation), (5) the formation of small nodules of cancer cells (micrometastases), and finally the growth of micrometastatic lesions into (6) macroscopic tumors (colonization).

The acquisition of the six functional capabilities allowing cancer cells to survive, proliferate, and disseminate is made possible by two enabling characteristics: genome instability, which generates random mutations, such as chromosomal rearrangements, driving tumor progression; and inflammation by innate immune cells, which results in tumor-promoting consequences. Indeed, the immune system both antagonizes and enhances tumor development and progression, playing dichotomous roles. In the last decade two emerging hallmarks have been added to this list: reprogramming of energy metabolism in order to most effectively support neoplastic proliferation and evading immune

destruction by T and B lymphocytes, macrophages, and natural killer (NK) cells. Particularly, the abilities to replicate in a chronically inflamed microenvironment, to evade immune recognition, and to suppress immune reactivity enable neoplastic cells to escape the immune responses [1].

The poor antitumor immunity and the escape to the innate and adaptive immune responses are based on the downregulation of tumor cell Major Histocompatibility Complex (MHC) I and costimulatory molecules, alteration of DCs and macrophages function in tumor tissue, regulatory T cells induction, and tumor-mediated immune cell death [4]. Besides, tumor resistance may also be a consequence of the altered expression of oncogene-coded proteins, as demonstrated in ovarian carcinoma-derived cells expressing low levels of HLA class I surface antigens and decreased or absent HLA-A2 expression [5]. Also dysregulation of various components of the MHC class I Antigen Processing Machinery (APM) may avoid the recognition of tumor cells by CD8+ T cells [6].

The long-standing concept of immunosurveillance implying the constant monitoring of cells and tissues by an ever-alert immune system able to recognize and remove nascent transformed cells [7] has been abandoned in favor of the cancer immunosurveillance acting as a component of the cancer immunoediting. Particularly, cancer immunoediting, which represents a refinement of the original cancer immunosurveillance hypothesis, plays a dual role in promoting host protection against cancer and facilitating tumor escape from immune destruction. It is responsible for both eliminating tumors and sculpting the immunogenic phenotypes of tumors as they develop. This process consists of three phases that are collectively denoted “the three Es of cancer immunoediting”: elimination, equilibrium, and escape. Elimination corresponds to immunosurveillance; equilibrium represents the process by which the immune system iteratively selects and/or promotes the generation of tumor cell variants with increasing capacities to survive immune attack; escape is the process wherein the immunologically sculpted tumor expands in an uncontrolled manner in the immunocompetent host [8].

In the clinical management of the neoplastic disease, the ideal therapeutic strategy should combine the restoration of cancer cell death and the enhancement of the immunological recognition of tumor cells [9]. This may be achieved by avoiding cancer modalities mediating immunosuppressive side effects and favoring therapies able to induce ICD, which represents a novel possibility to attack neoplasia with the specificity of the immune system [10].

It is still unclear under which circumstances cellular demise induces an immune response against dying tumor cells or rather it remains immunologically silent. The classical notion that apoptotic cell death is poorly immunogenic (or even tolerogenic), whereas necrotic cell death is truly immunogenic has been recently invalidated since it does not withstand experimental verification, at least in models of tumor vaccination [11, 12]. Indeed, tumor vaccination studies in mice demonstrate that some apoptosis-inducing regimens induce immune-dependent tumor regression whereas others do not, suggesting an unsuspected heterogeneity in

the biochemical pathways leading to apoptotic cell death [13].

The immunogenicity of dying cells is mediated by changes in the composition of the cell surface and the secretion of soluble molecules allowing the immune effectors, primarily dendritic cells (DCs), to sense immunogenicity [14]. Intracellular molecules, categorized as Damage-Associated Molecular Patterns (DAMPs), also known as alarmins, normally hidden within live cells, are released from or exposed at the surface of dying cell determining DCs activation and maturation, antigen processing, and T cell activation (see below).

The appealing idea of immunogenic cancer cell death demands screening of newer anticancer agents/modalities capable of sustaining a particular spectrum of DAMPs. Indeed, a chemotherapeutic agent-specific cancer ICD modality presents the potential to induce *in vivo* an “anticancer vaccine effect” by merging tumor cell kill and antitumor immunity within a single paradigm.

### **3. PhotoDynamic Therapy: Basic Principles and Applications**

One recent therapeutic modality endowed with a known association with certain DAMPs is PhotoDynamic Therapy (PDT), a Food and Drug Administration (FDA)-approved clinical protocol for the treatment of several malignant and nonmalignant diseases [15]. PDT presents multiple advantages over “classical” anticancer regimens, such as surgery, ionizing radiation, and chemotherapy: it is minimally invasive, it has low mutagenic potential, low systemic toxicity and it specifically targets tumor areas over normal tissue [16, 17].

It is a two-step procedure involving the administration of a tumor-localizing photosensitizer (PS) and its subsequent activation by light of specific wavelength. PDT utilizes the destructive power of Reactive Oxygen Species (ROS) generated *via* photophysical/photochemical reactions by the interaction between visible light, PS, and tissue molecular oxygen, the three main components of the photodynamic reaction, to elicit cancerous cells obliteration [18].

Efficient photosensitization primarily depends on the PS physico-chemical properties, including chemical purity, selectivity for cancer cells, chemical and physical stability, short time interval between the drug administration and its accumulation within tumor cells, activation at wavelength with optimal tissue penetration, and rapid clearance from normal tissues [19], and it is related to the amount of oxygen within the tumor area that, in turn, depends on the tissue oxygen concentration [20]. Photodynamic treatment also strictly relies on the light source and light delivery, whose choice is affected by tumor location, light dose delivered, and PS used. Lasers, lamps, and Light Emitting Diodes (LED) are all light sources employed in PDT. Conversely to lamps, lasers are typically near-monochromatic enabling the exact selection of wavelengths and the precise application of light. On the other hand, the main characteristics of LED use are

price and versatility in light delivery on difficult anatomic area [21].

The photodynamic reaction is based on photophysical and photochemical processes [22]. Upon visible light irradiation, the PS in its ground state is activated to the short-lived single excited state and it can lose its energy by emitting fluorescence or vibrational energy (photophysical reaction). The excited singlet state PS may also undergo a process known as intersystem crossing to form a relatively long-lived excited triplet state (photochemical reaction), which may interact with surrounding molecules resulting in two types of photooxidative reactions exploited in PDT. In type I photochemical reaction, the PS excited triplet state directly reacts with a substrate, such as the cell membrane, and it transfers an electron or hydrogen atom producing radical forms. These intermediates may further react with oxygen to form peroxides, superoxides ions, and hydroxyl radicals (known as ROS), initiating free radical chain reactions. Alternatively, type II photochemical reaction involves the direct transfer of triplet PS energy to molecular oxygen to form excited-state singlet oxygen ( ${}^1\text{O}_2$ ), the most important reactive species in PDT-mediated cytotoxicity [23]. The two types of photochemical reactions can simultaneously occur and their ratio depends on the type of PS, substrate, and oxygen concentration.

PDT-mediated tumor destruction is multifactorial: (1) direct tumor cells killing, (2) vasculature damage, and (3) rapid recruitment and activation of immune cells favoring the development of antitumor adaptive immunity [18, 24, 25].

Particularly, cancer cells can respond to photodynamic injury by initiating a rescue response and/or succumbing to multiple cell deaths. Three distinct mechanisms have been recognized to contribute to PDT-mediated tumor destruction: apoptosis, necrosis, and autophagy [26]. Apoptosis is the preferential PCD induced by the exposure of many photosensitized cell types to toxic agents, such as ROS. Apoptosis can be activated both in a caspases-dependent and independent manner. Particularly, PDT leads to activation of the several apoptotic pathways: extrinsic or death receptor pathway, implying the binding of death ligands to their specific cell surface death receptors (e.g., FasL/FasR, TNF- $\alpha$ /TNFR1, Apo3L/DR3, Apo2L/DR4, and Apo2L/DR5) ending in caspase 8 activation; intrinsic or mitochondrial pathway, involving caspase 9 activation and release of cytochrome c into the cytosol; ER stress-mediated pathway, mediating the cleavage of caspase 12; and caspase independent pathway, triggered by mitochondrial proapoptotic proteins, for example, AIF (Apoptosis Inducing Factor) and EndoG (Endonuclease G), able to induce apoptosis without caspase involvement by translocating to the nucleus where they generate DNA fragmentation (reviewed in [18, 26]). Master regulators of apoptotic machinery are Bcl-2 family proteins, comprising both anti- and proapoptotic members [27]. PDT is able to induce photooxidation of Bcl-2 antiapoptotic proteins and activate the proapoptotic members of the family [28].

If apoptosis is the preferential cell death mechanism induced by PDT, a switch from apoptosis to necrosis strictly

depends on PDT dose in term of light dose and PS concentration. Indeed, high PDT dose tends to cause cell death by necrosis, while low photodynamic regimen induces apoptotic cell death.

The role of autophagy in PDT-treated cells is controversial, since it plays a role in either inhibiting or stimulating cell death following photodynamic treatment [29, 30]. Although autophagy is generally thought of as a cell survival strategy, the high reactivity of photogenerated ROS can commit tumor cells to their final demise [31]. Generally, autophagy plays a prosurvival role in tumor cells capable of apoptosis; conversely, it promotes cancer cell death in apoptosis-deficient cells.

In some experimental PDT protocols, the specific inhibition of one of these three cell death mechanisms does not impair the activation of the others, suggesting their independent onset in PDT, which is able to ensure a long-term tumor photokilling [32, 33].

Tumor eradication is also mediated by strong PDT-induced inflammatory and immune reactions ending in the rapid recruitment of immune cells to neoplastic sites. Several reports suggest the infiltration of lymphocytes, leukocytes, and macrophages into the photosensitized tissue activating an immune response that consequently eliminates surviving cancer cells escaped to the direct PDT effects [34, 35].

The considerable beneficial immunomodulatory potential of PDT represents an exploitable plot in terms of cancer disease management. High-inflammatory PDT regimens induce acute inflammation characterized by increased expression of proinflammatory cytokines [36], adhesion molecules E-selectin and ICAM-1, and the rapid accumulation of leukocytes into the treated tumor area [37]. PDT enhancement of antitumor immunity appears to involve the stimulation of DCs by dying tumor cells [38]. Indeed, the incubation of photosensitized tumor cells with immature DCs induces an enhanced DC maturation, activation, and ability to stimulate T cell activation [39].

#### **4. Immunogenic Cell Death: New Concept in Cancer Therapy Outcome**

The intrinsic Achille's heel of conventional cancer regimens, that is, chemotherapy or radiotherapy, relies on their inability to eradicate all tumor cells. However, the knowledge of cancer immune hallmarks could be exploited to stimulate immune system and, consequently, to favour the patient, by designing therapeutic regimens able to elicit the immune reactivity and counteract immune suppression. In fact the immunogenicity of the succumbing tumor cells could drive a strong immune response against cancer cells survived to therapy [40]. Indeed, in response to anthracyclines (e.g., doxorubicin and mitoxantrone), oxaliplatin and ionizing irradiation, cancer cells die triggering a tumor-specific immune responses [41]. Altogether, these observations support the Immunogenic Cell Death (ICD) concept [42]. Particularly, the signals delivered by immunogenic dying cells function as antigens stimulating the crosstalk between DCs and T cells, that in turn mediates immunogenic *impetus* [43].

**4.1. The Effectors of ICD: Alarmins.** The relocation, release, and/or plasma membrane exposure of intracellular proteins by dying cancer cells are the key mechanisms in ICD. These intracellular molecules, known as alarmins, are categorized as DAMPs, and they are functionally similar to Pathogen-Associated Molecular Patterns (PAMPs), including bacterial and viral nucleic acids, fungal  $\beta$ -glucan and  $\alpha$ -mannan cell wall components, the bacterial protein flagellin, components of the peptidoglycan bacterial cell wall, and lipopolysaccharide (LPS) from Gram-negative bacteria [44].

DAMPs are normally retained within healthy cells and are extracellularly relocated in damaged/dying cells acquiring immunostimulatory/immunomodulatory properties when they interact with both intracellular and membrane-bound Pattern-Recognition Receptors (PPRs), for example, RIG-I-like receptors (RLRs), the NOD-like receptors (NLRs), and Toll-like receptors (TLRs). The diversity of DAMPs is related to the type of cell death, cell type, and tissue injury [44].

A multitude of immunogenic factors has been identified: Endoplasmic Reticulum (ER) protein calreticulin (CRT) [45], several members of the Heat Shock Proteins (HSPs) family [46–48], High-Mobility Group Box 1 (HMGB1) [49], end-stage degradation products (e.g., ATP, DNA and RNA, uric acid) [50–52], S100 proteins [53], and sphingosine [54]. Depending on the stage and relocation place, these molecules can be divided in three groups: (1) DAMPs exposed on plasma membrane; (2) DAMPs secreted extracellularly and (3) DAMPs produced as end-stage degradation products.

Secreted DAMPs can be in turn classified on the basis of the release mechanism: DAMPs passively released (e.g., during necrotic cell death), DAMPs released in a pulsatile manner (e.g., during apoptotic cell death), and DAMPs released by a noncanonical pathway, upon induction by activated immune cells [55].

Moreover, considering origin and mechanisms of action, the proinflammatory DAMPs can be classified as those that directly stimulate the immune cells and those that induce DAMPs generation exerting a bystander effect on extracellular molecules [56].

Furthermore, other signals, regarded as "atypical DAMPs", are being studied as alarmins, like "whole organelle-based danger signals", for example, complete mitochondria able to activate the NLRP3 inflammasome [57, 58], extracellular matrix compounds (e.g., hyaluronan, heparin sulphate, and degraded matrix constituents) [59], and signals/structures not yet fully characterized, for example, tumor cell-derived exosomes [60, 61].

In the following paragraphs, we summarize the characteristics and the translocation/release mechanisms of the main DAMPs acting as effectors of ICD.

**Calreticulin.** Calreticulin (CRT) is a 46 kDa  $\text{Ca}^{2+}$ -binding protein prevalently located in ER lumen, where it acts in proper folding of proteins, by interacting with ER-resident disulfide isomerase ERp57 and calnexin (CNX) [62], and in  $\text{Ca}^{2+}$  homeostasis/signaling regulation [63]. Moreover, in ER, the CRT participates in MHC class I molecule assembly

and loading of the antigen peptides onto the MHC class I molecule [64].

The CRT also resides in the nuclear envelope lumen, where it regulates nuclear protein transport [65] and signaling *via* nuclear steroid receptors and integrins [66], and in the cytoplasm. Probably, cytoplasmic CRT regulates cell adhesion, translation, gene expression, and nuclear export [67].

It has been demonstrated that CRT is also involved in cardiac development and adipocyte cells differentiation [68].

The dynamic exposure of CRT (ecto-CRT) on plasma membrane marks the cell for ICD [45]. Few studies have been performed in order to understand the mechanism regulating CRT translocation, that seems to unfold through three modules, that is, ER stress induction, apoptosis trigger, CRT translocation. Ecto-CRT translocation depends on the stress inducer and it certainly involves ROS-based ER stress; however, the ER can be or not the main inducer target, as it has been recently speculated by Garg et al. [69]. The well-known inducers of Immunogenic Cell Death, that is, anthracyclines, mitoxantrone, and oxaliplatin [45], primarily localize in the nucleus, inducing apoptosis upon DNA replication and repair damage, and only a fraction of them targets ER, producing ROS-based ER stress, indispensable to elicit immunogenicity during cell death. Similarly, inducers that preferentially localize ER, such as Hypericin, a molecule used in a particular cancer protocol, that is, PDT, are able to induce immunogenic apoptosis upon photo-oxidative ER (phox-ER) stress [70].

In anthracycline-based ecto-CRT translocation, ER stress induction ignites the ER stress response through Phosphorylation of eukaryotic Initiation transcription Factor 2 $\alpha$  (eIF-2 $\alpha$ -P) *via* serine/threonine kinase PERK (Protein kinase RNA-like Endoplasmic Reticulum Kinase) activation. The consequent apoptosis induction involves caspase 8, Bax and Bak Bcl-2 family members and the ER protein BAP31. CRT translocation on plasma membrane occurs in a SNARE-dependent exocytosis, based on a “CRT/Erp57 cotranslocation module” [71].

Conversely, the pathway orchestrating ecto-CRT translocation in phox-ER stress-induced immunogenic apoptosis only requires PERK and Bax/Bak, but it is independent on eIF-2 $\alpha$ -P and caspase-8 [70].

Moreover, the cotranslocation of CRT with Erp57, that has been described in immunogenic apoptosis, is probably not a universal phenomenon strictly necessary for the immunogenic outcome in cancer therapies. In fact, Garg et al. [70] describe the first Erp57-independent CRT exposure upon phox-ER stress.

Plasma membrane exposed CRT facilitates the engulfment of tumor dead cells by DCs, ensuring their immunogenicity [45]. A series of studies suggests that ecto-CRT exposure occurs in apoptotic anthracyclines, oxaliplatin, UVC, and  $\gamma$ -radiation induced cancer cells [45, 72]. These cells subcutaneously injected into syngenic immunocompetent mice boost a strong anticancer immune responses, also protecting against recurrence [45, 72, 73]. Immunogenic response to tumor antigens increases in patients with Acute Myeloid Leukemia (AML) expressing CRT on cell surface of

malignant blasts [74]. Moreover, Pekarikova and coworkers [75] demonstrate the presence of high percentage of anti-calreticulin antibodies in the serum of patients with gastrointestinal cancer pathology suggesting the cell surface expression of CRT on cancer cells, able to act as a target for B-cell immunogenic response.

The role of anti-CRT antibodies in cancer is enigmatic, since the translocation of CRT onto cell surface and/or its release in extracellular environment elicit an autoimmune reaction also in cancer pathologies [76–78] playing a negative role in antitumor defence [79, 80]. Probably, anti-CRT antibodies can interact with CRT engaging peptides exposed on the tumor cells surface with consequent decrement of cell immunogenicity or they can bind CRT peptides presented by MHC on APC preventing T cell response. The ecto-CRT exposure occurs before the specific morphological, that is, phosphatidylserine (PtdS) exposure on the outer leaflet of plasma membrane, and biochemical, that is, mitochondrial transmembrane potential depolarization, apoptotic signs [71]. The identity of the surface receptor docking ecto-CRT is not well known. The ecto-CRT colocalizes with PtdS on plasma membrane cholesterol rich GM-1 ganglioside-containing rafts [81]. It has been demonstrated that, in phox-ER stress, ecto-CRT surface docking does not depend on the correct organization of lipid rafts and occurs *via* the Low-density Receptor-related Protein 1 (LRP1) or CD91 molecule [70].

The extracellular CRT interacts with professional phagocytes plasma membrane CD91 internalization receptor forming a functional complex that drives engulfment of apoptotic died cells by stimulation of Rac-1 in phagocytes [82]. Further, ecto-CRT can also interact with thrombospondin [83], C1q and mannose binding lectin (MBL) [84], ficolin-2 and ficolin-3 [85, 86], and Surfactant Proteins (SP) A and D [82].

The receptor on DCs surface mediating engulfment by binding CRT-exposed on cancer cells is still obscure. Candidate receptors include scavenger receptor A [87], scavenger receptor class F, member 2 [88], and CD91 [89].

Intriguingly, ecto-CRT has been observed on the plasma membrane of the immune system cells, that is, monocyte-derived macrophages [90], DCs [91], resting and activated T-cells [92]. For instance, ecto-CRT on DCs plasma membrane interfaces cancer cells and hosts innate immune system by interacting with tumor-associated antigens like NY-ESO-1 [91]. If the presence of CRT on surface of immune cells, that is, macrophages, DCs and T-cells, effectively mediates an “anticancer vaccine effect” is still unclear.

**Heat Shock Proteins Family Members.** Inducible Heat Shock Proteins (HSPs) are a class of chaperone proteins ensuring the correct folding and subcellular compartments transport of newly synthesized proteins and the refolding or degradation of stress-accumulated misfolded ones [93].

Under stress conditions, intracellularly located HSPs are overexpressed and they can be translocated to plasma membrane surface and/or they can be also released into the extracellular environment. At least two members of HSPs

family, HSP70 and HSP90, can be expressed at the surface of plasma membrane and the change of cellular localization plays a dual role in cancer [94]. In fact, intracellular, cytoplasmic and organelles-located, overexpressed HSP70 and HSP90 exert a cytoprotective role by apoptosis inhibition [93], augmenting cancer cell survival; conversely, HSPs exposure suppresses tumor by attracting innate immune system cells.

Particularly, HSP70 inhibits (a) the apoptosome complex formation needful for postmitochondrial caspase activation by interacting with Apaf-1 [95]; (b) the caspase-independent apoptosis by blocking AIF translocation from mitochondria to the nucleus [96]; (c) the proapoptotic transcription factor p53 [97] or JNK1 or ERK stress kinases [98]; (d) the Bax-dependent release of proapoptotic factor from mitochondria by blocking mitochondrial outer membrane Bax translocation [99]. On the other hand, HSP90 can negatively affect apoptosis by (a) interacting with Apaf-1 and consequently blocking the apoptosome formation [100]; (b) interacting with Akt that, in turn, leads to inactivation of proapoptotic Bad protein and caspase-9, and to activation of NF $\kappa$ B apoptosis inhibition mechanism [101]; (c) inhibiting the action of calpains.

When HSP70 and 90 move from intracellular side to plasma membrane upon stress conditions, for example, oxidative stress, irradiation, serum deprivation, and chemotherapeutics drugs, they elicit a potent immunostimulatory activity. Particularly, in hyperthermia induced surface HSP enriched melanoma [102] and colon carcinoma cells [103], HSP70 and 90 act as DAMPs determining the immunogenicity of dying cancerous cells. HSP70 tightly associates with PtdS on plasma membrane, accelerating apoptosis as reported in PC12 tumor cells [104]. The immunostimulatory effect of ecto-HSP70 and 90 is based on their ability to interact with several APC surface receptors [105], for example, CD91, LOX1, and CD40 [106], and to elicit CD8+ T-cell response by participating in cross-presentation of tumor-derived antigens on MHC class I molecules [107]. This process is very needful in mouse models [108]. Antigen processing and cross-presentation of HSP-linked peptide involve a complex formed by Toll-Like Receptors (TLRs) and CD14 [109, 110]. TLR4 activates NF $\kappa$ B pathway in DCs that in turn induces the release of proinflammatory cytokines, such as TNF $\alpha$ , IL-1 $\beta$ , IL-12 and IL-6, and Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) [109–112]. Besides, HSP70 can promote DCs maturation by upregulating CD86 and CD40 [113] and NKs activation by interacting with various NKs surface localized inhibitory/activating receptors, such as CD94/NKG2A [114] and CD94, respectively, [115].

A number of human and animal tumor cells, that is, colon, pancreas, breast neck and head tumors [102, 103], and acute myeloid leukemia cells [116], express HSPs on their plasma membrane upon heat shock treatment.

The immunogenic potential of HSPs also occurs when they are secreted in the extracellular environment. In fact, high levels of HSP70 and 90 have been detected both *in vitro* and *in vivo*. Particularly, in humans, several stress conditions, such as inflammation, bacterial and viral infections, and

cancer diseases, lead to HSP70 and 90 presence in the serum of cancer patients. *In vitro*, the supernatants of the cultured of APCs and tumor cell lines contain members of HSPs family whose release occurs following exogenous stress, that is, proinflammatory cytokines [117].

HSPs surface translocation mechanisms and exposed HSPs derivation, that is, ER, cytosol or both, are still unknown. Also the membrane anchorage and the release process are not completely understood. Several mechanisms have been suggested to explain these phenomena including tumor cell necrosis and apoptosis and, recently, active release from viable tumor cells.

Since cytosolic HSPs do not contain the leader peptides enabling membrane localization, it has been speculated that other proteins possessing transmembrane domain shuttle HSPs from the cytosol to the plasma membrane or that HSPs directly interact with lipid components of plasma membrane. It has been demonstrated, in PC12 cells, that HSP70 associates with PtdS, supporting the hypothesis that the transport of HSP70 from inside the cell to the outer plasma membrane leaflet involves a flip flop mechanism similar to the PtdS one [104].

An active release of HSP70 along with BAG family molecular chaperon regulator 4 (Bag-4) *via* binding on surface of exosomes, endosome-derived vesicles 30–100 nm sized playing a dual role in cancer pathogenesis [118], has been observed in viable human colon and pancreatic carcinoma cells. Hsp70/Bag-4 surface-positive exosomes elicit migratory and cytolytic activity of NKs [119]. Also 4T1 breast adenocarcinoma and K562 erythroleukemic cells stimulated with IFN- $\gamma$  increase exosomal export of HSP70 inducing IL-12 release by DCs [120]. Similarly to tumor cell lines, human peripheral blood mononuclear cells (PBMCs) in both basal and stress-induced (heat shock at 40 or 43 degrees C for 1 h) states release HSP70-containing exosomes [121].

Taking into account all the above reported contents, HSPs are reported as DAMPs. Recently, Eden and coworkers [122] argue against the role of HSPs as DAMPs on the basis of the criteria suggested by Kono and Rock in 2008 [123]. These criteria are considered essential in order to classify a particular molecule as DAMP in terms of biological outcomes: a DAMP should be active and a highly purified molecule; its biological effect should not be owing to contamination with microbial molecules; it should be active at concentrations present in pathophysiological status; its selective elimination or inactivation should ideally inhibit dead cells biological activity both *in vitro* or *in vivo* assays. HSPs do not satisfy the first two criteria, since, due to their chaperone nature, HSPs engage other molecular structures and they are easily contaminated with microbial products [122].

On the other hand, TLR2 and TLR4, the most credited receptors for HSP60 and HSP70, respectively, are not strictly proinflammatory [124]. Moreover, experiments performed with DCs cultured in the presence of HSPs, both in murine [125] and human models [126, 127], demonstrated no stimulatory effects on DCs activation.

Then, it has been speculated that the HSPs might function as carriers of DAMPs rather than DAMPs, [116, 128].

*High Mobility Group Box 1 (HMGB1)*. The HMGB1 is a 29 kDa nucleus localized and nonhistone chromatin bound protein, also known as amphoterin. It affects different nuclear functions, that is, transcription and nucleoprotein complexes assembly. When it is actively secreted by inflammatory cells or passively released by necrotic cells [129], it acquires immunological potential based on its redox status [130] constituting a crucial step in the activation of APCs [49]. Three types of HMGB proteins exist: HMGB1 ubiquitously expressed [131], and HMGB2 and HMGB3 mostly expressed during embryogenesis and restrictively in adult-stage [132, 133]. Moreover, HMGB1 actively secreted by IL-1 $\beta$ , TNF or LPS-activated macrophages and monocytes is molecularly different from that passively released by the necrotic cells, since it is acetylated on several specific lysine residues [134].

Extracellular HMGB1 activates macrophages and DCs [135, 136] and burst neutrophil recruitment [137] by binding to a range of receptors, including TLR2, TLR4, and RAGE (Receptor for Advanced Glycation Endproducts) [135].

HMGB1 has several unique roles in cancer. Its expression is very important in lymphoma, melanoma and breast, cervix, colon, liver, lung, and pancreas cancer cells; further, its serum level significantly increases [138].

The HMGB1 release as DAMP is widely demonstrated in necrotic cancer cells [139]. Recently, also apoptotic [41] and autophagic [140] cancer cells might release HMGB1 at some points in their respective execution phases.

The HMGB1-DNA binding dictates the time and the occurrence of release. Since nuclear DNA is released in a time-dependent manner following apoptosis induction [141] and since during apoptosis HMGB1-DNA binding increases, apoptotic cells can release DNA as well as HMGB1 during later stages, for example, secondary necrosis [130, 142, 143]. In contrast to inflammatory response initiated by necrotic cells [139], ROS produced and HMGB1 released by apoptotic cells promote tolerance [130].

Recently Liu and coworkers [144] found that HMGB1 release after vincristine (VCR), cytosine arabinoside, arsenic trioxide adriamycin (ADM) chemotherapy treatment is a critical regulator of autophagy in HL-60 and Jurkat leukemia cells and a potential drug target for therapeutic intervention. In fact, HMGB1 contributes to chemotherapy resistance through autophagy regulation [145]. It has been demonstrated that leukemia cells sensitivity to chemotherapeutic agents increases by inhibiting HMGB1 release; conversely, leukemia cells resist to cell death by overexpressing HMGB1; finally, pretreatment with exogenous HMGB1 increases leukemia cells drug resistance [145]. Moreover, HMGB1-mediated autophagy depends on Beclin-1 regulation carried out by HMGB1 itself and it requires PI3KC3-MEK-ERK pathway. In fact, Liu et al. [144] found that HMGB1 increases Beclin 1/class III phosphatidylinositol 3-kinase (PI3KC3) and suppresses Beclin 1/Bcl-2 interaction, promoting vesicle nucleation. HMGB1 also contributes to phagophore membrane elongation and autophagosome formation by promoting Atg12-Atg5-Atg16 complex formation and by enhancing the accumulation of the Atg12-Atg5-Atg16 complex with LC3.

Finally, the redox state controls HMGB1's function in promotion of autophagy. In particular, reducible HMGB1 decreases cell injury/death in cancer cells by interfering with Beclin 1, whereas oxidized HMGB1 enhances cell injury/death in response to anticancer agents [146].

*End-Stage Degradation Products*. Upon loss of membrane integrity during primary or secondary necrosis, intracellular substances normally retained within cells are released. These end-stage degradation products, including uric acid, RNA, genomic double-stranded DNA, nucleotides (ATP) and nucleosides (adenosine), exert immunostimulatory effects on macrophages and DCs [50, 147, 148]. The uric acid is the end product of purine metabolism in uricotelic mammals, identified as a major alarmin released by injured cells since it elicits inflammation by enhancing CD8+ [149] and CD4+ [150] T cell responses towards particulate antigens. Plasma membrane collapse leads to release of (1) RNA molecules, interacting with TLR3 on DCs [51]; (2) double-stranded DNA, stimulating both macrophages and DCs [50]; (3) nucleotides, triggering maturation of DCs [147] through activation of the NF $\kappa$ B signaling [151]. Parallel to passive release, ATP can be liberated into the extracellular space by voltage-gated hemichannels such as pannexin 1 or connexin [152] and/or vesicular exocytosis [153].

*Inflammatory Cytokines*. Necrotic cells can also elicit an inflammatory response by active or passive secretion of inflammatory cytokines, that is, IL-1 $\alpha$  e IL-6. In particular, Eigenbrod et al. [154] demonstrated that IL-1 $\alpha$  is passively released by necrotic cells and it induces, in peritoneum mesothelial cells, secretion of CXCL1, leading to neutrophilic inflammation. Conversely, necrotic cells actively release proinflammatory cytokine IL-6 by upregulating NF $\kappa$ B and p38MAPK [155]. End-stage degradation products modulate their recognition by the immune system cells via the pentraxins, a family of innate immunity receptors [156].

**4.2. ICD: Immunogenic Signals Delivery Spatiotemporal Pattern.** ICD can be triggered by a panoply of anticancer stimuli, including Photodynamic Therapy, anthracycline- and oxaliplatin-based chemotherapy, and  $\gamma$ -radiotherapy. The spatiotemporal sequence responsible or not for the stimulation of the immune system, dictating the success of cancer therapy, includes three phases: decision phase, processing phase, and effector phase [43].

In the decision phase, CRT early (within few hours) translocates on plasma membrane of dying tumor cells colocalizing in patches with PtdS in concomitance with ER stress response. CRT acts as an “eat me signal”, driving the engulfment of dying cancer cells and in parallel the uptake of tumor antigens by DCs [45]. The uptake, via a CRT-dependent manner, is ensured by loss/redistribution of CD47, a “don't eat me signal” that avoids the accidental phagocytosis of viable cells [157], occurring within 30 minutes after the induction of apoptosis [81] and hence displaying a pattern similar to CRT translocation. Late exposure on cell surface of HSP70 and 90 cooperates in the tumor antigen chaperoning

and in the adhesion of tumor cells to DCs, respectively, [106, 158].

However, the DC-mediated uptake of tumor antigens is not sufficient to ignite immune response. In fact, the fusion antigen-containing phagosomes with lysosomes causes the destruction of antigens [159], that can be inhibited by recognition of DAMPs by TLRs localized on DCs surface [160]. Thus, other signals are required during processing phase to stimulate antigen processing. Among TLR4 ligands, the release of HMGB1, occurring within 18 hours after ICD induction, plays a key role. It has been suggested that the physical interplay between TLR4 and HMGB1 [49] triggers an immunogenic signal operating downstream of the DC-mediated antigen uptake that enables the optimal presentation of tumor antigens to T lymphocytes [73]. In addition, the antitumor immune response is controlled by NLR family, pyrin domain containing 3 (NLRP3) inflammasome [161]. ATP, released into the extracellular space during cellular stress, is the most abundant factor activating NLRP3 [162], that is essential for processing pro-IL-1 $\beta$  and secretion of IL-1 $\beta$  [163], that, in turn, primes CD4+ and CD8+ lymphocytes and elicits the antitumor response. ATP also functions as a “find me signal” promoting monocytes recruitment and activation [164].

In effector phase, matured and activated DCs elicit an IFN- $\gamma$ -polarized T cell response, essential for efficient antitumor immunity [45, 49, 161].

In Figure 1, we represent the temporal exposure and/or release of DAMPs and the relative response of immune cells in *in vitro* conditions.

## 5. The Response of Immune System to Photodynamic Treatment

The ability of PDT to induce activation of the immune system and specific antitumor immunity is a well-known phenomenon, recently reviewed in Pizova et al. [165]. Indeed, the ignition of anticancer immunity is as important as tumor cell death in designing an optimal cancer therapy [12, 40, 166]. In terms of ICD, various DAMPs can mediate antitumor immunity in PDT and a broad spectrum of photosensitizers localizing different subcellular organelles, especially ER, is very important in ICD triggering upon PDT [167].

**5.1. How PDT Elicit Antitumor Immunity.** Three mechanisms interplay to reduce and/or eradicate tumors after PDT: cancer cell death *via* ROS generation, ischemia of tumor area *via* destruction of tumor-associated vasculature depleting cancer cells of oxygen and nutrients, recruitment of inflammatory and immune mediators contributing to tumor destruction, and recognition of cancer cells *via* leukocytes invasion [168].

The triggered inflammatory responses are fundamental to achieve long-term tumor control [169].

The innate immunity against cancer is a step-by-step process involving initiation of inflammation, cytokine release, neutrophil infiltration of tumor site and neutrophilia and, finally, complement activation. On the other hand,

acute inflammation can supply bioactive molecules to the tumor microenvironment, including growth factors that sustain proliferative signaling, survival factors that limit cell death, proangiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis, and inductive signals that support tumorigenesis [170–173]. Antitumor effect of cancer PDT involves both innate and adaptive immune system.

PDT alters the tumor microenvironment by causing oxidative stress which triggers a vast array of signal pathways through TLRs, stimulating inflammation by expression of HSPs, NF $\kappa$ B, and AP-1 [169]. The increased activity of these factors has been reported in several cancer cell lines photodynamically treated with different PSs. For example, NF $\kappa$ B activation has been observed in L1210 mouse leukemia cells after Photofrin-PDT [174], in lymphocytes or monocytes infected with HIV-1 after proflavine-PDT [175], in human colon carcinoma cells phototreated with pyropheophorbide-a methyl ester (PPME) [176], and in human HL-60 cells after PDT with benzoporphyrin-derivative-(BPD-) verterporfin [177]. Similarly, AP-1 activation occurs in cervical carcinoma HeLa cells [178] and in epithelial PAM 212 cells [179] photosensitized with Photofrin.

NF $\kappa$ B and AP-1 activation causes release by macrophages of different immunoregulatory and proinflammatory proteins, such as interleukins (IL-1 $\alpha$ , -1 $\beta$ , -2, -6, -8, -11, -12, -15), tumor necrosis factor (TNF), chemokines (inflammatory protein IP-10, keratinocytes-derived chemokines KC, Macrophage Inflammatory Proteins MIP-1 $\alpha$  and  $\beta$ , MIP-2, eotaxin, Methyl-accepting Chemotaxis Protein MCP-1, Regulated on Activation Normal T cell Expressed and Secreted, RANTES), and interferons (IFN- $\alpha$  and  $\beta$ ) (reviewed in [169]).

Other cell types eliciting innate immunity in PDT are neutrophils. They play a fundamental role both in the direct killing of tumor cells and in the activation of other immune cells. Moreover, they are also a source of proinflammatory mediators [180]. Neutrophils do not only accumulate in PDT-treated tumors, but they are also present in the blood of the host (so-called neutrophilia) [181]. Cecic et al. [182] reported that, in mammary carcinoma EMT6 tumors, Photofrin-PDT induces in the host mice a significant increment of neutrophils in blood persisting for at least 10 hours after treatment. Also Gollnick et al., [34] report that 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide- $\alpha$  (HPPH)-mediated-PDT causes neutrophil migration into the tumor area as well as Krosl and colleagues [183] report a 200-fold rise in neutrophils in the cellular infiltrate in SCCVII tumor treated with Photofrin-PDT. Finally, treatment of rat rhabdomyosarcoma tumors with 5-aminolevulinic acid (5-ALA)-PDT induces a blood neutrophils increase during the first few days after illumination [184].

Thus, PDT prompts a powerful acute inflammation leading to activation of complement cascade likely by the alternative pathway [180, 182, 185, 186]. In particular, *in vitro* studies indicate that PDT induces fixation of complement C3 protein to tumor cells [187] that, in turn, marks the cells to be destructed by the innate immune system [188–190]. The complement system can directly promote T-cell

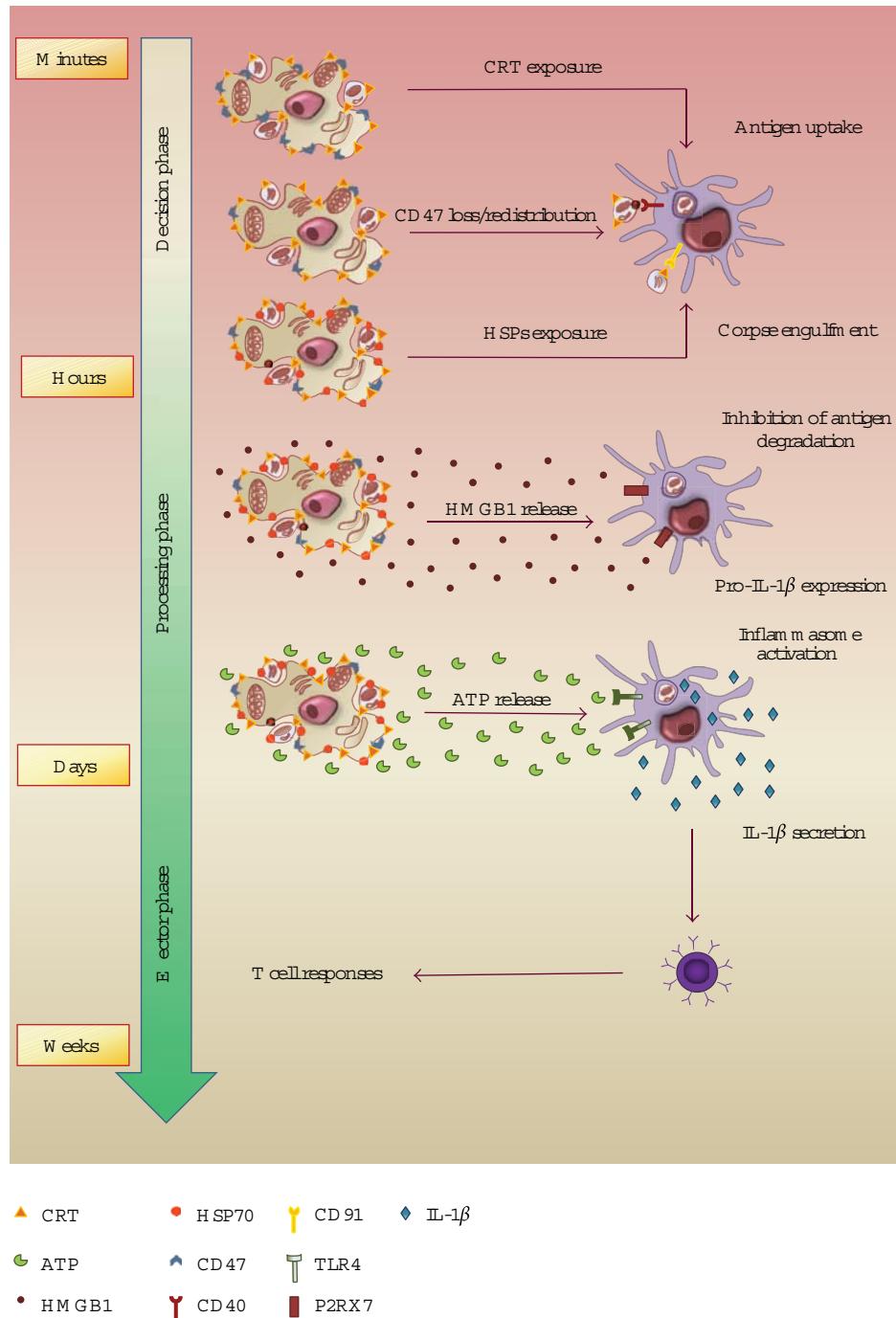


FIGURE 1: Spatiotemporal immunogenic signals delivery pattern. Decision phase, processing phase, and effector phase represent the spatiotemporal sequence determining the activation of immune system in *in vitro* conditions.

mediate response, playing a role in the adaptive immunity [191] carried out by antigen-specific B and T cells.

The growth inhibition of murine EMT6 tumors dependent on the presence of CD8+ T cells has been demonstrated after Photofrin-PDT by Kabingu and coworkers [192]. Preise et al. [38], by transferring CD8+ and CD4+ T cells from mice survived to cancer 3 months after vascular targeted PDT with bacteriochlorophyll derivative WST11, found that

the transferred mice are protected from subsequent challenge with viable cancer cells. Similar results have been obtained in human studies. For example, Thong et al. [193] demonstrated increased CD8+ T cell infiltration into multifocal angiosarcoma of the head and neck carcinoma area phototreated with Fotolon, a PS comprising 1:1 chlorin e6 and polyvinylpyrrolidone. Photodynamic treatment with Photofrin or 5-ALA in patients with basal cell carcinoma

provides an enhanced recognition of MHC-I-antigen complexes by immune cells and the activation of tumor specific CD8+ T cells [194].

The activity of CD8+ cells upon PDT is correlated to the presence of tumor antigens eliciting a potent antitumor effect. In fact, Mroz and coworkers [195], demonstrated that BPD-PDT can induce a strong antigen specific immune response capable to incite the memory immunity enabling BALB/c mice to reject a tumor rechallenge obtained with the same antigen positive tumor from which they were cured. The importance of tumor antigen presence is confirmed by the observation that BPD-PDT low level distant metastasis destruction correlates with a loss of tumor antigen expression.

It is worth mentioning that, certain PDT regimens systematically suppress immune reactivity [169]. Cutaneous Photofrin-PDT induces elevated levels of systemic IL-10, correlating to a prolonged suppression of contact hypersensitivity (CHS) reaction of at least 28 days following treatment. Since the major effector cell in CHS is the IFN- $\gamma$  secreting CD8+ cell and IL-10 suppresses cell-mediated immune response *via* inhibition of CD4+ cells activation, it is possible that PDT negatively influences the CD4+ or CD8+ development. Moreover, the inhibition of CD4+ or CD8+ activity can be caused by induction of systemic IL-4 [196]. Yusuf et al. [197] demonstrated that silicon phthalocyanine (Pc4)-PDT causes immunosuppression in cancer ill mice by involving of CD4+ and CD8+ T cells. Moreover, immunosuppression can be adoptively transferred with spleen cells from Pc4-PDT treated donor mice to syngenic naive recipients and it is primarily mediated by T cells, although macrophages were also found to participate. Among CD4+ cells, a special population that functionally suppresses an immune response is Tregs. They mediate their immunosuppressive effects by multiple pathways [198]. Particularly, Tregs express TGF- $\beta$ , an immunosuppressive cytokine [199] participating in further proliferation of Tregs [200], and suppress DCs activation [201]. Castano and colleagues [202] observed that Tregs can be depleted by cyclophosphamide (CY) that, in combination with PBD-PDT, leads a long-term J774 reticulosarcoma cure and resistance to tumor rechallenge.

### 5.2. Involvement of ICD in Photodynamic Cancer Therapy.

The link between PDT and HSPs, and especially HSP70, the best characterized DAMPs in PDT, [203] is already known as well as the immunogenicity of PDT treatment and its ability to elicit an antitumor immunity [204].

PDT is able to trigger the main types of cell death, that is, apoptosis, autophagic cell death, and necrosis [26]. It is interesting to understand if there exists a relation between PDT-induced cell death and DAMPs and between DAMPs and ICD. In case of necrosis, DAMPs spectrum does not change relatively to agents inducing them, including PDT. For example, the release of HMGB1 has been demonstrated both in serum of Photofrin-treated mice with subcutaneous Lewis Lung Carcinoma (LLC) and in LLC Photofrin-treated cells [205]. On the other hand, HMGB1 has been reported

to be the only DAMP involved in autophagy [144–146], but nothing is known about the DAMPs associated with autophagic cell death induced by photodynamic treatment. Moreover, PDT-induced apoptotic cells have been predominantly associated with HSPs [204] and only recently it has been suggested the involvement of CRT and ATP in apoptotic cell death upon photodynamic sensitization [70].

Interestingly, we found, for the first time, that Rose Bengal Acetate (RBAc) is able to induce the release and translocation of HSP70 and 90 and the exposure of CRT on plasma membrane of both apoptotic and autophagic RBAc-PDT induced HeLa cells (unpublished data). However, it is still under investigation the involvement of these DAMPs in RBAc-PDT induced immunogenicity.

The DAMPs spectrum observed in PDT is reported in Figure 2.

In the following section, the link between DAMPs-associated PDT and their involvement in the elicitation of immune cells have been discussed in detail.

*Interplay between DAMPs Involved in PDT-Induced Cell Death and Immunogenicity.* The best characterized DAMPs involved in PDT-triggered cell death able to confer immunogenicity are HSPs proteins and especially HSP70, as described for squamous cells carcinoma SCCVII [203], murine mammary tumor cells C127 [206] during Photofrin-PDT, EMT6 cells during meso-tetrahydroxyphenyl chlorin (mTHPS, Foscan)-PDT [207] and glioblastoma cell lines U87 and U251 during 5-ALA-PDT [208].

It has been observed that almost instantaneously HSP70 can be translocated onto the outer leaflet of plasma membrane of SCCVII cells apoptotically committed with Photofrin-based PDT. The authors reported that several other HSPs, that is, HSP60 and Glucose regulated Protein 94 (GRP94) are also exposed at the surface of tumor SCCVII cells. A fraction of HSP70 is promptly (within 1 hour) released by cells after high treatment doses, whereas lower PDT doses induce a HSP70 release at later time intervals, suggesting that the release is a consequence of membrane permeabilization upon necrosis. The induction of cell surface expression and release of HSPs stimulate macrophages coincubated with PDT-treated SCCVII cells to produce TNF- $\alpha$ . This study also suggests that DAMPs exposed onto the surface in response to PDT stress based on *in vitro* or *in vivo* settings, are probably related to tumor microenvironment. In fact, when authors induce SCCVII tumor in mice, they observe that cancer cells expose GRP78 rather than HSP60 and GRP94 [203]. Moreover, ecto-HSP70 participates in the opsonization of cancer cells by C3 complement protein [209]. Similarly, Zhou and coworkers [206] demonstrated that HSP70 secreted and released by C127 cells induced to apoptosis by Photofrin-PDT orchestrates an immunological regulatory mechanism towards murine Raw 264.7 macrophages. In fact, macrophages incubated with apoptotic cells as well as necrotic tumor cells showed a high level of TNF- $\alpha$  secretion. Also EMT6 cells photosensitized with Foscan expose and release HSP70 resulting in long-term tumor growth control [207]. In 5-ALA spheroids of

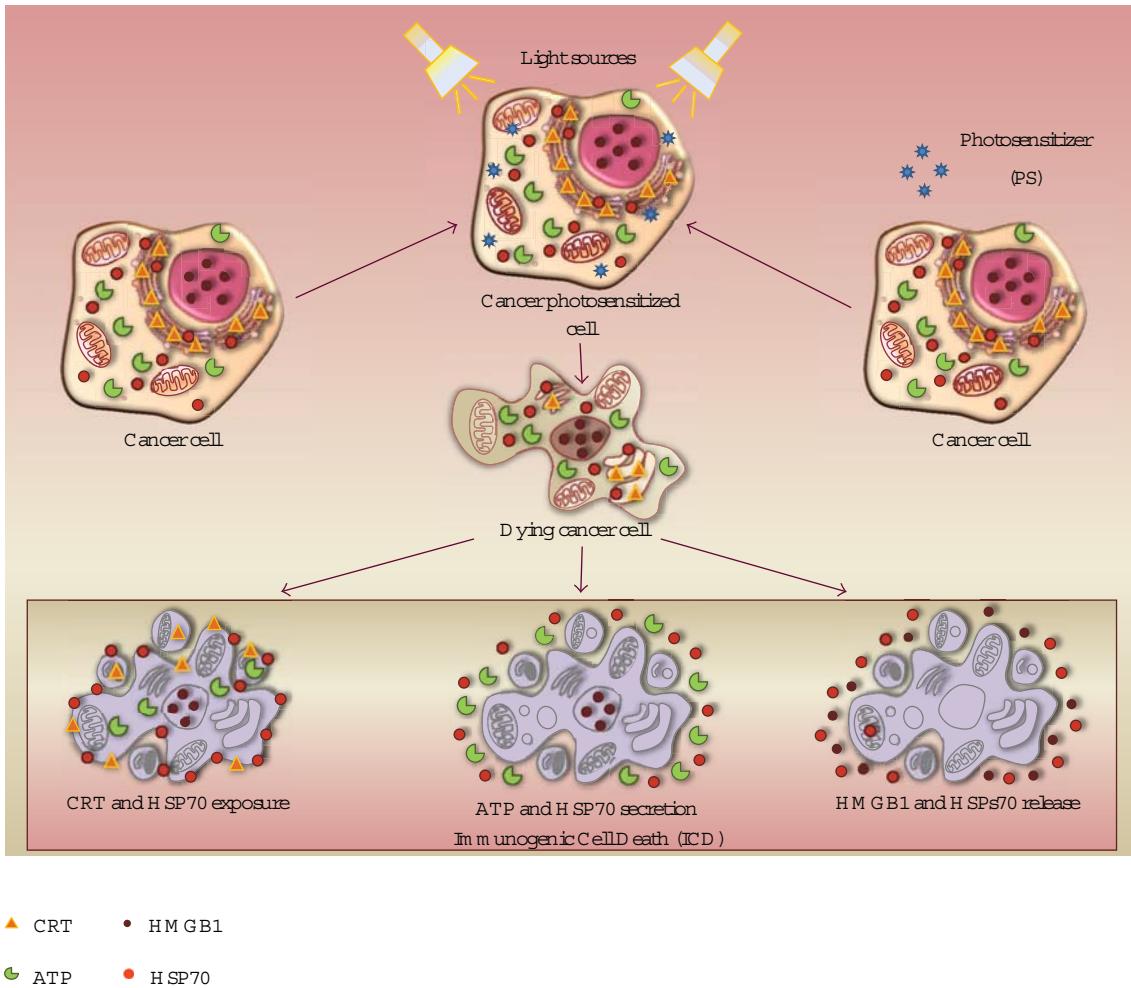


FIGURE 2: Molecular mechanisms of PDT-induced Immunogenic Cell Death (ICD). The three main processes characterizing the ICD are the preapoptotic cell surface exposure of calreticulin (CRT) and HSP70, the secretion of ATP and HSP70, and the postapoptotic release of HMGB1 and HSP70.

glioblastoma cell lines U87 and U251, HSP70 is expressed on the surface of cancerous cells and induces both attraction and maturation of DCs and antigen uptake by upregulation of CD83 and costimulatory molecules as well as increasing T-cell stimulatory activity of DCs [208].

Recently, experiments performed in the Agostinis laboratory reveal that the CRT exposure and ATP secretion during PDT elicit ICD, adding Hypericin-(Hyp-) based PDT to the list of ICD inducers [70, 210].

Hyp-PDT causes, soon after 30 minutes after irradiation, the precocious exposure on surface of T24 human bladder carcinoma and colon carcinoma CT26 cells of both CRT and HSP70 [210]; moreover, T24 cells secrete ATP in response to Hyp-PDT [70]. Ecto-CRT exposure depends on the PDT dose, both in PS concentration and in light fluence; surprisingly, conversely to the literature data reporting cotranslocation of ERp57 and CRT on plasma membrane, after Hyp-PDT, T24 cells expose CRT in the absence of ERp57 and it

does not require eIF2 $\alpha$  phosphorylation, caspase 8 activity, and increased cytosolic Ca $^{2+}$  concentration [70, 210]. In terms of immune cells ignition, only ecto-CRT influences phagocytosis of T24 and Ct26 dead cells. In fact, T24 cells succumbing to Hyp-PDT were engulfed by murine Mf4/4 macrophages and human DCs [70]; similarly, CT26 Hyp-PDT photokilled cells were preferentially phagocytosed by JAWSII murine dendritic cells [210]. Moreover, CT26 Hyp-PDT photokilled cells immunized syngenic BALB/c mice against a recidive in the presence of living CT26 cancer cells [70]. DCs cultured in the presence of Hyp-PDT treated T24 cells produce high levels of Nitric Oxide (NO) and IL-1 $\beta$ , but they do not secrete the anti-inflammatory IL-10 [70]. Since IL-1 $\beta$  is involved in polarization of IFN- $\gamma$ -producing antineoplastic CD8+ T cells [211], Garg et al. [70] found the antitumor immune response elicited by Hyp-PDT. The authors [70] also suggest the processes and molecules sustaining CRT exposure, that is, ROS production,

class I phosphoinositide-3-kinase (PI3K) activation, actin cytoskeleton, ER-to-Golgi anterograde transport, PERK, Bax and Bak proapoptotic proteins and CRT cell surface receptor CD91, and ATP release, that is, ER-to-Golgi anterograde transport, PI3K and PERK.

## 6. Concluding Remarks

The knowledge of DAMPs involved in cell death inducing cancer settings could help in the prompt choice of the better therapeutic design for a particular cancer condition. In fact, cancer therapies associated with DAMPs expression, in term of both exposure on plasma membrane and release on extracellular environment, have been shown to be able to fuse efficient cell death induction and activation of antitumor immune response. DAMPs can be also exploited as tool to mark disease's stage and to identify the extent of inflammation associated with the disease. Further, they can be used to prepare highly immunogenic vaccines. Due to its uniqueness to efficiently induce cell demise, antitumor effect by involving both innate and adaptive immune system and DAMPs expression, PDT is very promising to optimize cancer protocol. In fact, by inducing ICD, PDT could be capable to counteract cancer recurrence by instructing immune system.

In this context, it is a priority for the future oncology practice to clarify the molecular mechanisms associated with the immune response triggered by immunogenic tumor cell death.

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## Research Article

# In Vitro Analysis of Photosensitizer Accumulation for Assessment of Applicability of Fluorescence Diagnosis of Squamous Cell Carcinoma of Epidermolysis Bullosa Patients

Patrick Larisch,<sup>1</sup> Thomas Verwanger,<sup>1</sup> Kamil Onder,<sup>2</sup> and Barbara Krammer<sup>1</sup>

<sup>1</sup> Division of Molecular Tumorbiology, Department of Molecular Biology, University of Salzburg, Hellbrunnerstrasse 34, 5020 Salzburg, Austria

<sup>2</sup> Division of Molecular Dermatology, Department of Dermatology, Paracelsus Medical University, Muellner Hauptstrasse 48, 5020 Salzburg, Austria

Correspondence should be addressed to Barbara Krammer; barbara.krammer@sbg.ac.at

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Epidermolysis bullosa (EB) is a group of inherited skin disorders characterized by blistering following mechanical trauma. Chronic wounds of EB patients often lead to tumors such as squamous cell carcinoma (SCC). Early diagnosis may prevent its invasive growth—frequently the reason of premature mortality of EB-patients. Early detection of tumors is achieved by fluorescence diagnosis (FD), where photosensitizers localize selectively in tumors and fluoresce upon illumination. Excessive accumulation of photosensitizers in inflamed areas, as occasionally found at chronic wounds and tumors due to inflammatory processes, leads to false-positive results in FD. This study analyzed accumulation kinetics of the photosensitizers hypericin and endogenous protoporphyrin IX (PpIX) in different skin cell lines including the three EB subtypes under normal and proinflammatory conditions (stimulated with TNF-alpha). The aim was to assess the applicability of FD of SCC in EB. All cell lines accumulate hypericin or PpIX mostly increasing with incubation time, but with different kinetics. SCC cells of recessive dystrophic EB (RDEB) accumulate less hypericin or PpIX than nonmalignant RDEB cells. Nevertheless, tumor selectivity *in vivo* might be existent. Non-EB cell lines are more active concerning photosensitizer enrichment. Proinflammatory conditions of skin cell lines seem to have no major influence on photosensitizer accumulation.

## 1. Introduction

Epidermolysis bullosa (EB) is a group of skin disorders which are genetically determined. They are characterized by blistering of the skin and mucosa following mechanical trauma [1–3]. EB can be divided into three classes.

EB simplex (EBS) is the most common form of EB. Its inheritance is normally autosomal dominant but in some cases an autosomal recessive trait can be found. The blister formation begins intraepidermally with a subnuclear disruption of the basal keratinocytes. The reason for this is mutations in specific genes encoding for keratin 5 and keratin 14 (KRT5 and KRT14) [4, 5] and for plectin (PLEC1) [6].

EB junctionalis (EJ) is a group of autosomal recessive disorders. There are two main categories within this group of EB, the Herlitz (lethal) and non-Herlitz (nonlethal) form.

The tissue separation of these forms is through the lamina lucida of the basement-membrane zone beneath the plasma membrane of epidermal basal cells. Nonscarring blistering is the result of this separation. Mutations in genes encoding for laminin 5 subunits (LAMA3, LAMC2, and LAMB3) and collagen, type XVII, alpha 1 (COL17A1) are causative for this form of EB [7].

EB dystrophica (EBD) has an autosomal recessive or dominant inheritance. The blistering level of this type of EB lies below the lamina densa of the epidermal basement membrane. Mutations are occurring in COL7A1, the gene encoding for collagen, type VII, alpha 1 [8].

All these forms of EB are resulting in the pain of blistering, inflammation, and in some cases scarring and cancer because of loss of the skin's barrier function [9].

The chronic wounds of EB patients are accompanied by inflammatory processes, which may promote induction and growth of skin tumors such as squamous cell carcinoma (SCC), especially when the inflammation lasts for a long period or is derailed [10]. Early diagnosis of SCC is important, since early stages of SCC can be treated more easily than invasively growing SCC, which often is the main reason of premature mortality of the EB patients. To this purpose, a new, effective, and noninvasive technique for early detection of SCC would be offered by fluorescence diagnosis (FD) using a photosensitizer. The latter localizes selectively in tumor tissue and is able to fluoresce upon irradiation with visible light of a wavelength matching the absorption spectrum of the substance. This modality can be applied for tumor diagnosis, even in early stages, and it is especially helpful in fluorescence-guided resection [11].

Beyond diagnosis, the tumor-localizing photosensitizer is able to kill the target cells when light activated. In the presence of oxygen, most photosensitizers generate either superoxide radicals, that might form peroxides and hydroxyl radicals in a type I reaction, or singlet oxygen molecules ( $^1\text{O}_2$ ) in a type II reaction. The tumor destruction occurs finally due to reactive oxygen species (ROS) [12] or reactive nitrogen species [13]. This treatment is called “photodynamic therapy (PDT)” and was already used for basal cell carcinoma treatment of an RDEB-patient [14].

Chronic wounds, especially a problem for EB patients, as well as tumors are often accompanied by inflammatory processes, which may lead to false-positive results in FD, decreasing the specificity. The reason for this is unclear, but some clinical studies supposed local immune cells such as macrophages, which invade inflamed areas, as source for an excessive accumulation of the photosensitizer [15–18]. Nevertheless, it cannot be excluded that nonimmune cells accumulate the photosensitizer at a higher rate under inflammatory conditions, and that proinflammatory cytokines could play a role in this process.

Proinflammatory cytokines control inflammation and modulate neovascularisation, cell proliferation, and migration [19]. Inflammation is an essential part of wound healing, but it can turn to a problem, when this controlled process is switching to an uncontrolled or excessive one. This is often seen in diseases like chronic wounds, tumor metastasis, psoriasis, and arthritis [20]. Most of all deregulated wound healing is caused by an increase of interleukin 1 (IL-1alpha and IL-1beta) and tumor necrosis factor-alpha (TNF-alpha) levels [21–24]. Interleukin 6 (IL-6) also seems to play an important role in the pathogenesis of inflammation [25]. On the other hand, secretion of these cytokines was found to be upregulated by PDT [26–28].

As indicated before, detection of early stages of SCC in EB-patients via fluorescence diagnosis would be a new approach to prevent invasive SCC growth by early intervention. Therefore, the aim of the present study should be the analysis of the fluorescence kinetics of photosensitizers in EB cell lines to assess the applicability of FD on SCCs of EB-patients.

TABLE 1: Cell lines, EB subtype, and mutations.

Cell line	EB Subtype	Mutation
RDEB-CL	RDEB-sev gen	COL7A1 (7786DG/R578X)
SCCRDEB4	RDEB-sev gen	COL7A1 (8244dupC/8244dupC)
GABEB	JEB-nH gen	COL17A1 (4003delTC/4003delTC)
EBS-MD	EBS-MD	PLEC1 (1287ins3/Q1518X)
HaCat	—	Wild type keratinocytes
Skin	—	Wild type fibroblasts
A431	—	epidermoid squamous cell carcinoma

The uptake of an externally applicable photosensitizer such as hypericin and accumulation of the endogenously formed photosensitizer protoporphyrin IX (PpIX) should be analysed in the malignant EB-cell line SCCRDEB4 and compared to nonmalignant EB cell lines and a malignant non-EB cell line. To induce endogenous PpIX formation, its precursor ALA (5-aminolevulinic acid) in the heme biosynthesis will be applied. PpIX is currently successfully used in tumor diagnosis [11]. Hypericin is a plant constituent from St. John's wort with excellent fluorescent properties that can modulate several signaling pathways [29, 30].

To prove the hypothesis that inflammation of tissues causes excessive accumulation of photosensitizers often leading to false-positive results in FD, the effect of proinflammatory conditions on uptake or accumulation of hypericin or PpIX, respectively, in normal and malignant EB-cell lines and their respective reference cell lines should also be analysed. The question here is whether FD is influenced by the proinflammatory state of EB cells.

In order to address these issues, we performed analysis of uptake kinetics on seven different skin cell lines (GABEB, EBS-MD, RDEB-CL, SCCRDEB4 as EB cell lines, representative of the three main subtypes, and HaCat, Skin, A431 as keratinocyte, fibroblast, and SCC cancer cell lines as control and reference). For establishment of a proinflammatory milieu, we stimulated the cells with TNF-alpha (tumor necrosis factor-alpha) to activate proinflammatory pathways [31].

## 2. Material and Methods

**2.1. Cell Lines.** GABEB and EBS-MD (immortalized fibroblasts) cells were obtained from Dr. Johann Bauer (Division of Molecular Dermatology and EB House Austria, Department of Dermatology, Paracelsus Medical University, Salzburg, Austria). RDEB, exactly RDEB-CL cells, were obtained from Professor Guerrino Meneguzzi, Nice, France, and SCCRDEB4 [32] cells were kindly provided by Dr. Andrew South, Dundee, UK. Control cell lines (HaCat, Skin, and A431) were available in our laboratory. For cell lines, EB subtypes and mutations see Table 1.

The SCCRDEB4 cell line was routinely grown in Keratinocyte-SFM (Life Technologies, Vienna, Austria) and RDEB-CL and GABEB cell lines in Keratinocyte-SFM with 100 U/mL penicillin and 0.1 mg/mL streptomycin. Keratinocyte-SFM was always supplemented with bovine pituitary extract and recombinant epidermal growth factor.

EBS-MD, Skin, HaCat, and A431 cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose supplemented with 10 mM HEPES, 4 mM l-glutamine, 1 mM Na-pyruvate, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10% (v/v) or 5% (v/v) for A431, respectively, foetal bovine serum (FBS) (all from PAA-Laboratories, Linz, Austria or LONZA, Basel, Switzerland) in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>.

**2.2. Photosensitizers.** In preliminary experiments, cell proliferation characteristics were analysed by the MTT assay in order to determine the respective cell number for each cell line, which should be used to yield comparable results due to comparable cell mass and monolayer density. Twenty-four hours after seeding the cells to confluence of about 80% cell culture medium was replaced with medium containing hypericin or the PpIX precursor ALA.

Hypericin is a secondary metabolite predominantly extracted from the *Hypericum perforatum* (St. John's wort). It was purchased from Planta Natural Products (Vienna, Austria) and added to the serum-free medium of the cell cultures in final concentrations of 3 and 5 μM. These concentrations were chosen according to previous work [33]: irradiation of A431, HaCat, and SCCRDEB4 cells with a diagnostic protocol using 3 μM hypericin was sublethal, but using 5 μM hypericin was phototoxic with 40% to 70% survival having therapeutic impact.

Endogenous PpIX is the last molecule in the heme biosynthesis prior to heme and depends on its precursor 5-aminolevulinic acid (ALA). If ALA is given in excess, PpIX is accumulated in cells and can be used as a very effective photosensitizer for many hours. ALA was purchased from Sigma-Aldrich (Vienna, Austria). It was applied to the serum-free medium of the cell cultures in final concentrations of 0.5 and 1 mM. Also, these concentrations were chosen according to a previous study [34], in which 0.5 mM and 1 mM ALA was used to induce PpIX efficiently in a linear relationship for fluorescence detection of mononuclear and circulating tumor cells.

Handling with photosensitizers was performed under subdued light conditions.

**2.3. Induction of Proinflammatory Milieu.** Fibroblast and keratinocyte cell lines were incubated with lipopolysaccharide (LPS) or TNF-alpha (both Sigma-Aldrich, Vienna, Austria) in preliminary tests to induce inflammation. Based on these tests TNF-alpha was then chosen as most applicable inductor. The culture medium was replaced by corresponding serum-free medium containing TNF-alpha (5 ng/mL) 4 h before photosensitizer treatment was started.

IL-6 and IL-1beta Ready-Set-Go! ELISAs (eBioscience, Vienna, Austria) were performed according to the manufacturer's protocol to identify the best procedure for inducing a proinflammatory milieu, which was repeatedly checked throughout the study.

**2.4. Uptake Experiments.** After incubation with hypericin or ALA, respectively, for 1 h up to 8 h, cells were washed twice with 100 μL DPBS and lysed for 10 min by addition of 50 μL

1% Triton X-100. Subsequently, the fluorescence intensity of the photosensitizers hypericin and PpIX in the cells was measured in 96-well plates (Greiner, Kremsmuenster, Austria) using a microplate reader (Infinite M200pro, Tecan, Groedig, Austria). Hypericin fluorescence was detected at λ(ex) = 340 nm and λ(em) = 604 nm and PpIX fluorescence at λ(ex) = 435 nm and λ(em) = 635 nm. The fluorescence signals were related to the protein content of each sample (BCA assay; Fisher Scientific, Vienna, Austria) to correct for variations in the cell mass. Measurements were performed in triplicates and series were repeated independently for at least two more times.

**2.5. Data Analysis, Statistics.** Comparisons between data points were statistically evaluated by the Student's *t*-test for independent samples. At least three independent series were included in the analysis.

### 3. Results

The results show the analysis of the fluorescence kinetics of EB and non-EB cell lines under normal and proinflammatory conditions. In order to check for the proinflammatory state of the selected cell lines after TNF-alpha induction, the IL-6 level of cells was checked at random (data not shown).

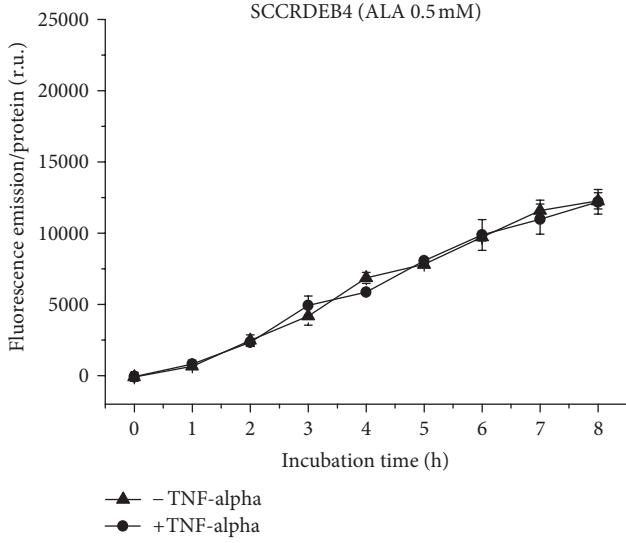
All cell lines take up hypericin or generate PpIX, respectively, at the selected concentrations.

SCCRDEB4 cells generate PpIX from 0.5 mM ALA with linear kinetics, equally under normal and proinflammatory conditions (Figure 1(a)), and from 1 mM ALA with linear kinetics and moderately higher in the proinflammatory state (Figure 1(b)). However, these variations are not significant. This differs from normal state, which shows a final fluorescence intensity similar to the lower concentration but with a curve shape forming a plateau.

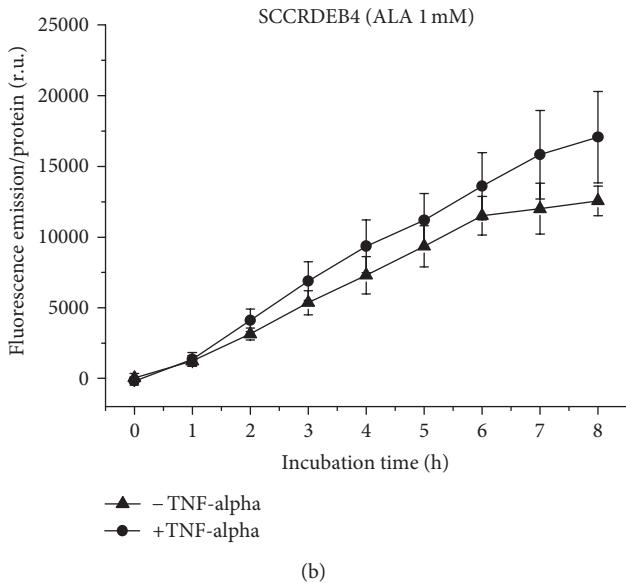
SCCRDEB4 cells take up hypericin at a concentration of 3 μM with linear kinetics, equally under normal and proinflammatory conditions (Figure 2(a)), and at a concentration of 5 μM almost equally under normal and proinflammatory conditions with curve shapes showing the onset of a plateau. The final fluorescence level at the proinflammatory state is marginally higher than the fluorescence after 3 μM hypericin (Figure 2(b)).

PpIX is generated by RDEB-CL cells from 0.5 (Figure 3(a)) and 1 mM ALA (Figure 3(b)) equally under normal and proinflammatory conditions, with more or less linear kinetics for 0.5 mM and with a beginning flattening to a plateau for 1 mM ALA. The fluorescence intensity is slightly lower at the higher concentration.

RDEB-CL cells take up hypericin at a concentration of 3 μM in a moderately flattening curve course, statistically nonsignificantly higher under proinflammatory than normal conditions (Figure 4(a)). Uptake of 5 μM hypericin occurs in a curve forming a plateau also more or less equally under normal and proinflammatory conditions (Figure 4(b)). Under normal conditions, the fluorescence increases with the hypericin concentration, but under TNF-alpha pretreatment, the final fluorescence values at 8 h incubation time with



(a)



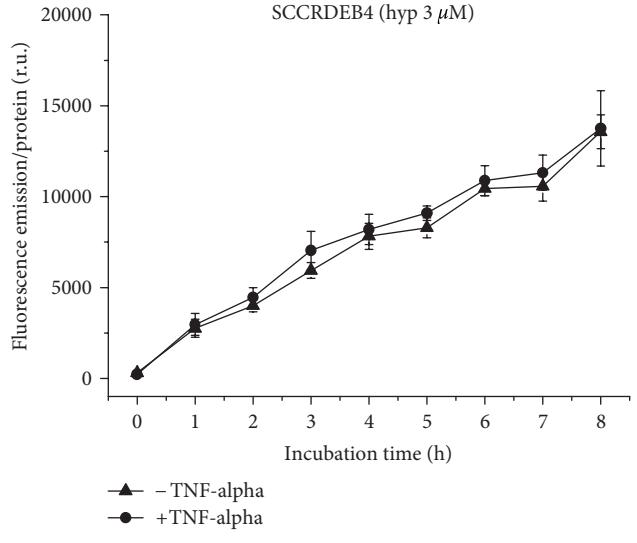
(b)

FIGURE 1: PpIX formation in SCCRDEB4 cells over 8 h, with and without TNF-alpha induction. (a) Application of 0.5 mM (b) of 1 mM ALA.

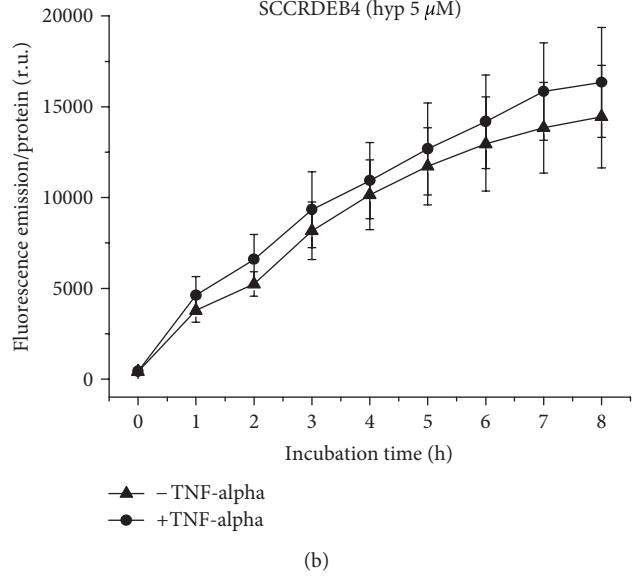
3 and 5  $\mu$ M hypericin are similar (see also Figures 15(c) and 15(d)).

GABEB cells generate PpIX from 0.5 mM ALA in a linear correlation with the incubation times, with a delay of 2 h. Under proinflammatory conditions, the fluorescence between 5–7 h is significantly increased ( $P \leq 0.05$ , Figure 5(a)), in contrast to 1 mM ALA, which induces about equal PpIX fluorescence in a linear relation with a delay of 1 h (Figure 5(b)). Fluorescence intensity increases with ALA concentration and is about double without TNF-alpha induction.

Hypericin at a concentration of 3  $\mu$ M is taken up by GABEB cells in a linear curve course, equal under proinflammatory and normal conditions (Figure 6(a)). While the



(a)



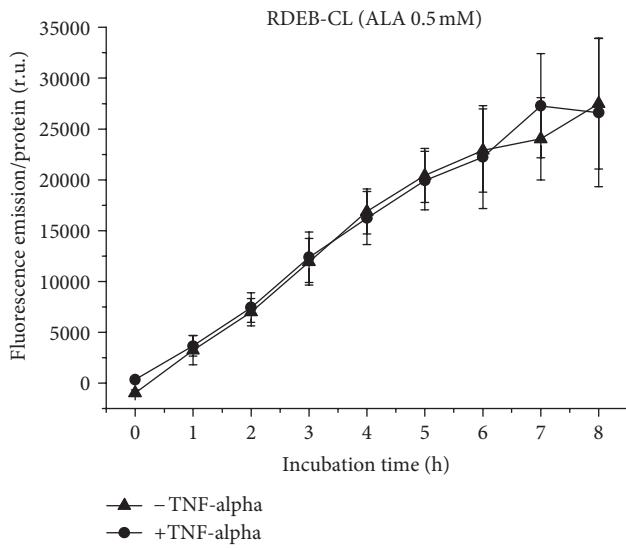
(b)

FIGURE 2: Hypericin uptake to SCCRDEB4 cells over 8 h, with and without TNF-alpha induction. (a) Application of 3  $\mu$ M, (b) of 5  $\mu$ M hypericin.

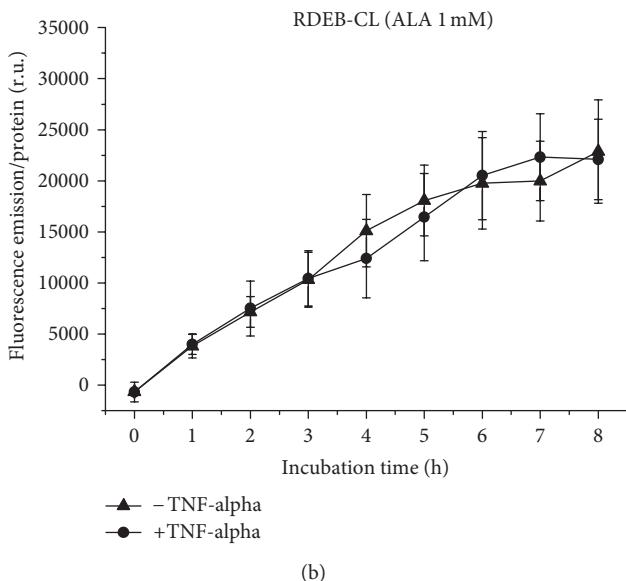
uptake of 5  $\mu$ M hypericin occurs also more or less equally under normal and proinflammatory conditions, the curve shape shows here a plateau (Figure 6(b)). Under both conditions, fluorescence increases with hypericin concentration.

EBS-MD cells generate PpIX from 0.5 mM ALA with linear kinetics up to 8 h (Figure 7(a)). The course is equal under proinflammatory and normal conditions, for 0.5 mM, and more or less also for 1 mM ALA (Figure 7(b)). However, PpIX formation after application of 1 mM ALA leads to a curve shape with a plateau. Noteworthy is the fact that the fluorescence decreases with increasing ALA concentration.

EBS-MD cells take up hypericin at a concentration of 3  $\mu$ M in a moderately flattening curve course, equal under proinflammatory and normal conditions (Figure 8(a)). While



(a)

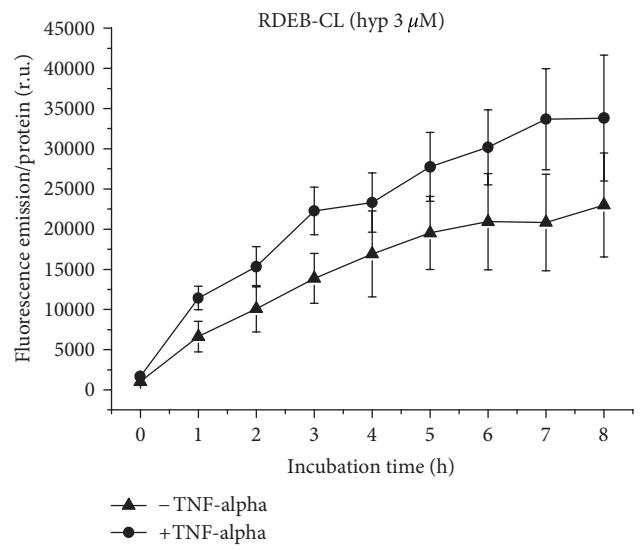


(b)

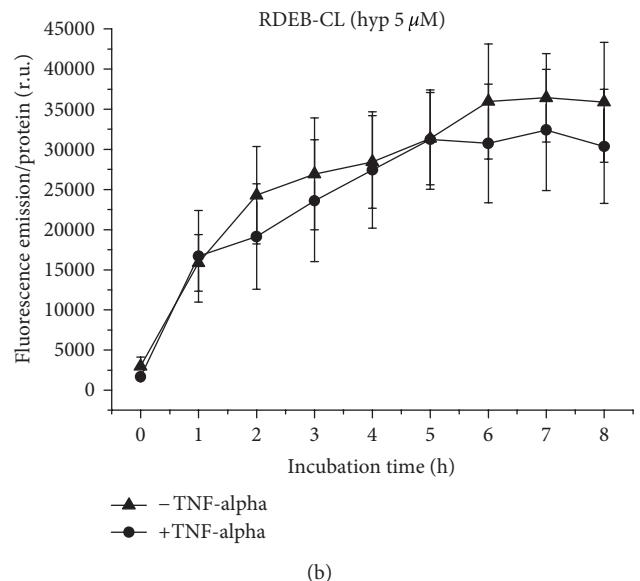
FIGURE 3: PpIX formation in RDEB-CL cells over 8 h, with and without TNF-alpha induction. (a) Application of 0.5 mM (b) of 1 mM ALA.

the uptake of 5  $\mu$ M hypericin occurs also more or less equally under normal and proinflammatory conditions, the curve shape shows a distinct plateau and a very rapid increase already within the first two hours (Figure 8(b)). Under both conditions, fluorescence increases with hypericin concentration.

PpIX is generated by A431 cells from 0.5 mM ALA with linear kinetics up to 8 h under noninflammatory conditions (Figure 9(a)). Under proinflammatory conditions, the final fluorescence intensity is significantly ( $P \leq 0.05$ ) reduced, and the curve reaches a plateau. PpIX formation after application of 1 mM ALA shows a linear curve under proinflammatory conditions, which is flattened under normal conditions with lower fluorescence endpoints. A significant difference can be



(a)

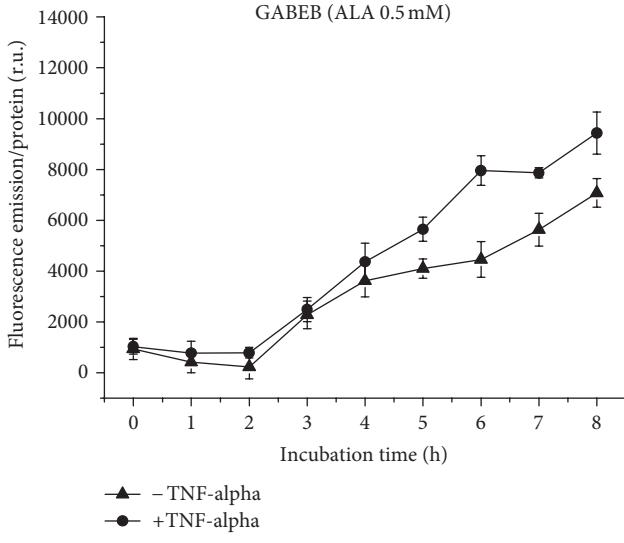


(b)

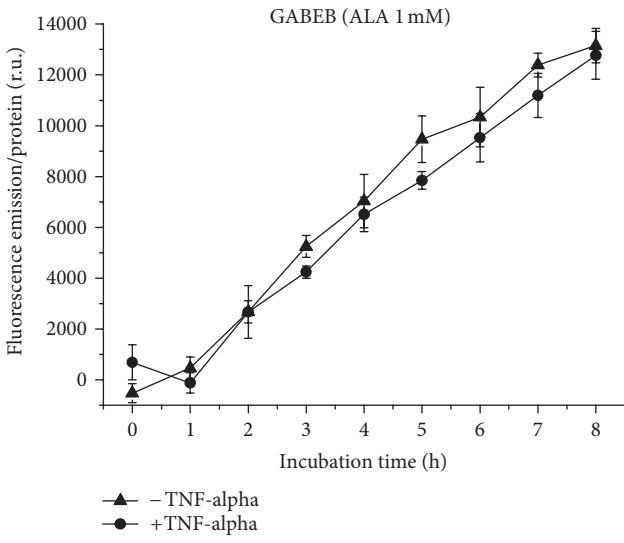
FIGURE 4: Hypericin uptake to RDEB-CL cells over 8 h, with and without TNF-alpha induction. (a) Application of 3  $\mu$ M, (b) of 5  $\mu$ M hypericin.

found for 2 and 3 h ( $P \leq 0.05$ ) (Figure 9(b)). Fluorescence increases with increasing ALA concentration.

A431 cells take up hypericin at a concentration of 3  $\mu$ M in a moderately flattening curve course, equal under proinflammatory and normal conditions until 5 h. Between 5 and 8 h, the fluorescence increase is reduced under proinflammatory conditions with statistical significance at 7 h ( $P \leq 0.05$ ) (Figure 10(a)). Uptake of 5  $\mu$ M hypericin shows a similar curve course under proinflammatory and normal conditions, which is linear until 7 h. After 7 h, a reduced fluorescence increase after TNF-alpha application leads to a moderate difference in both conditions (Figure 10(b)). However, under both conditions, fluorescence increases more than doubles with increasing hypericin concentration.



(a)

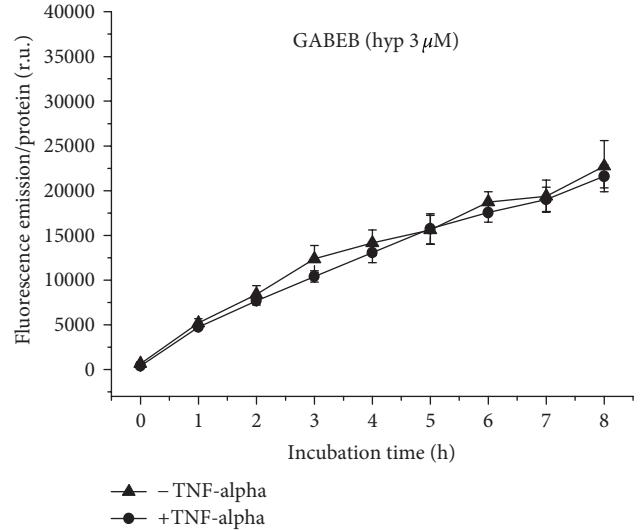


(b)

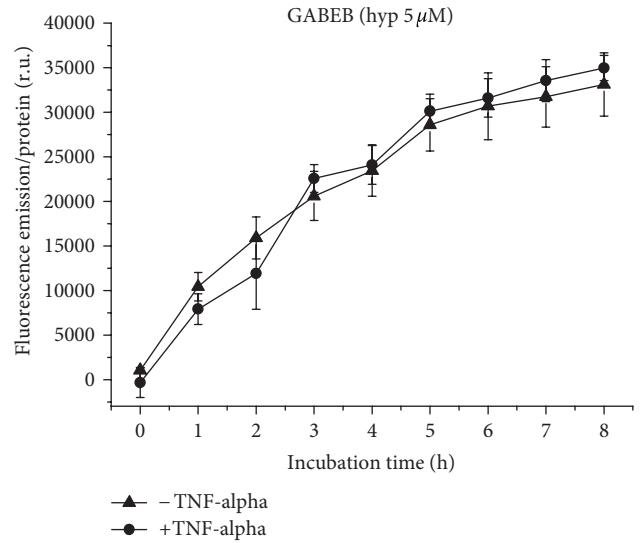
FIGURE 5: PpIX formation in GABEB cells over 8 h, with and without TNF-alpha induction. (a) Application of 0.5 mM (b) of 1 mM ALA.

HaCat cells generate PpIX from 0.5 (Figure 11(a)) and 1 mM ALA (Figure 11(b)) equally under normal and proinflammatory conditions with more or less linear kinetics. The only difference is that the fluorescence of PpIX induced by 1 mM ALA shows no further increase from 7 to 8 h. The fluorescence intensity shows almost double the amount with the higher concentration.

Hypericin at a concentration of 3  $\mu$ M is taken up by HaCat cells in a moderately flattening curve shape, equal under proinflammatory and normal conditions (Figure 12(a)). While the uptake of 5  $\mu$ M hypericin occurs also more or less equally under normal and proinflammatory conditions, the curve shows a distinct plateau (Figure 12(b)) as well as a steep increase within the first hour. Under



(a)



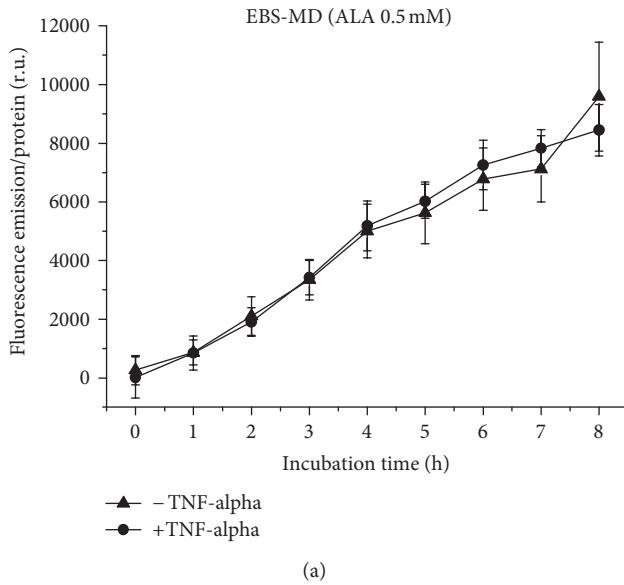
(b)

FIGURE 6: Hypericin uptake to GABEB cells over 8 h, with and without TNF-alpha induction. (a) Application of 3  $\mu$ M, (b) of 5  $\mu$ M hypericin.

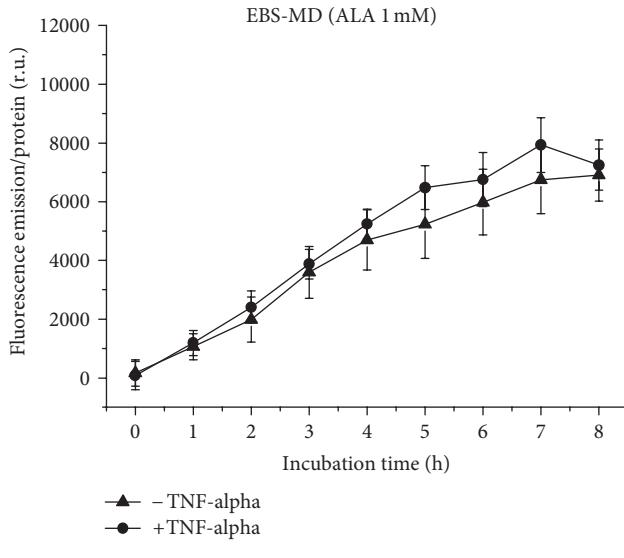
both conditions, fluorescence increases with hypericin concentration.

PpIX formation in Skin cells is about linear after incubation with 0.5 mM ALA (Figure 13(a)) and almost linear after incubation with 1 mM ALA (Figure 13(b)). Differences in fluorescence between inflammatory and normal conditions are not significant. Fluorescence decreases with increasing ALA concentration.

Skin cells take up hypericin at concentrations of 3  $\mu$ M (Figure 14(a)) and 5  $\mu$ M (Figure 14(b)) in curve courses with plateaus under proinflammatory as well as normal conditions. Under proinflammatory conditions, the fluorescence of hypericin, 3 or 5  $\mu$ M, respectively, is lowered, highly significant for 6 and 7 h incubation with 3  $\mu$ M hypericin



(a)



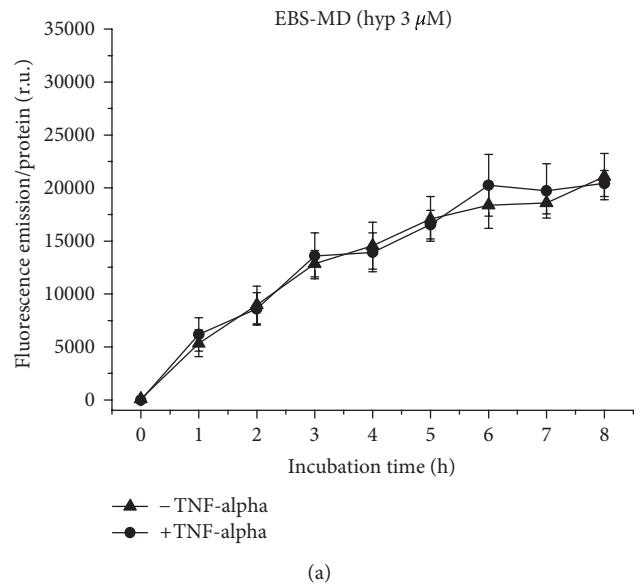
(b)

FIGURE 7: PpIX formation in EBS-MD cells over 8 h, with and without TNF-alpha induction. (a) Application of 0.5 mM (b) of 1 mM ALA.

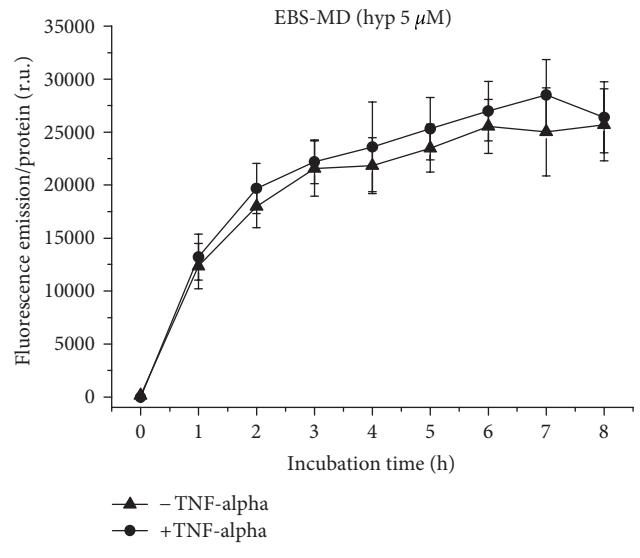
( $P \leq 0.01$ ), and significant for the final value at 8 h ( $P \leq 0.05$ ) for 5  $\mu$ M hypericin. Under both conditions, fluorescence increases with hypericin concentration (see also Figures 15(c) and 15(d)).

#### 4. Discussion

The aim of this study was the analysis of fluorescence kinetics of photosensitizers in EB cell lines representing the three types of EB, to assess the suitability of FD for squamous cell carcinoma of EB patients. Since proinflammatory conditions due to the chronic wounds [10] are often present in EB-patients, and excessive accumulation of photosensitizers in



(a)



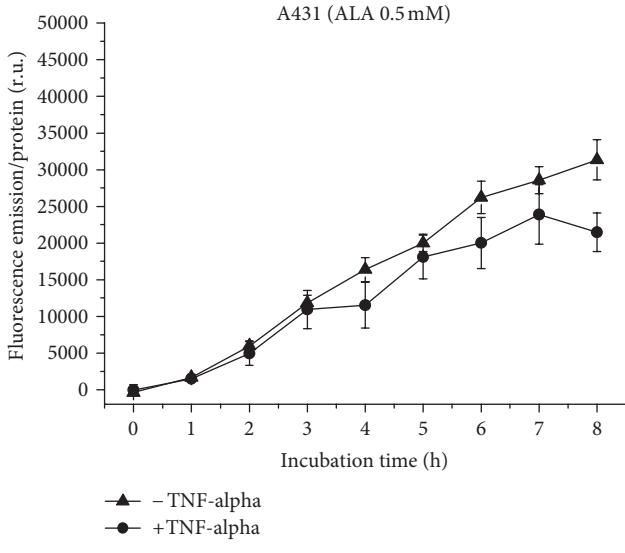
(b)

FIGURE 8: Hypericin uptake to EBS-MD cells over 8 h, with and without TNF-alpha induction. (a) Application of 3  $\mu$ M, (b) of 5  $\mu$ M hypericin.

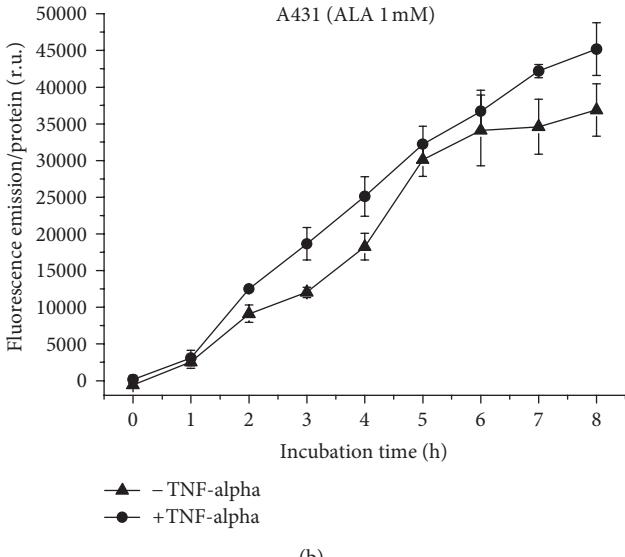
inflamed areas had been occasionally observed in the clinical situation, the fluorescence kinetics were also measured in all cell lines after TNF-alpha induction.

For this purpose, the photosensitizer hypericin and the precursor ALA were externally applied; the latter one to induce the endogenous generation of the photosensitizer PpIX in the cells.

All cell lines produce PpIX and take up hypericin, as demonstrated by the fluorescence measurements, but with different accumulation kinetics. Since all cell lines represent fibroblasts or keratinocytes, this is in line with other studies [35–37].



(a)



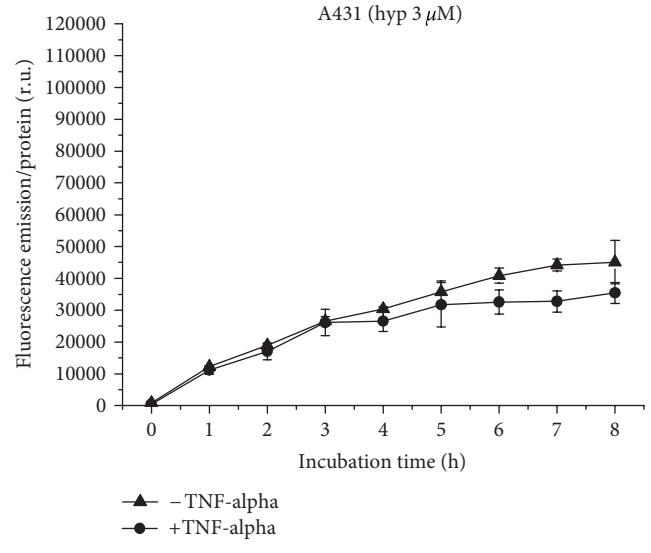
(b)

FIGURE 9: PpIX formation in A431 cells over 8 h, with and without TNF-alpha induction. (a) Application of 0.5 mM (b) of 1 mM ALA.

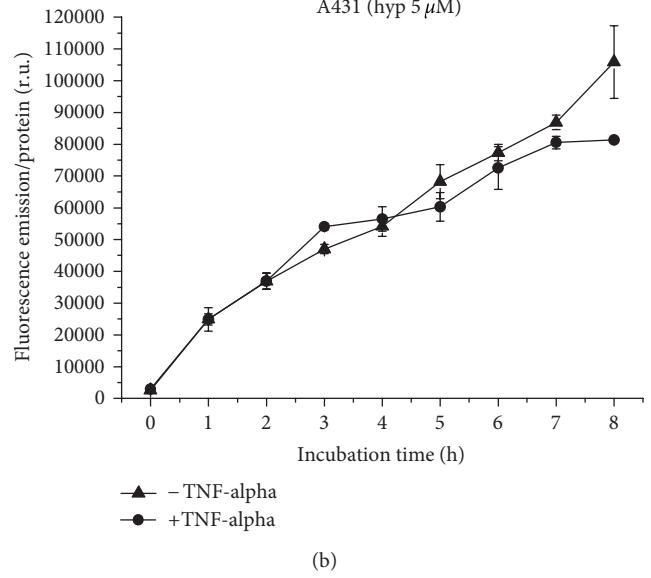
Based either on the fluorescence kinetic curves or their endpoints at 8 h (Figures 15(a)–15(d)), the following comparisons can be made with special emphasis on the applicability of FD for SCC of EB-patients.

**4.1. Curve Shape and Specific Accumulation of Each Photosensitizer in All Cell Lines.** A moderate-curve flattening until to a distinct plateau is found in all cell lines after 5  $\mu$ M hypericin incubation and in most cases also after 3  $\mu$ M hypericin. In cell lines where it is found after ALA incubation, it is limited to 1 mM.

Curves with a plateau normally indicate saturation with the photosensitizer but could in some cases be based on



(a)

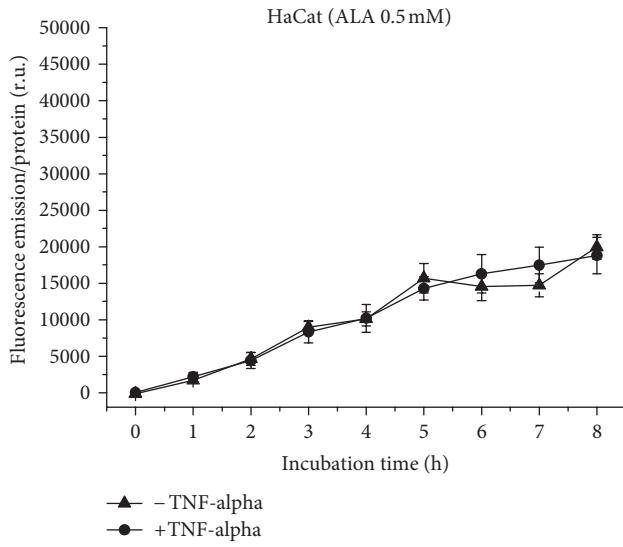


(b)

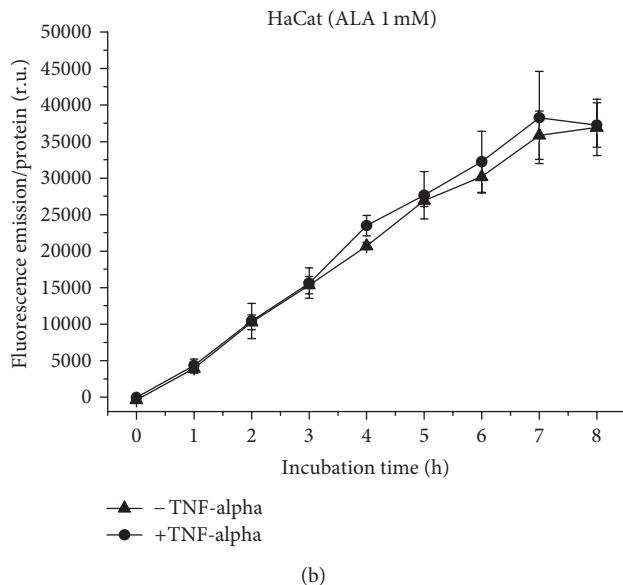
FIGURE 10: Hypericin uptake to A431 cells over 8 h, with and without TNF-alpha induction. (a) Application of 3  $\mu$ M, (b) of 5  $\mu$ M hypericin.

aggregation of the molecules, which quenches the fluorescence [38]. Especially the application of 5  $\mu$ M hypericin seems to be at the upper limit, and incubation with a higher concentration cannot be recommended for the used cell lines.

A retarded fluorescence increase was observed after ALA incubation in GABEB cells and a rapid initial increase after 5  $\mu$ M hypericin incubation of EBS-MD, HaCat, and Skin cells. The latter phenomenon points to a rapid initial uptake of hypericin mainly to fibroblast cell lines, which are known to carry out increased receptor-mediated endocytosis of low-density lipoprotein (LDL) in contrast to keratinocytes [39]. Lipophilic photosensitizers such as hypericin bind reportedly to LDL [40, 41]. However, uptake kinetics soon reach a distinct plateau in most cell lines, including Skin fibroblasts.



(a)

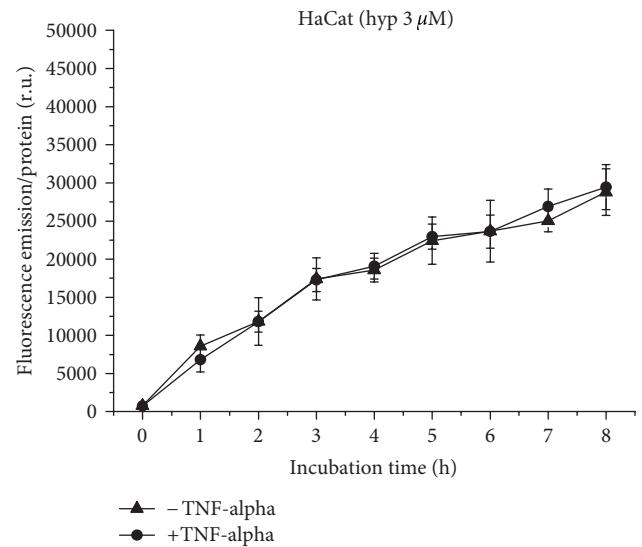


(b)

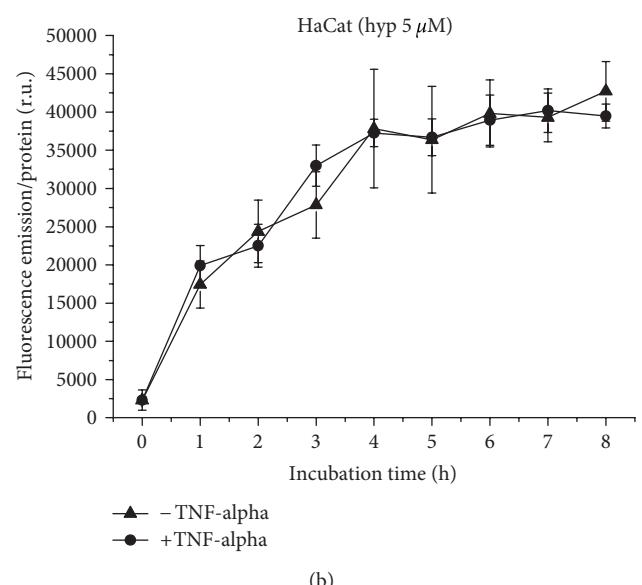
FIGURE 11: PpIX formation in HaCat cells over 8 h, with and without TNF-alpha induction. (a) Application of 0.5 mM (b) of 1 mM ALA.

**4.2. Effect of Concentration on the Photosensitizer Accumulation.** In most cell lines, higher photosensitizer or precursor concentrations induce higher or—in the case of saturation—at least equal fluorescence (Figures 15(a)–15(d)). The observed seeming decrease in a few cell lines is not statistically significant.

**4.3. Tumor Selectivity of EB Cell Lines (SCCRDEB4 versus RDEB-CL).** SCCRDEB4 cells show lower fluorescence levels than RDEB-CL cells, independent of TNF-alpha induction. This negative selectivity is the highest (more than double) after 0.5 mM ALA incubation, about double after hypericin treatment, with both concentrations, and reduced after 1 mM ALA (Figures 15(a)–15(d)). However, a statistically significant fluorescence reduction ( $P \leq 0.05$ ) was only detected



(a)



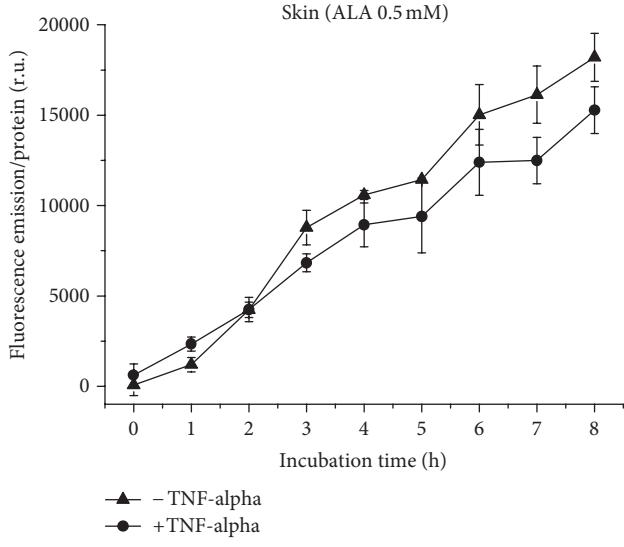
(b)

FIGURE 12: Hypericin uptake to HaCat cells over 8 h, with and without TNF-alpha induction. (a) Application of 3  $\mu$ M, (b) of 5  $\mu$ M hypericin.

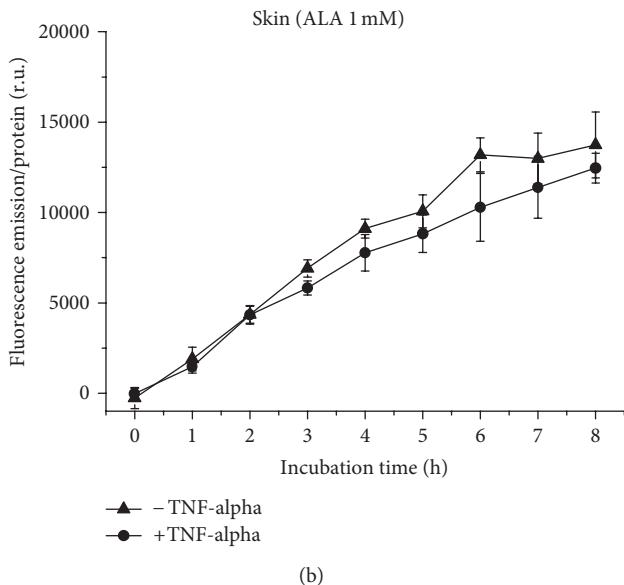
with 5  $\mu$ M hypericin. In spite of its negative *in vitro* selectivity, hypericin tumor selectivity *in vivo* might well be existent [42].

RDEB-CL cell fluorescence is equal to or higher than that of the other EB cell lines.

**4.4. EB-Specific Photosensitizer Accumulation in Malignant Cells (SCCRDEB4 versus A431).** The comparison of squamous cell carcinoma cells of an RDEB patient with those of a non-EB patient shows a 2–3 times higher fluorescence of A431 cells after PpIX formation and after 3  $\mu$ M hypericin accumulation, and an up to 8 times higher fluorescence after 5  $\mu$ M hypericin, for both pretreatment conditions (with and without TNF-alpha induction) (Figures 15(a)–15(d)).



(a)

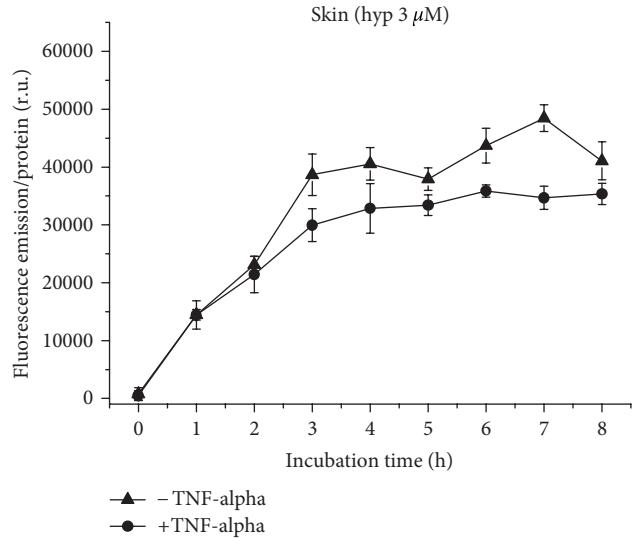


(b)

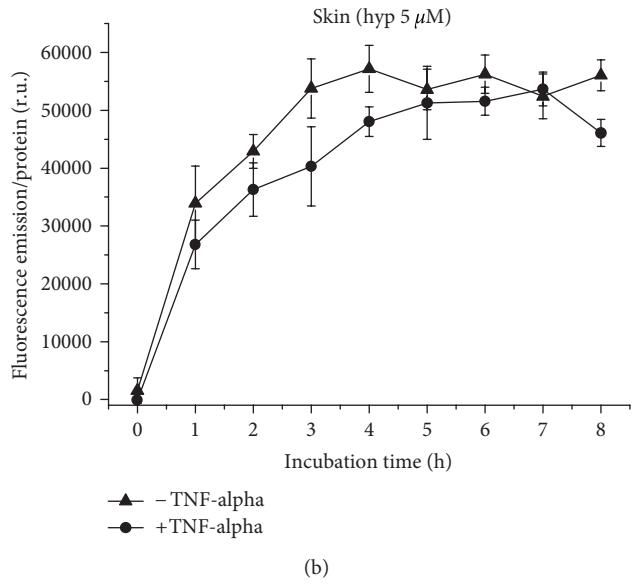
FIGURE 13: PpIX formation in Skin cells over 8 h, with and without TNF-alpha induction. (a) Application of 0.5 mM (b) of 1 mM ALA.

All differences are highly significant ( $P \leq 0.01$ ) with the only exception of a  $P \leq 0.05$  significance for 0.5 mM ALA in the proinflammatory state. The EB background of the malignant cells leads to drastically reduced fluorescence by decreased PpIX formation or hypericin uptake. Due to constant deficiency compensation processes of the recessive dystrophic EB cell line [43], other physiological activities could be limited.

**4.5. Tumor Selectivity of Non-EB Cell Lines (A431 versus HaCat and Skin Cells).** The fluorescence of PpIX is significantly higher in A431 cells than in HaCat and Skin cells under noninflammatory conditions when incubated with 0.5 mM ALA (both:  $P \leq 0.01$ ) and significantly higher than in Skin cells under both conditions when incubated with 1 mM ALA



(a)



(b)

FIGURE 14: Hypericin uptake to Skin cells over 8 h, with and without TNF-alpha induction. (a) Application of 3  $\mu$ M, (b) of 5  $\mu$ M hypericin.

(both:  $P \leq 0.01$ ). All three cell lines take up 3  $\mu$ M hypericin in a similar rate. When the cells are incubated with 5  $\mu$ M hypericin, the dye is accumulated to a much higher degree in A431 cells than in HaCat and Skin cells (highly significant with  $P \leq 0.01$ ), independent of TNF-alpha pre-treatment, (Figures 15(a)–15(d)). Except for 3  $\mu$ M hypericin and restricted for 1 mM ALA, the protocols are suitable to generate tumor selectivity in non-EB cell lines, at least under noninflammatory conditions.

**4.6. EB-Specific Photosensitizer Accumulation in Non-Malignant Cells (GABEB versus HaCat and EBS-MD versus Skin).** Cells of junctional EB patients (GABEB), compared to keratinocytes (HaCat) as well as cells of an EB

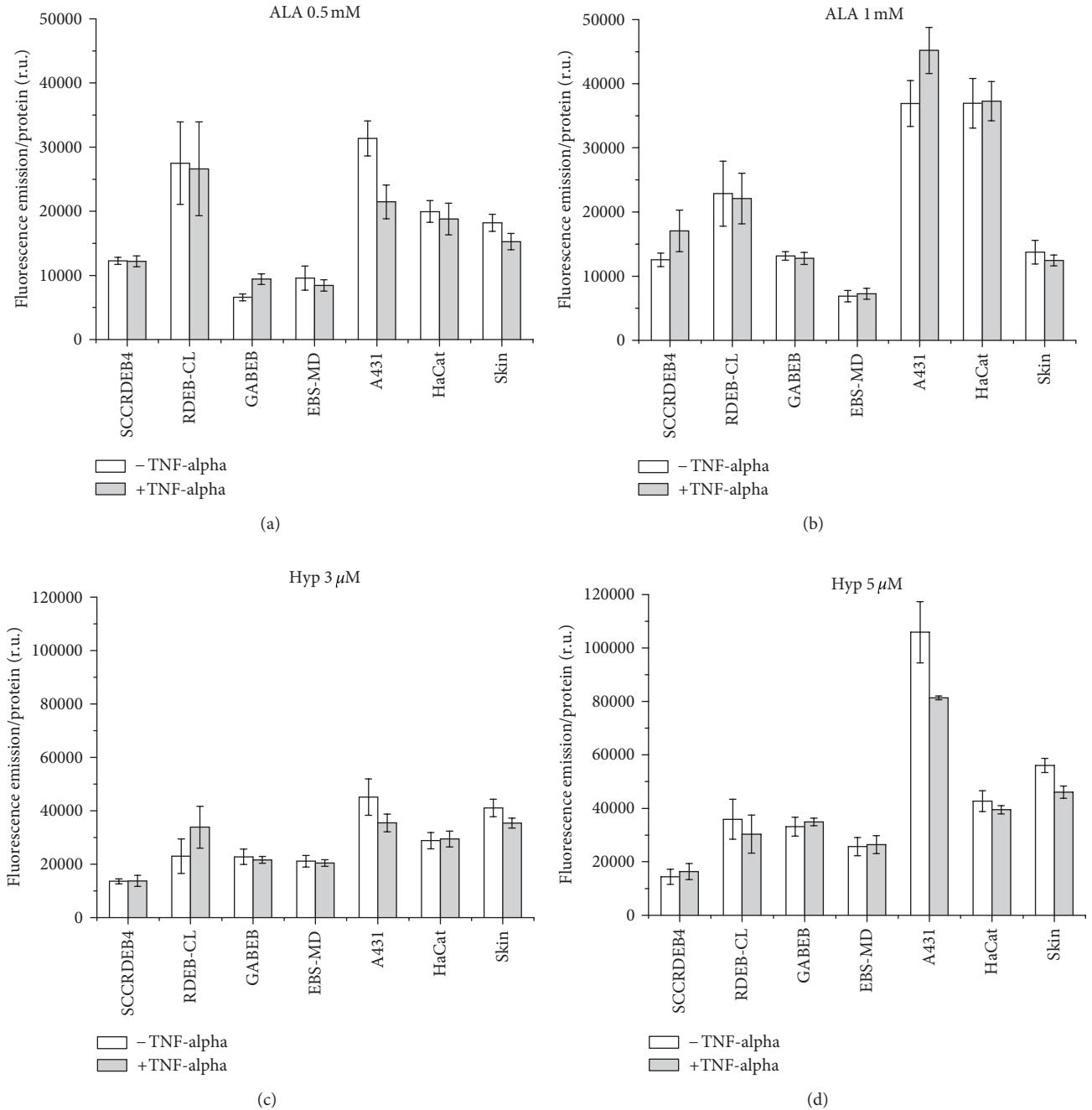


FIGURE 15: Relative photosensitizer fluorescence intensity in all cell lines at 8 h incubation time, with and without TNF-alpha. (a) Application of 0.5 mM ALA, (b) 1 mM ALA, (c) 3  $\mu$ M hypericin, and (d) 5  $\mu$ M hypericin.

simplex patient compared to primary skin fibroblasts as their respective non-EB reference cell lines, show similar properties in the differential accumulation of photosensitizers (Figures 15(a)–15(d)): PpIX formation is significantly reduced in GABEB and EBS-MD cells for both conditions (all:  $P \leq 0.01$ , except for GABEB, 0.5 mM ALA and TNF-alpha, and EBS-MD, 1 mM ALA without TNF-alpha: both:  $P \leq 0.05$ ). Hypericin uptake is also significantly reduced in GABEB cells when applied at a concentration of

3  $\mu$ M after TNF-alpha induction ( $P \leq 0.05$ ) and in EBS-MD fibroblasts under all treatments (all:  $P \leq 0.01$ ).

This confirms the evaluation made before concerning the comparison of the malignant cell lines (SCCRDEB4 versus A431 cells): EB cell lines seem to be physiologically less active resulting in reduced photosensitizer uptake or formation. Besides that, the underexpression of the low-density-lipoprotein receptor expression in the GABEB, EBS-MD and RDEB-CL cell lines, which was described by Knaup et al. [44],

could also play a role—at least with regard to the decreased hypericin uptake.

**4.7. Effect of TNF-Alpha-Induced Proinflammatory State on Photosensitizer Accumulation.** In general, the proinflammatory state shows no major influence on photosensitizer accumulation as only a few nonsignificant differences to the noninflammatory state were found. Significantly increased fluorescence after TNF-alpha induction is restricted to the presence of PpIX in GABEB cells (5–7 h with 0.5 mM ALA) and A431 cells (2–3 h with 1 mM ALA). Reduced fluorescence was measured in A431 cells (incubated with 0.5 mM ALA and 3 μM hypericin) and in Skin cells after hypericin application.

Concomitant measurements for checking the proinflammatory state of the cells showed that GABEB cells generate high levels of IL-6 and Skin cells even double the amount whereas A431 and HaCat cells present a low IL-6 level. Based on these data, it is hardly possible to correlate an influence of TNF-alpha induction on the fluorescence of the photosensitizers in the studied cell lines with the amount of released IL-6.

However, with our data, we support the hypothesis that the increased fluorescence found in inflamed tissue during FD of tumors is not due to higher accumulation of photosensitizers in nonimmune cells with a proinflammatory status but might rather be due to additional photosensitizer accumulation in the extracellular matrix and/or in infiltrating immune cells such as neutrophils, mast cells, monocytes, and macrophages [45, 46].

## 5. Conclusions

Following conclusions can be drawn from the results above.

- (1) All cell lines take up hypericin or generate PpIX mostly increasing with the incubation time, but with different kinetics.
- (2) SCCRDEB4 cells take up less hypericin and generate less PpIX than the nonmalignant RDEB-CL cells. This is in contrast to the non-EB cell lines, which show tumor selectivity.

From the here found *in vitro* results, we cannot conclude whether fluorescence diagnosis of SCC in EB patients will be feasible. Even though the applied photosensitizers exhibit no tumor selectivity *in vitro*, their tumor selectivity *in vivo* might well be existent.

- (3) EB cell lines are less active than non-EB cell lines concerning uptake of hypericin or formation of PpIX.
- (4) Since uptake of hypericin or formation of PpIX is hardly modified under proinflammatory conditions, the proinflammatory state of the cells seems to have no influence on the fluorescence detection of the photosensitizers. Therefore, the higher fluorescence of inflamed areas in tissue might rather be due to photosensitizer accumulation in infiltrating immune cells.

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## Research Article

# Size-Dependent Photodynamic Activity of Gold Nanoparticles Conjugate of Water Soluble Purpurin-18-N-Methyl-D-Glucamine

Byambajav Lkhagvadulam, Jung Hwa Kim, Il Yoon, and Young Key Shim

PDT Research Institute, School of Nano System Engineering, Inje University, Gimhae 621-749, Republic of Korea

Correspondence should be addressed to Il Yoon, [yoonil71@inje.ac.kr](mailto:yoonil71@inje.ac.kr) and Young Key Shim, [ykshim@inje.ac.kr](mailto:ykshim@inje.ac.kr)

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Gold nanoparticles (GNPs) conjugates of water soluble ionic photosensitizer (PS), purpurin-18-N-methyl-D-glucamine (Pu-18-NMGA), were synthesized using various molar ratios between HAuCl<sub>4</sub> and Pu-18-NMGA without adding any particular reducing agents and surfactants. The PS-GNPs conjugates showed long wavelength absorption of range 702–762 nm, and their different shapes and diameters depend on the molar ratios used in the synthesis. *In vitro* anticancer efficacy of the PS-GNPs conjugates was investigated by MTT assay against A549 cells, resulting in higher photodynamic activity than that of the free Pu-18-NMGA. Among the PS-GNPs conjugates, the GNPs conjugate from the molar ratio of 1:2 (Au(III): Pu-18-NMGA) exhibits the highest photodynamic activity corresponding to bigger size (~60 nm) of the GNPs conjugate which could efficiently transport the PS into the cells than that of smaller size of the GNPs conjugate.

## 1. Introduction

Photodynamic therapy (PDT) is a promising noninvasive cancer treatment by using a combination of photosensitizer (PS), light, and oxygen [1–3]. For excellent photodynamic activity, PS should be penetrated into the tumor cells sufficiently [4]. Most of PSs are hydrophobic and thus locate preferentially in the lipid bilayers of organelle membranes in cancer cells. However, the hydrophobic nature of PSs makes them insoluble under physiological conditions and hinders to reach the accumulation in the tumor sites [5]. Therefore, for both hydrophobic and hydrophilic (amphiphilic) environments of PSs, introduction of water soluble PS with suitable carrier is a one potential method [6–8]. On the other hand, highly water soluble (hydrophilic) PSs allow poor cellular uptake based on a short pharmacological half-life which may have limit to penetrate through the tissue and cell membranes [9–11].

Nanoparticles (NPs) [12–14] are promising carrier system of PSs that could be protected from being taken up by the reticuloendothelial system and extended the circulation time of NPs in the blood, and, finally, preferentially

accumulated in tumor sites through the so-called “enhanced permeability and retention (EPR)” effect [15–17]. Among the NPs, gold nanoparticles (GNPs) are highly efficient PDT drug delivery platform with good advantages based on their chemical inertness and minimum toxicity that has potential applications in biomedicine such as photothermal therapy (PTT) [18–21] of cancer, gene and drug delivery, biological imaging, and biosensing [22–26]. In addition, GNPs have large surface-to-volume ratios and easy tuning of the NPs size, resulting in penetration into tumor cells and intracellular localization at endosomes/lysosomes of the cells, and finally targeting at mitochondrial of cancer cells induces apoptosis to destroy the cancer cells [27–31]. It is noted that the size of the GNPs plays a big role in their uptake at the cellular level leading to different PDT activity. However, to the best of our knowledge, there are few reports for relationship between GNPs and its size effect on photodynamic activity [28].

Previously, we developed new synthesis of PS-GNPs conjugate using water soluble ionic purpurin-18-N-methyl-D-glucamine (Pu-18-NMGA, PS1, Figure 1) and this conjugate showed better *in vitro* anticancer efficacy than that of free PS1 against A549 lung cancer cells [32].

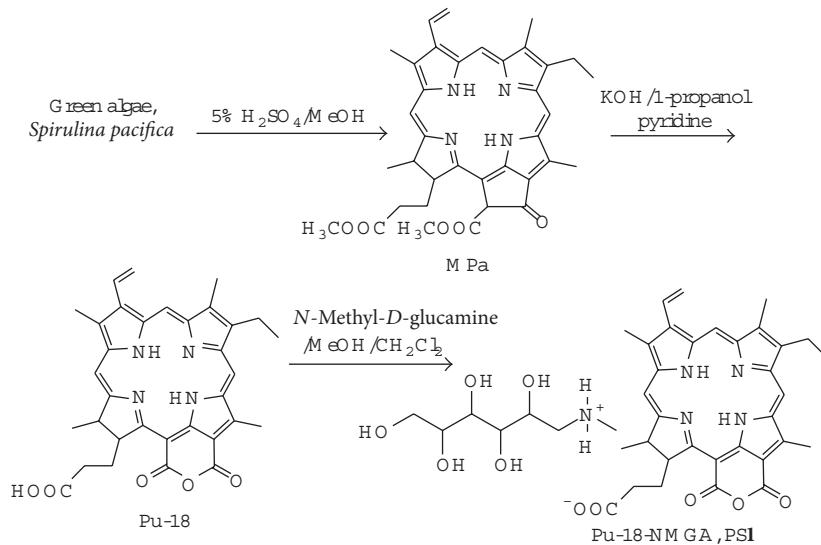


FIGURE 1: Synthetic method of *N*-methyl-*D*-glucamine salt of purpurin-18 (Pu-18-NMGA, PS1).

In this paper, we have synthesized various sizes of PS-GNPs conjugates using a simple single-step synthesis from different molar ratios of HAuCl<sub>4</sub>/PS1 without adding any particular reducing agents and surfactants, and showed size effect allowed different photodynamic activity results of the conjugates as an important factor for PDT. We evaluated *in vitro* anticancer efficacy of the PS-GNPs conjugates against A549 cells using MTT assay.

## 2. Materials and Methods

**2.1. Materials.** All reagents were purchased from Aldrich and used without further purification. All aqueous solutions were made using triply distilled water. All reactions were monitored by thin-layer chromatography (TLC) using Merck 60 silica gel F254 precoated (0.2 mm thickness) glass-backed sheets. Silica gel 60A (230–400 mesh, Merck) was used for column chromatography. The <sup>1</sup>H NMR spectra were obtained using a Varian spectrometer (500 MHz) at Biohealth Products Research Center (BPRC) at Inje University. The chemical shifts ( $\delta$ ) are given in parts per million (ppm) relative to tetramethylsilane (TMS, 0 ppm). High-resolution fast atom bombardment mass (HRFABMS) spectra were obtained with a Jeol JMS700 high-resolution mass spectrometer at the Daegu center of KBSI, Kyungpook National University, Korea.

The PS1 and PS-GNPs conjugates **2a–2e** were characterized by a combination analysis of <sup>1</sup>H-NMR and UV-vis spectroscopies, transmission electron microscopy (TEM), and infrared (IR) spectroscopy. UV-vis absorption spectra were recorded using a SCINCO S-3100 UV-vis spectrophotometer using 1 cm quartz cuvette. TEM images were performed on a JEOL, JEM 2011. A typical sample for TEM was prepared by drying of a drop of the solution at room temperature on a carbon-coated copper grid. IR spectra were measured on a Varian-640 FT-IR spectrometer.

**2.2. Synthesis.** Methyl pheophorbide-a (MPa) [33], purpurin-18 (Pu-18) [34], and *N*-methyl-*D*-glucamine salt of purpurin-18 (Pu-18-NMGA, PS1) [32] were prepared according to the procedures in literature, and all analytical data are identical with those in the literatures.

***N*-Methyl-*D*-Glucamine Salt of Purpurin-18 (Pu-18-NMGA, PS1)** [32]. To a solution of Pu-18 (56.4 mg, 0.1 mmol) in MeOH/CH<sub>2</sub>Cl<sub>2</sub> (3 : 1, 10 mL), a solution of NMGA (39.0 mg, 0.2 mmol) in MeOH/water (1 : 2, 20 mL) was added and the mixture was stirred for 4 h. The organic solvents were evaporated under vacuum and the resulting aqueous solution was filtered through a membrane (20  $\mu$ m) and freeze-dried to give PS1. Yield: 68.0 mg (87%). UV-vis (water):  $\lambda$ , nm (log  $\epsilon$ ) 282 (0.34), 379 (0.55), 388 (0.58), 405 (0.61), 501 (0.13), 561 (0.11), 652 (0.28), 702 (0.21). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD, 25°C, TMS)  $\delta$ , ppm 9.68 (1H, s, H-5), 9.39 (1H, s, H-10), 8.95 (1H, s, H-20), 8.69 (2H, s, H-NH gluc), 7.80 (1H, m, H-3<sup>1</sup>), 6.24 and 6.12 (2H, dd, H-3<sup>2</sup>), 5.07 (1H, m, H-18), 4.49 (1H, m, H-17), 4.05 (2H, m, H-1 gluc), 3.83 (4H, m, OH-2,3,4,5 gluc), 3.69 (4H, m, H-2,3,4,5 gluc), 3.63 (3H, s, H-12), 3.48 (2H, m, H-8<sup>1</sup>), 3.21 (3H, s, H-2<sup>1</sup>), 3.18 (1H, m, OH-1 gluc), 3.07 (2H, dd, H-6 gluc), 3.03 (3H, s, H-7<sup>1</sup>), 2.74 (3H, s, H-7 gluc), 2.46 (2H, m, H-17<sup>1</sup>), 2.26 (3H, m, H-17<sup>2</sup>), 1.95 (3H, m, H-18<sup>1</sup>), 1.83 (3H, d, H-8<sup>2</sup>), 1.69 (1H, br s, NH), 1.32 (1H, br s, NH). HRFABMS: calcd for C<sub>40</sub>H<sub>50</sub>N<sub>5</sub>O<sub>10</sub> ([M + H]<sup>+</sup>) 760.3558, found 760.3554.

**Synthesis of PS-GNPs Conjugates **2a–2e**.** The GNPs were synthesized according to the seed growth method [25] with some modifications. PS-GNPs conjugates **2a–2e** were synthesized from different molar ratios between Au(III) and PS1 through reduction of chloroauric acid (HAuCl<sub>4</sub>) with no use of reducing agent or surfactant.

**Preparation of Seed Solution of PS-GNPs Conjugate.** 0.002 M solution of PS1 (5 mL) was mixed with 0.001 M HAuCl<sub>4</sub>

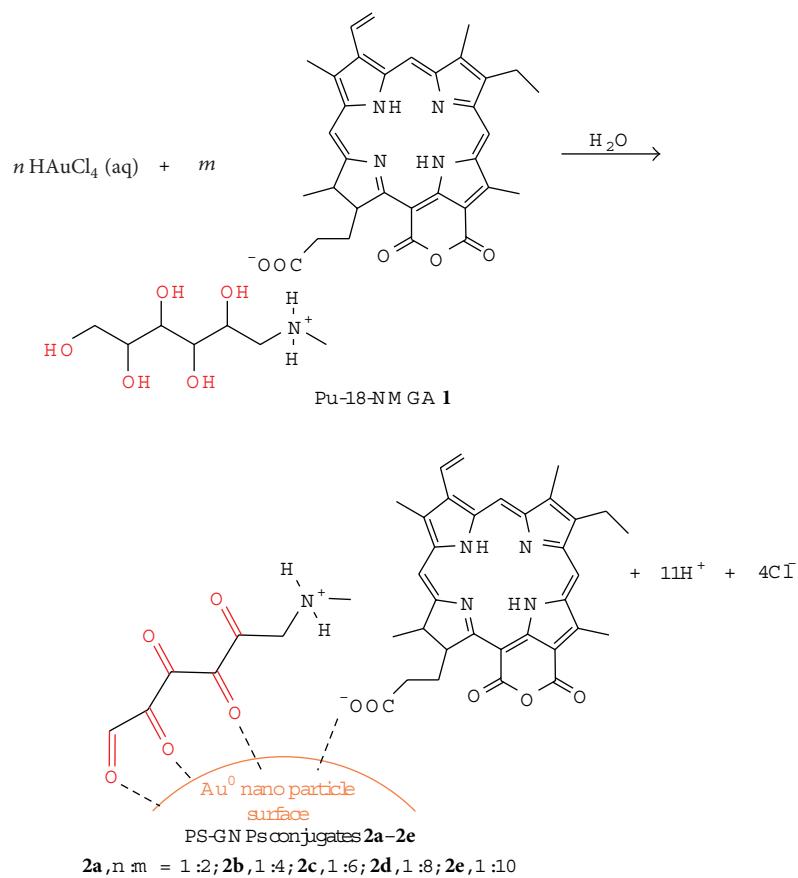


FIGURE 2: Synthetic method of PS-GNPs conjugates **2a–2e** with various molar ratios between gold and Pu-18-NMGA ( $n$  and  $m$  are molar ratios).

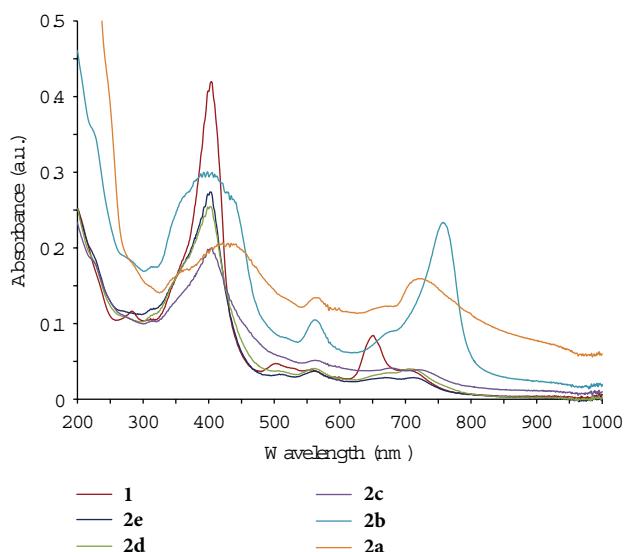


FIGURE 3: UV-vis spectra of (a) PS1 and PS-GNPs conjugates **2a–2e** with various molar ratios between gold and Pu-18-NMGA in water (**2a**, Au:Pu-18-NMGA = 1 : 2; **2b**, 1 : 4; **2c**, 1 : 6; **2d**, 1 : 8; **2e**, 1 : 10).

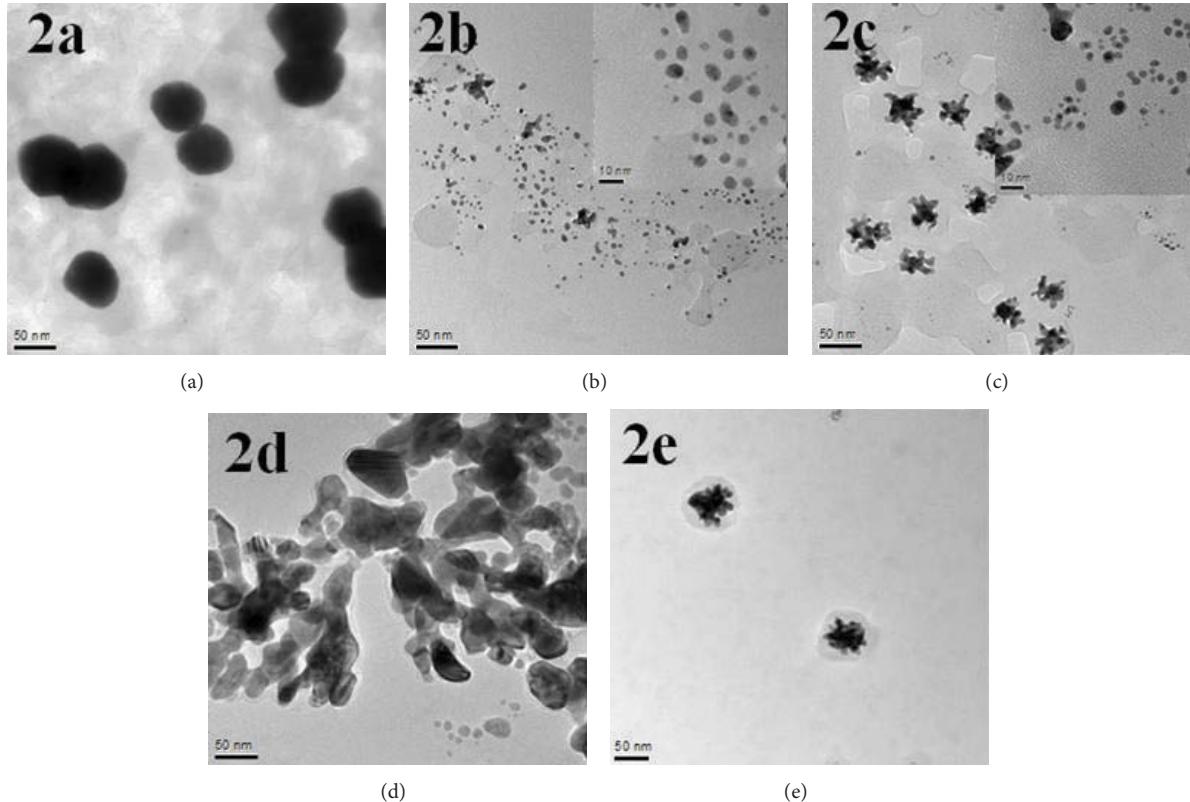


FIGURE 4: TEM images of the PS-GNPs conjugates **2a–2e**. The scale bars are 50 nm and the inset scale bars are 10 nm.

TABLE 1: Absorption properties of **PS1** and the PS-GNPs conjugates **2a–2e**.

Compound	Absorption $\lambda_{\text{max}}$ (nm) (log $\epsilon$ ) Soret	Qy
<b>1</b>	435 (0.64)	702 (0.67)
<b>2a</b>	440 (0.34)	724 (0.28)
<b>2b</b>	434 (0.69)	762 (0.77)
<b>2c</b>	435 (0.62)	719 (0.34)
<b>2d</b>	434 (0.69)	702 (0.50)
<b>2e</b>	434 (0.69)	702 (0.44)

(2.5 mL) in a 50 mL flat bottom flask and was stirred at room temperature for 2 h. The solution color was changed from yellow to greenish black, and then the solution was stored at room temperature.

**Growth of PS-GNPs Conjugate.** 0.001 M solution of HAuCl<sub>4</sub> (25 mL) is added to suitable concentration (for various molar ratios between Au(III) and **PS1**) of **PS1** solution (25 mL) in a 250 mL flat bottom flask (the color of solution was changed from yellow to green). Then 0.005 M AgNO<sub>3</sub> solution (1 mL) was added to the mixture. To this mixture, the seed solution (100  $\mu$ L) was added to the center of the solution. Then the flask was never moved, so that the seed started to grow in the growth solution. After few minutes, the each PS-GNPs conjugate was obtained and washed with water for several

times and was centrifuged at 10,000 rpm for 10 min and resuspended in water. Selected data for **2a**: UV-vis (water):  $\lambda$ , nm (log $\epsilon$ ) 275 (0.36), 373 (0.43), 388 (0.46), 393 (0.48), 524 (0.21), 560 (0.25), 672 (0.23), 714 (0.35). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD, 25°C, TMS)  $\delta$ , ppm 8.91 (2H, s, H-5 and H-1 gluca), 8.74 (1H, s, H-10), 8.64 (3H, s, H-20 and H-NH gluc), 7.82 (1H, m, H-3<sup>1</sup>), 6.21 and 6.12 (2H, dd, H-3<sup>2</sup>), 4.95 (1H, m, H-18), 4.41 (1H, m, H-17), 3.62 (3H, s, H-12<sup>1</sup>), 3.41 (2H, d, H-8<sup>1</sup>), 3.21 (3H, s, H-2<sup>1</sup>), 3.16 (2H, d, H-6 gluc), 2.82 (6H, s, H-7 gluc and 7<sup>1</sup>), 2.63 (2H, m, H-17<sup>1</sup>), 2.30 (2H, m, H-17<sup>2</sup>), 1.75 (3H, d, H-18<sup>1</sup>), 1.33 (3H, m, H-8<sup>1</sup>), 0.88 (1H, br s, NH), 0.08 (1H, br s, NH). ATR IR ( $\text{cm}^{-1}$ ): 3400 (w, stretching  $-\text{NH}_2^+$ ), 1760 (s, stretching C=O), 1525 (s), 1300–1100 (stretching, bending C=O).

**2.3. Cell Culture and Photo Irradiation.** A549 human lung carcinoma cell lines were obtained from the cell line bank at Seoul National University's cancer research center and were grown in medium RPMI-1640 (Sigma-Aldrich) with 10% fetal bovine serum, glutamine, penicillin, and streptomycin at 37°C in humidified atmosphere of 5% CO<sub>2</sub> in air. Phosphate-buffered saline (PBS) (Sigma-Aldrich), microscope (Olympus, CK40-32 PH), ELISA-reader (BioTek, SynergyHT), trypsin-EDTA, solution and incubator (37°C, 5% CO<sub>2</sub>) were used. The PDT was carried out using a diode laser generator apparatus (BioSpec LED, Russia) equipped with a halogen lamp, a bandpass filter (640–710 nm), and

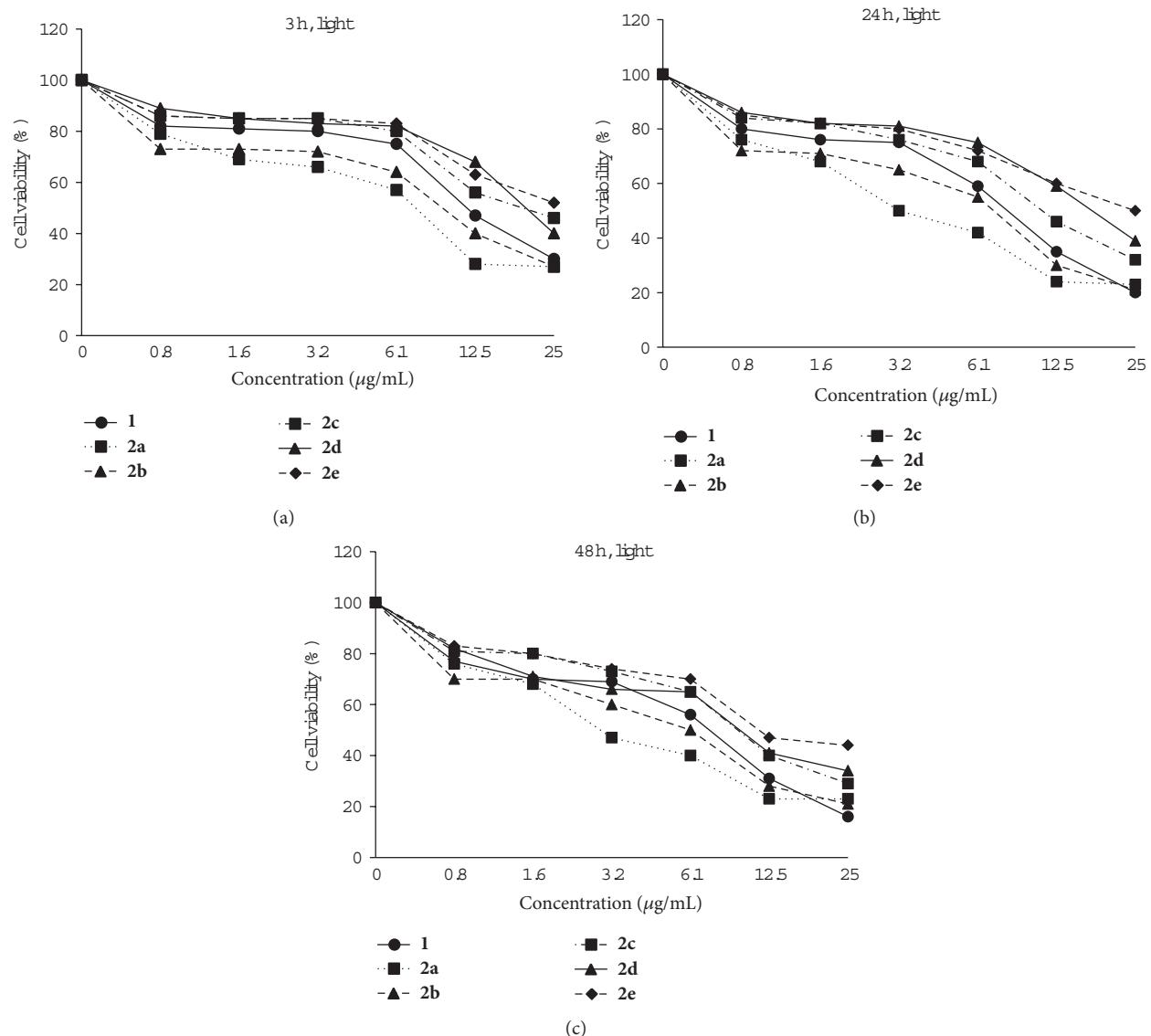


FIGURE 5: Cell viability (%) of PS1 ( $0\text{--}25 \mu\text{g/mL}$ ) and the PS-GNPs conjugates 2a–2e against A549 cells by exposure to an irradiation after 3 h, 24 h, and 48 h incubation times at  $670\text{--}710 \text{ nm}$  ( $2 \text{ J}\cdot\text{cm}^{-2}$ ) for 15 min.

a fiber optics bundle. The duration of light irradiation, under PDT treatment, is calculated taking into account the empirically found effective dose of light energy in  $\text{J}\cdot\text{cm}^2$ .

**2.4. MTT Assay and Cell Viability.** A549 Cells ( $1 \times 10^5$  cells/well) in  $100 \mu\text{L}$  of the mixed medium were placed in a 96-well plate and incubated for 48 h ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ). The medium was removed and the cultures were washed 3 times with physiologic saline. And the Pu-18-NMGA PS1 ( $0.8\text{--}25 \mu\text{g/mL}$ ) or corresponding amount of the PS-GNPs conjugates 2a–2e (constant amount of the PS1) in  $100 \mu\text{L}$  of the mixed medium was added in each well. 24 h later, the Pu-18-NMGA PS1 or each PS-GNPs conjugate solution was discarded, and the cultures were washed 3 times with physiological saline and then medium ( $100 \mu\text{L}/\text{well}$ ) was added. The cultures were then subjected to the irradiation

( $2 \text{ J}\cdot\text{cm}^{-2}$ ) at the distance of 20 cm for 15 min, followed by an 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay to evaluate their sensitivity to PDT. For the MTT assay, MTT solution ( $10 \mu\text{L}$ ) was added to each cell-culture well and cultured in the incubator for 3 h. Detergent solution (TACS, Trevigen,  $200 \mu\text{L}$ ) was added to the culture, shaken for 10 min, and the absorbance was measured with an ELISA reader at 570 nm. Measurements were performed 3 h, 24 h, and 48 h incubation time after the irradiation, respectively. Each group consisted of 3 wells.

### 3. Results and Discussion

**3.1. Preparation of Gold Nanoparticles Conjugates.** The commonly used synthetic way of GNPs is a reduction method of Au(III) salt (usually from  $\text{HAuCl}_4$ ) using sodium citrate

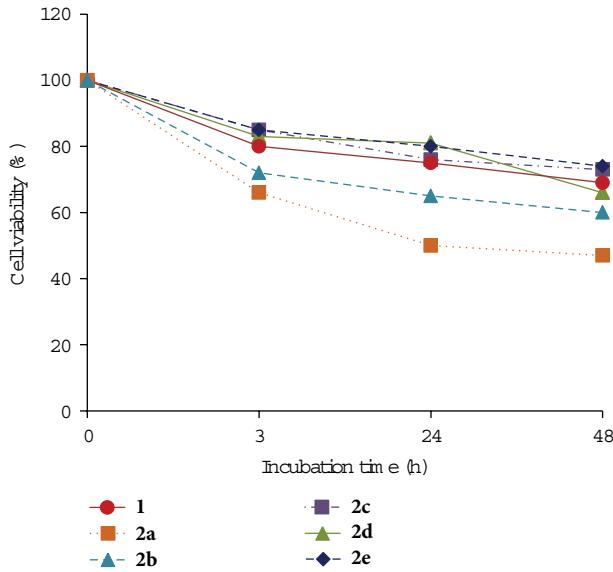


FIGURE 6: Comparative cell viability (%) of PS1 ( $3.2 \mu\text{g}/\text{mL}$ ) and the PS-GNPs conjugates **2a–2e** against A549 cells by photo irradiation after 3 h, 24 h, and 48 h incubation times.

in water [35]. In this method, sodium citrate has a double role as a weak reducing agent as well as a capping agent that stabilizes the NPs. The particle size is controlled by a ratio between citrate and  $\text{AuCl}_4^-$  ions. Higher concentration of citrate afforded smaller particle size [35].

However, in this work, we used hydrophilic PS1 and Au(III) without any additional reducing agents and surfactants [32]. The hydroxyl groups of NMGA in PS1 have important roles as a reducing agent as well as a stabilizer through the electrically charged functional groups (i.e., carboxylate and amine groups) in forming the PS-GNPs conjugates [9]. PS1 was obtained from the carboxyl group of purpurin-18 (Pu-18) and the amine group of NMGA by simple and effective method (Figure 1). Pu-18 was synthesized from a conversion of methyl pheophorbide *a* (MPa) by air oxidation in *n*-propanol with KOH [34]. MPa was obtained from *Spirulina pacifica* algae by the procedure reported by Smith et al. [33]. PS-GNPs conjugates were prepared from the reaction of different molar ratios between Au(III) and PS1 (**2a**, 1 : 2; **2b**, 1 : 4; **2c**, 1 : 6; **2d**, 1 : 8; **2e**, 1 : 10) in water to afford different particle sizes (Figure 2). The structures of the water soluble PS1 and the PS-GNPs conjugates were confirmed by  $^1\text{H-NMR}$  spectroscopy, mass spectrometry, and UV-vis spectroscopy (Figure 3).

The water soluble PS1 acts not only as a reducing agent, but also as a capping agent in the reduction of  $\text{HAuCl}_4$  for synthesis of PS-GNPs conjugates. The formation of PS-GNPs conjugates is stable in the aqueous solution due to the adsorption of oxidized PS1 on the surface of the GNPs through a strong coordinate-covalent bond between carboxylate on PS1 and gold metal. So the binding strength of PS1 on the GNPs surface is enough to allow accumulation of PS1 in culture medium or *in vivo* [8, 24, 36, 37]. Therefore, a large amount of water soluble PS was generally used in

order to get stable GNPs. Hence, we have used five different concentration ratios between Au(III) and PS1 in order to find suitable concentration ratio that gives optimal size of the PS-GNPs conjugates for best photodynamic activity result.

**3.2. UV-Vis Spectroscopic Investigation and Size Analysis by TEM Images.** Figure 3 shows the UV-vis absorption spectra of the PS-GNPs conjugates **2a–2e** in water. In each conjugate, typical plasmon resonance band of the GNPs was appeared at 506–525 nm, respectively [24]. In **2a–2c**, the longest wavelength absorption ( $\lambda_{\text{max}}$ ) is longer (719–762 nm) than that of PS1 (702 nm), while  $\lambda_{\text{max}}$  of **2d–2e** is the same with that of PS1 (Table 1). Among the conjugates, **2b** showed the longest wavelength absorption at 762 nm. In **2a–2b**, the Soret band at about 330–450 nm was broadened, indicating the formation of stacking structure of the chlorin ring on the gold surfaces [25].

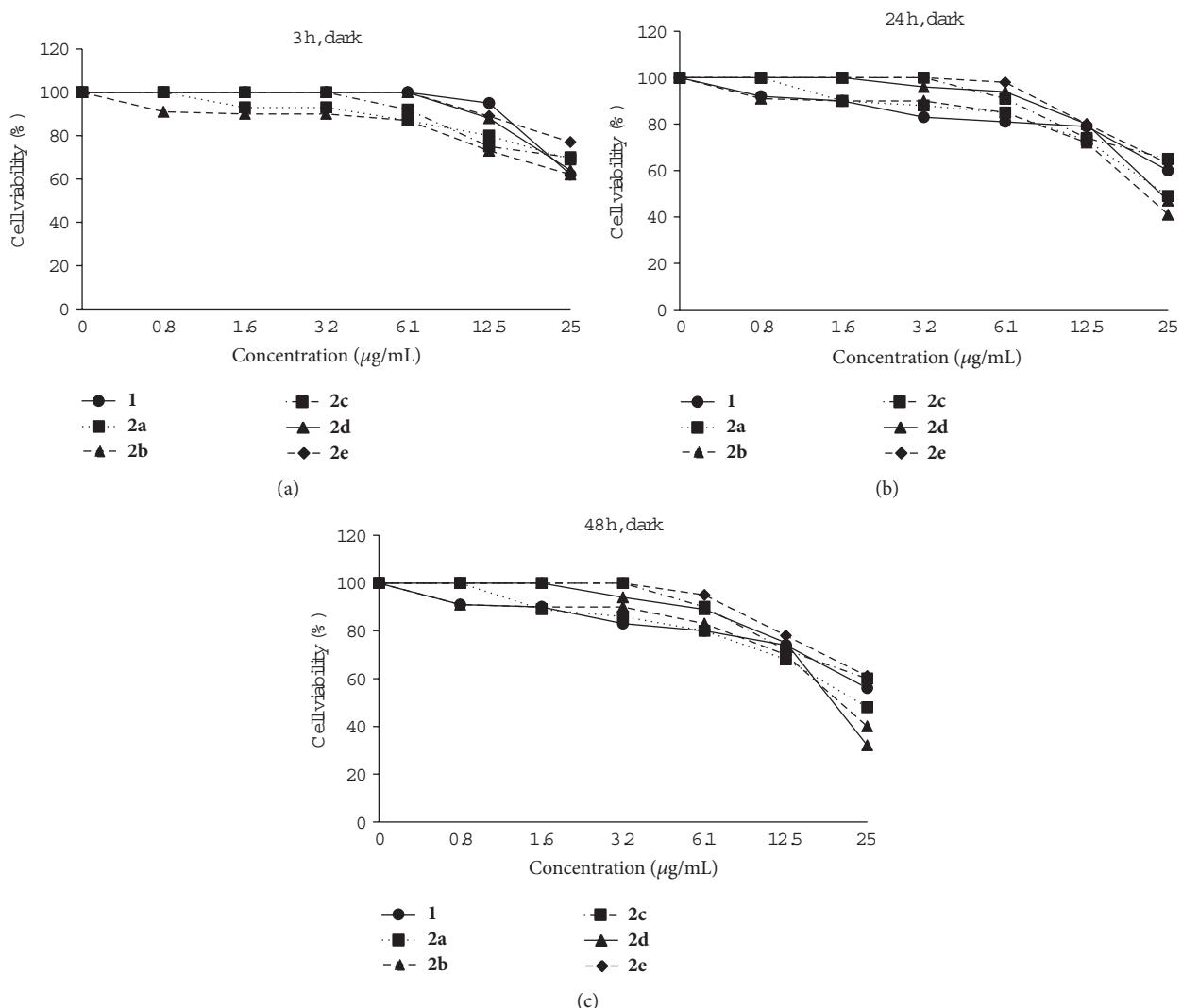
Figure 4 shows the typical TEM images of the PS-GNPs conjugates **2a–2e** prepared by using different concentration ratios between Au(III) and PS1. The images of the conjugates are different from each other in size and shape corresponding to the different molar ratios used in the preparation of the conjugates (Table 2). In **2a**, when molar ratio was 1 : 2 for Au(III) : PS1, the GNPs are mainly peanut-shaped nanocrystals in water. And some spheres have diameters around 60 nm and are well dispersed with no aggregation between the GNPs in water. In **2b**, when molar ratio was 1 : 4, the GNPs are nanospheres have diameters around 5–11 nm. And some GNPs are closely placed each other and have a chain-like appearance with branching. In **2c**, when molar ratio was 1 : 6, the GNPs are nanospheres have diameters around 5–10 nm. However, some GNPs are aggregated together to form many bundles of GNPs, resulting in bigger diameters around 27–44 nm. In **2d**, when molar ratio was 1 : 8, the GNPs are mainly aggregated bundles and shape was not spheres with length around 50–90 nm and width around 25–50 nm. And yield of the GNPs was low and some aggregated GNPs have size around 200 nm. In **2e**, when molar ratio was 1 : 10, the GNPs are mainly aggregated and yield of the GNPs was very low, and some aggregated bundles of GNPs were around 50–70 nm size. When relatively lower molar ratio of PS1 (2 or 4) made stable GNPs conjugate, however, higher molar ratio allowed unstable GNPs conjugate and remains continuous aggregation [35]. Consequently, the molar ratio between Au(III) and PS1 is an important driving force to control GNPs size, shape, and aggregation degree of the GNPs in aqueous media.

Based on the UV-vis spectra and TEM images, there is a good relationship between absorption intensity and particle size. Higher absorption intensity of the conjugate corresponds to bigger particle size. In **2a**, absorption intensity at over than 450 nm ranges is the highest among all the conjugates, which corresponds to the biggest size (about 60 nm) in the conjugates.

Compound **2b** shows the longest wavelength absorption at 762 nm which is included in NIR wavelength region (PTT therapeutic window, 750–1100 nm), so there is a potential for using PTT. We are considering that the GNPs conjugate

TABLE 2: Summary of TEM images of the PS-GNPs conjugates **2a–2e**.

Compound	Shape	Diameter (nm)	Dispersion
<b>2a</b>	Sphere, peanut	~60	GNPs are well dispersed and showed no aggregation.
<b>2b</b>	Sphere, chain-like appearance with branching	5–11	GNPs are well dispersed and showed no aggregation.
<b>2c</b>	Sphere	5–11	Some aggregated GNPs have diameter sizes of 27–44 nm.
<b>2d</b>	Not sphere	—	GNPs have a lot of aggregation.
<b>2e</b>	Not sphere	—	Very few aggregated GNPs have sizes of 50–70 nm.

FIGURE 7: Cell viability (%) of PS1 (0–25  $\mu\text{g/mL}$ ) and the PS-GNPs conjugates **2a–2e** against A549 cells without photo irradiation (dark cytotoxicity) after 3 h, 24 h, and 48 h incubation times.

for a combination (synergy effect) therapy of PDT and PTT [38, 39].

**3.3. Photodynamic Activity and Size Effect by In Vitro.** *In vitro* activity of the GNPs conjugates was evaluated by comparison with PS1 against A549 human lung adenocarcinoma cells at 0.8, 1.6, 3.2, 6.1, 12.5, and 25  $\mu\text{g/mL}$ . In this case, PS1 was dissolved in a mixed solvent of ethanol and water (1:1 volume ratio) and conjugates **2a–2e** were resuspended in

water. The dark cytotoxicity and phototoxicity of **2a–2e** and PS1 were measured by MTT assay at 3 h, 24 h, and 48 h incubation times, respectively.

In all the compounds, upon photo irradiation, the cell viability was decreased corresponding to the increased incubation time after PDT as well as increased concentration (Figure 5), for example, at 48 h incubation and 3.2  $\mu\text{g/mL}$ , 80% at 3 h, 75% at 24 h, and 69% for PS1, and 66% at 3 h, 50% at 24 h, and 47% for **2a** (Figure 6), respectively.

TABLE 3: IC<sub>50</sub> ( $\mu\text{g}/\text{mL}$ ) values of PS1 and 2a–2e against A549 cells at various incubation times. IC<sub>50</sub> values were determined by MTT assay at 3 h, 24 h and 48 h incubation after photo irradiation.

Compound	3 h	24 h	48 h
1	14.8	10.5	8.72
2a	7.14	5.32	4.32
2b	12.06	7.24	6.38
2c	20.53	14.43	13.05
2d	20.52	18.51	12.95
2e	24.23	22.12	11.82

Dark cytotoxicity of PS1 and conjugates 2a–2e is shown in Figure 7. At highest concentration (25  $\mu\text{g}/\text{mL}$ ) with 48 h incubation time, all compounds showed high dark cytotoxicity (cell viability 32–61%).

PS1 showed slightly higher photocytotoxicity (IC<sub>50</sub>, 10.5  $\mu\text{g}/\text{mL}$  = 14  $\mu\text{M}$  at 24 h incubation time) than that of the purpurin-18-choline derivative (IC<sub>50</sub>, 15  $\mu\text{M}$  at 24 h incubation time) that has been previously reported by us [25]. Conjugates 2a and 2b showed higher photocytotoxicity than that of PS1. At high concentration (e.g., at 25  $\mu\text{g}/\text{mL}$ ), 2a showed higher dark cytotoxicity (69% at 3 h, 49% at 24 h, and 48% at 48 h incubation time, Figure 7) as compared to PS1 (62% at 3 h, 60% at 24 h, and 56% at 48 h incubation time), which might be attributed to large amount of PS1 molecules on the GNPs surface in 2a. However, conjugates 2c–2e showed lower photocytotoxicity than that of PS1. This result demonstrates that photodynamic activity significantly depends on size and aggregation degree of the GNPs. For example, in 2a and 2b there is no aggregation between each other and 2a has about 60 nm size, while in 2c–2e there are some aggregated bundles of the GNPs with small size. Chithrani et al. [29] studied a relationship between particles size (14–100 nm) and cellular uptake of the GNPs in HeLa cells, in which the maximum uptake was occurred at a size of 50 nm. Jiang et al. [28] have reported that cellular uptake strongly depends on the size of the GNPs, in which the GNPs having 2–100 nm size range were coated with Herceptin and were evaluated for cell internalization against breast cancer cell lines by the ErbB2 receptor. The most efficient cellular uptake was observed with particles range of 20–50 nm. Apoptosis was also enhanced by the GNPs having 40–50 nm size [28]. From the high dark cytotoxicity at high concentration, we confirmed that the PS-GNPs conjugate 2a and 2b showed better photodynamic activity at low concentration (3.2  $\mu\text{g}/\text{mL}$ ) having low dark cytotoxicity (Figure 7).

In addition, 2a and 2b have higher absorbance at irradiated wavelength range, which allowed good photodynamic activity results. In 2c–2e, absorption intensity was lower than that of PS1, resulting in lower photocytotoxicity as compared to PS1. Table 3 shows the IC<sub>50</sub> values for PS1 and its PS-GNPs conjugates 2a–2e. At 48 h incubation time, 2a and 2b showed better IC<sub>50</sub> value, 4.32 and 6.38  $\mu\text{g}/\text{mL}$ , respectively, as compared to PS1 (8.72  $\mu\text{g}/\text{mL}$ ). Therefore, as we pointed out above, photodynamic *in vitro* activity of synthesized PS-GNPs conjugates (2a and 2b) is much higher than that of

the free PS1. This result indicates that optimal size and well-dispersed nanoparticles are important for photodynamic effect in aqueous media. Especially, bigger size (~60 nm) of nanoparticles 2a could be useful to transport more chlorine molecules into the cancer cells by endocytosis [28, 29, 40].

## 4. Conclusions

In summary, a simple single-step synthesis of PS-GNPs conjugates from different molar ratios of Au(III)/water soluble ionic PS1 (purpurin-18-N-methyl-D-glucamine) has been studied without adding any particular reducing agents and surfactants. *In vitro* anticancer efficacy of the PS-GNPs conjugates against A549 lung cancer cell lines was evaluated. We revealed that PDT *in vitro* activity of synthesized PS-GNPs conjugates was higher as compared to free PS1 because of good transport of the PS into the cells by using size effect. Conjugate 2a based on molar ratio between HAuCl<sub>4</sub> and PS was 1:2 that exhibits best PDT efficiency than other conjugates having different molar ratios. This result could be useful for synthesis of new PS and PS-GNPs conjugates having different size as well as for developing good relationship between PDT activity and size effect of GNPs in aqueous media.

## Conflict of Interests

We do not have any conflict of interests to Sigma-Aldrich and BioSpec LED.

## Acknowledgments

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## Research Article

# Hydrogen Bond Acceptors and Additional Cationic Charges in Methylene Blue Derivatives: Photophysics and Antimicrobial Efficiency

Ariane Felgenträger,<sup>1</sup> Tim Maisch,<sup>1</sup> Daniel Dobler,<sup>2</sup> and Andreas Späth<sup>2</sup>

<sup>1</sup> Department of Dermatology, University Hospital Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany

<sup>2</sup> Department of Organic Chemistry, University of Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany

Correspondence should be addressed to Andreas Späth; [andreas.spaeth@chemie.uni-regensburg.de](mailto:andreas.spaeth@chemie.uni-regensburg.de)

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Photodynamic inactivation of bacteria (PIB) by efficient singlet oxygen photosensitizers might be a beneficial alternative to antibiotics in the struggle against multiresistant bacteria. Phenothiazinium dyes belong to the most prominent classes of such sensitizers due to their intense absorption in the red-light region ( $\lambda_{\text{abs}, \text{max}}$  ca. 600–680 nm,  $\epsilon > 50000 \text{ L mol}^{-1} \text{ cm}^{-1}$ ), their low toxicity, and their attachment/penetration abilities. Except simple substituents like alkyl or hydroxalkyl residues, nearly no modifications of the phenothiaziniums have been pursued at the auxochromic sites. By this, the properties of methylene blue derivatives and their fields of application are limited; it remains unclear if their potential antimicrobial efficacy may be enhanced, also to compete with porphyrins. We prepared a set of six mainly novel methylene blue derivatives with the ability of additional hydrogen bonding and/or additional cationic charges to study the substituents' effect on their activity/toxicity profiles and photophysical properties. Direct detection of singlet oxygen was performed at 1270 nm and the singlet oxygen quantum yields were determined. In suspensions with both, Gram-positive and Gram-negative bacteria, some derivatives were highly active upon illumination to inactivate *S. aureus* and *E. coli* up to 7 log<sub>10</sub> steps (99.9999%) without inherent toxicities in the nonirradiated state.

## 1. Introduction

Owing to its structure, methylene blue (MB, 7) can penetrate cells and can be used as a staining agent in histology [1, 2] or as a chemotherapeutic [3–6]. Binding with cytoplasmic structures within the cell and interference with oxidation/reduction processes [7, 8] may lead to killing of bacteria, funguses, viruses, or parasites.

Methylene blue and its derivatives were proven to be versatile photosensitizers for the inactivation of pathogens in suspension [9–11], for example, *Candida* species [12, 13], *E. coli* [14, 15], *S. aureus* [16] and *MRSA* [17], tropical diseases [18], or several viruses [19, 20], and therefore finds its application in antimicrobial fields, like blood disinfection [21–24]. *In vivo* the phenothiazinium chromophore system is used with benefit against oral infections [25] performing photodynamic root channel disinfection [26–29] or

treatment of periodontitis, inactivating bacteria like *E. faecalis* [30], *P. gingivalis* [31], or *A. actinomycetemcomitans* [32]. As “state of the art” it may be given that methylene blue and its derivatives like 1,9-dimethylmethylene blue (DMMB), toluidine blue (TBO) and new methylene blue (NMB) can achieve a log reduction  $>6$  log steps of a bacterium at fluences ranging from 0 to  $30 \text{ J cm}^{-2}$ , using a fluence rate of  $125 \text{ mW cm}^{-2}$  in a concentration range of 2 to  $10 \mu\text{M}$  in suspension [33].

Although methylene blue and its alkyl- and aryl-derivatives are well studied [34–37], not many approaches followed variations of the structure beyond simple modifications [38]. For example, the effect of additional positive charges on the antimicrobial activity and the influence of such substituents on the singlet oxygen yield have not been investigated yet. A comparison of such photosensitizers with related hydrogen

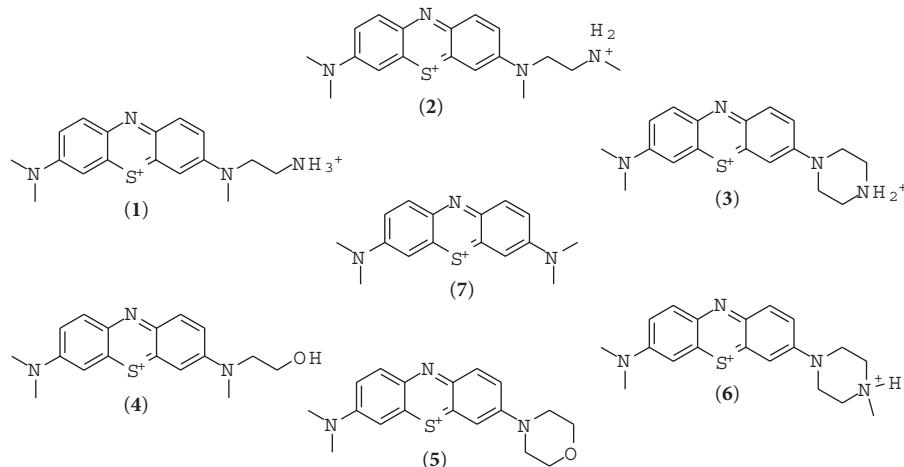


FIGURE 1: Compounds investigated (**1–6**) in comparison to the lead compound methylene blue (**7**); counterions are chloride in all cases and were avoided for clarity [40].

bond acceptor moieties, thus also strongly increasing polarity, is lacking as well as the direct spectroscopic determination of the group's influence on the singlet oxygen quantum yield. More polarity in the structure should cause the molecules to stay outside cell, causing only photodynamic damage of the cell wall. Positive charges in addition may lead to better attachment to the exterior of the cell, resulting in shorter process times and higher antimicrobial activity in comparison to methylene blue. Attack from outside the cell also overcomes the problem of reduced photosensitizer activity by efflux mechanisms [39].

The synthesis of methylene blue and its derivatives was summarized [41] and the preparation of phenothiazinium systems with additional positive charges for other purposes was demonstrated [42], but a straight forward, reliable purification protocol without the use of expensive HPLC methods is still missing.

We focused on the synthesis and study of methylene blue derivatives with highly polar and/or hydrophilic groups, to extend the field of highly hydrophilic phenothiazinium compounds in antimicrobial photodynamic therapy (aPDT). One substituent in the methylene blue lead structure (**7**) was changed (Figure 1) in order to achieve pursuable variations of the behaviour of the compound.

## 2. Material and Methodology

**2.1. Synthesis, Purification, and Analytics.** Methylene blue has been purchased by Sigma Aldrich and was purified by flash chromatography with silica gel using dichloromethane/ethanol 10:1 as the eluent mixture resulting in an overall pureness of >99% (HPLC-MS). Methylene blue and its derivatives were dissolved and diluted in  $\text{H}_2\text{O}$  and kept in the dark at 4°C until use. Further information on chemicals, analytics, description of the syntheses and purification protocols are given in the supporting information. See the supporting information available online at <http://dx.doi.org/10.1155/2013/482167>.

**2.2. Absorption Spectroscopy.** Absorption spectra were recorded at room temperature with a DU640 spectrophotometer (Beckman Instruments GmbH, Munich, Germany) in a concentration range of  $5 \cdot 10^{-6} \text{ M}$  to  $1 \cdot 10^{-4} \text{ M}$ . The transmission has been measured and the absorption cross-section  $\sigma [\text{cm}^2]$  was calculated according to the following equation:

$$\sigma = -\frac{\ln(T/100)}{c \cdot l \cdot N_A} \quad (1)$$

with  $\sigma$  being the absorption cross section,  $c$  the concentration of PS,  $l$  the length of light path through the solution,  $T$  the transmission in %, and  $N_A$  the Avogadro constant.

**2.3. Direct Detection of Singlet Oxygen Luminescence.** Solutions with the photosensitizer were filled in a quartz cuvette with a path length of 1 cm (QS-101, Hellma Optik, Jena, Germany) and were excited during magnetic stirring with an OPO tuneable laser (EKSPLA, Lithuania) at a wavelength of  $\lambda = 600 \text{ nm}$ , power output  $P = 90 \text{ mW}$ , frequency of  $f = 1 \text{ kHz}$ , and therefore an energy per pulse of  $E = 9 \cdot 10^{-5} \text{ J}$ . Every sample was illuminated with 20.000 pulses. Direct detection as described in previous papers was done by time resolved measurements at 1270 nm (10 nm FWHM filter) in near-backward direction with respect to the exciting beam using an infrared-sensitive photomultiplier (R5509-42, Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany). The luminescence intensity is given by

$$I(t) = \frac{c}{t_R^{-1} - t_D^{-1}} \left[ \exp\left(-\frac{t}{t_D}\right) - \exp\left(-\frac{t}{t_R}\right) \right], \quad (2)$$

where  $c$  was used to fit the singlet oxygen luminescence signal, and  $t_R$  and  $t_D$  are the rise and decay times [44–46]. Therefore, the Levenberg-Marquardt algorithm of Mathematica (Wolfram Research, Champaign, IL USA) was used. The luminescence signal was spectrally resolved using

TABLE 1: Characteristic values of methylene blue and its derivatives MB-1 to MB-6, where  $\lambda_{\text{max}}$  describes the maximum of the absorption; the dimerisation was detected in a concentration range between 10–200  $\mu\text{M}$ ; the photostability is described with the ratio of the height of the absorption maximum after irradiation to height of the maximum before irradiation with 180000 laser pulses;  $t_D$  and  $t_R$  are the rise and decay time of the time resolved singlet oxygen luminescence, respectively;  $\Phi_\Delta$  is the quantum yield of singlet oxygen generation relatively to the quantum yield of methylene blue, which is found in literature to be 0.52 [43];  $\Phi_\Delta^{1.00}$  is the quantum yield of MB set to 1.00, to simplify the comparison. For the values of the quantum yield an error of 10% in regard to the measurement procedure had to be estimated. “overlap” describes the uptake of the lamp emission spectrum by the different photosensitizers at a concentration of 10  $\mu\text{M}$ . “Eff.Tox.” describes therefore the predicted effective toxicity that was calculated by multiplication of  $\Phi_\Delta^{1.00}$  (ref. MB) with the value of the overlap.

PS	$\lambda_{\text{abs,max}}$	dimeriz.	photostab.	$t_D$ ( $\mu\text{s}$ )	$t_R$ ( $\mu\text{s}$ )	$\Phi_\Delta^{1.00}$ (ref. MB)	$\Phi_\Delta^{1.00}$ (ref. MB)	Overlap [%]	Eff.Tox. [%]
MB	664	Yes	95%	3.51	1.64	0.52*	1.00	54.4	54
MB-1	653.5	No	82%	3.47	1.93	0.45 $\pm$ 0.05	0.87	43.1	37
MB-2	650.5	No	81%	3.57	1.76	0.38 $\pm$ 0.04	0.73	38.7	28
MB-3	643.5	No	95%	3.44	1.92	0.47 $\pm$ 0.05	0.90	38.4	35
MB-4	663.5	Yes	97%	3.51	1.78	0.51 $\pm$ 0.05	0.98	44.5	44
MB-5	662	Yes	96%	3.46	1.70	0.41 $\pm$ 0.04	0.79	45.4	36
MB-6	649	No	95%	3.47	1.76	0.35 $\pm$ 0.04	0.67	29.6	20

interference filters in front of the photomultiplier tube at wavelengths ranging from 1150 nm to 1400 nm. The values show the integrated luminescence signals detected at a certain wavelength and are normalized to the maximal value. A Lorentz-shaped curve was fitted through the measurement points, with the maximum at  $\lambda = 1275$  nm [47].

**2.4. Quantum Yield of Singlet Oxygen Formation.** The quantum yields ( $\Phi_\Delta$ ) of the derivatives of MB were compared to the  $\Phi_\Delta$  of MB which is reported in the literature being  $\Phi_\Delta = 0.52$  in aqueous solution [48]. Therefore, a sample of each photosensitizer was diluted to a final absorption of  $A = 30\%$  at  $\lambda = 600$  nm in  $\text{H}_2\text{O}$ . 3 mL ( $\text{O}_2$  concentration at air-saturation at 25°C) of each sample was illuminated in a quartz cuvette (path length of 1 cm) with the OPO tuneable laser with the above given parameters, and the emitted singlet oxygen photons were determined by the integral over the luminescence curve.

**2.5. Photostability.** The photosensitizers were diluted to a final absorption of  $A = 30\%$  at the wavelength of  $\lambda = 600$  nm. The samples were irradiated in quartz cuvettes at a path length of 1 cm with the OPO tuneable laser at the given parameters with 180000 laser pulses during magnetic stirring. After the irradiation absorption spectroscopy was done in the range from 200 nm to 1000 nm and the data was compared to the nonilluminated samples.

**2.6. Bacterial Strains.** The biochemical analysis of each bacteria strain was done by a VITEK2 System (bioMérieux, Nürtingen, Germany) according to NCCLS (National Committee for Clinical Laboratory Standards) guidelines. The bacterial strains, *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922), were grown aerobically at 37°C in Mueller-Hinton broth (Gibco Life Technologies GmbH, Eggenstein, Germany). A 500  $\mu\text{L}$  portion of an overnight cell culture (5 mL) was transferred to 50 mL of fresh BHI media and grown at 37°C on an orbital shaker. When the cultures reached the stationary phase of growth, the cells were harvested

by centrifugation (200 g, 15 min), washed with phosphate-buffered saline (PBS; Biochrom, Berlin, Germany) at pH 7.4, containing 2.7 mM KCl and 0.14 M NaCl, and suspended in PBS at an optical density of 0.6 at 600 nm corresponding to  $\approx 10^8 - 10^9$  cells/mL for the use in the phototoxicity experiments.

**2.7. Light Source.** The bacteria were illuminated using an incoherent light source PDT1200 provided by Waldmann Medizintechnik (Villingen-Schwenningen, Germany) which covers partially the absorption spectrum of methylene blue and its derivatives (Figure 3). The normalized emission spectrum of the light source was provided by Waldmann Medizintechnik. The maximal fluence rate at the level of the illuminated samples was 50  $\text{mW cm}^{-2}$ . The samples were illuminated for 10 min (30  $\text{J cm}^{-2}$ ). In order to estimate the effectiveness of the uptake of the light energy by the different derivatives the values of the emission spectrum “Em” were folded with the values for the absolute absorption “Abs” for the spectral region between 500 and 800 nm. According to the following formula an effective toxicity “Eff.Tox.” was predicted for each derivative:

$$\text{Eff.Tox.} = \left( \sum_{i=500 \text{ nm}}^{800 \text{ nm}} \text{Em}_i \cdot \text{Abs}_i \right) \cdot \Phi_\Delta. \quad (3)$$

Here it has been taken into account that the effectively absorbed energy (i.e., the sum of the product of emission and absorption) of every photosensitizer is used partially to generate singlet oxygen. Therefore, also the quantum yield  $\Phi_\Delta$  was multiplied to the effectively absorbed energy. The results, given as percentaged values, are listed in Table 1.

**2.8. Phototoxicity Assay of the Bacteria.** A bacterial cell number of  $10^8$  to  $10^9$   $\text{mL}^{-1}$  was incubated for 10 min in the dark with different concentrations of methylene blue-based photosensitizers (0, 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , 50  $\mu\text{M}$ , and 100  $\mu\text{M}$ ). At the end of the incubation period the cells were transferred into a 96-well microtitre plate (200  $\mu\text{L}/\text{well}$ ) and illuminated

for 10 min ( $50 \text{ mW cm}^{-2}$ ;  $30 \text{ J cm}^{-2}$ ). Controls were neither sensitized with a photosensitizer nor exposed to the light source or were incubated with the photosensitizer only. After illumination, the survival of the bacteria was determined by CFU assay. Serially diluted aliquots of treated and untreated (no photosensitizer, no light) cells were plated on Mueller-Hinton agar and the numbers of  $\text{CFU mL}^{-1}$  were counted after 24 h of incubation at  $37^\circ\text{C}$ .

**2.9. Data Analysis and Statistics for Cell Experiments.** Each individual experiment was performed at least in triplicate. All primary data are presented as means with standard deviation of the mean. A reduction of at least 3 orders of magnitude of  $\log_{10}$  viable median numbers of bacteria cells was considered biologically relevant with regard to the guidelines for hand hygiene [49].

### 3. Results

In order to cover the field of hydrophilic phenothiaziniums in our studies, we selected a small library of methylene blue derivatives to investigate the influence of hydrogen bond acceptors (**4** and **5**) and/or an additional cationic charge (**1**, **2**, **3**, and **6**) located in one of the systems side chains on the photophysical characteristics and the antimicrobial efficiency. This selection allows us to study the effect of the substituent's structure on the stability of the methylene blue derivative comparing cyclic (compounds **3**, **5**, and **6**) with acyclic (compounds **1**, **2**, and **4**) moieties. The effect of the nature of the additional charge, being either a tertiary (**6**) or a secondary (**2** or **3**) or a primary (**1**) ammonium group, can be compared in this selection. All photosensitizers were supplied in their chloride form to ensure comparability of the photosensitizer salts and avoiding disadvantageous influence of the counterion on the phototoxicity studies. As trifluoroacetate is known to be toxic against microorganisms, it has to be exchanged with a nontoxic counterion. Iodide salts readily react with singlet oxygen to form triiodide, which has a negative influence on the antimicrobial efficacy of the photosensitizer [50]. Figure 1 summarizes the studied compounds.

**3.1. Synthesis.** 3-Dimethylamino-phenothiazinium triiodide (**10**) was proven to be a suitable starting material for synthesis of the chromophore library. It was prepared using known conditions from literature starting from phenothiazine (**8**), as can be seen in Scheme 1 [40].

The compound was converted to the desired products (**14**, **15**, and **16**) in good yields using an excess of the appropriate boc-protected amine (**11**, **12**, or **13**) in presence of triethylamine in dichloromethane. After deprotection with TFA using standard conditions, the counterion was exchanged versus chloride using amberlite IRA958. Both steps resulted in quantitative yields (Scheme 2).

The second set of phenothiazinium compounds was prepared using similar conditions and reacting 2-(N-methylamino)ethanol to give **4-I**, morpholin to yield **5-I** or 4-N-methyl-piperazine to give **6-I**, respectively, with moderate

to good yields. After purification by flash chromatography and crystallisation, the counterion was exchanged with chloride in quantitative yield following the same protocol as before (Scheme 3).

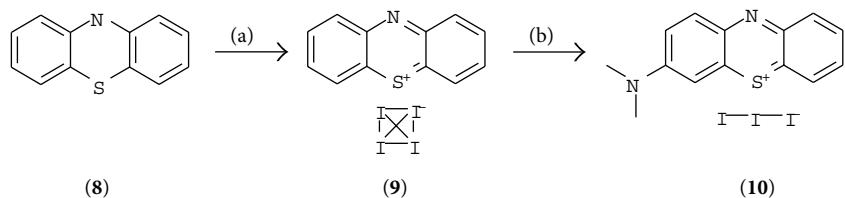
For detailed synthesis and purification protocols, see the supporting information.

### 3.2. Photophysical Data

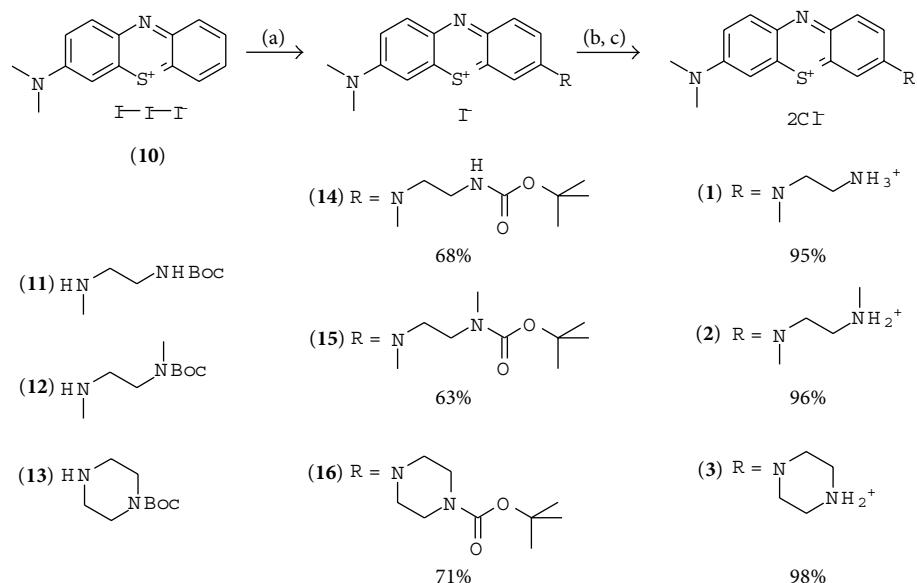
**3.2.1. Absorption Spectra for Different Photosensitizer Concentration.** Phototoxic reactions of methylene blue on microorganisms can involve redox reactions between the dye and the pathogen, or the generation of reactive oxygen species (ROS) via type-I mechanism or type-II mechanism, for example, direct energy transfer from excited triplet state of the photosensitizer to oxygen, resulting in the formation of singlet molecular oxygen [21, 51]. Both mechanisms are described to be important for an antibacterial effect. The photoinactivation of bacteria might therefore be dependent on the aggregation state of the molecule (dimerisation) [52–55] that can be influenced also by the presence of bacteria [56, 57] or other influences like the pH value of the surrounding [13, 58, 59]. Dimerization of methylene blue and some of its derivatives like toluidine blue (TBO) has been described [60, 61] and also has an influence on the photophysical properties of the dye resulting in different phototoxic efficacies [56]. This has been investigated for the new methylene blue derivatives in  $\text{H}_2\text{O}$  within a concentration range from 10 to  $200 \mu\text{M}$ .

In Table 1 the absorption maximum of each derivative is shown. MB-4 and MB-5 closely match the peak of methylene blue. In the given concentration range the derivatives MB-4 and MB-5 show the formation of an absorption peak at 613 nm (Figure 2). The peak between 662 and 664 nm is diminished with increasing the dye concentration (hypochromicity). The evolving local maximums at 613 nm show each a hypsochromic effect indicating aggregation processes. The peak at 613 nm is considered to be the dimer, as described for methylene blue [60, 61].

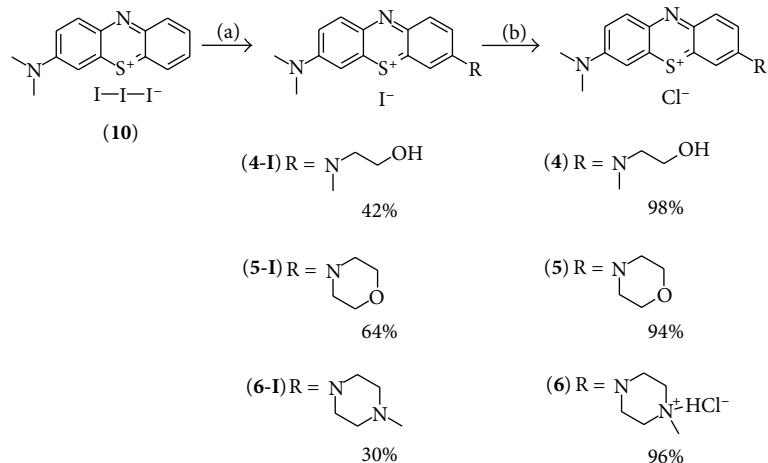
**3.2.2. Absorption of the Lamp Emission by the Different Derivatives of MB.** The emission spectrum of the incoherent light source PDT1200 covers partially the absorption spectrum of methylene blue and its derivatives (Figure 3). The effectiveness of light absorption at the same molar concentration of  $10 \mu\text{M}$  was calculated with (3) and the results are listed in Table 1. There one finds the “overlap” of emission and absorption and the effective toxicity “Eff.Tox.,” which estimates the phototoxic effect on microorganisms via singlet oxygen by taking into account the relative singlet oxygen quantum yield  $\Phi_\Delta^{1,00}$ , which describes the part of absorbed energy that generates singlet oxygen. “Eff.Tox.” describes therefore the predicted effective toxicity that was calculated by multiplication of  $\Phi_\Delta^{1,00}$  with the value of the overlap. With this method we assume methylene blue being most active and in descending manner MB (54%) > MB-4 (44%) > MB-1 (37%) > MB-5 (36%) > MB-3 (35%) > MB-2 (28%) > MB-6 (20%).



SCHEME 1: Synthesis of the precursors; conditions: (a) DCM, I<sub>2</sub>, RT, 2 h, quant.; (b) HNMe<sub>2</sub>, MeOH, RT, 14 h, 63%.



**SCHEME 2:** Synthesis of the boc-protected derivatives (**14–16**) and their transformation to the deprotected chromophores as chloride salts (**1–3**); conditions: (a) DCM, boc-protected amine (**11**, **12**, or **13**), NEt<sub>3</sub>, RT, 5 h; (b) DCM, TFA, RT, 4 h; (c) ion exchanger Amberlite IRA958, water.



SCHEME 3: Synthesis of the second set of methylene blue derivatives as chloride salts (**4–6**); conditions: (a) DCM, secondary amine: 2-(N-methylamino)ethanol, morpholin or 4-N-methyl-piperazine, RT, 5 h; (b) ion exchanger Amberlite IRA958, water, then HCl.

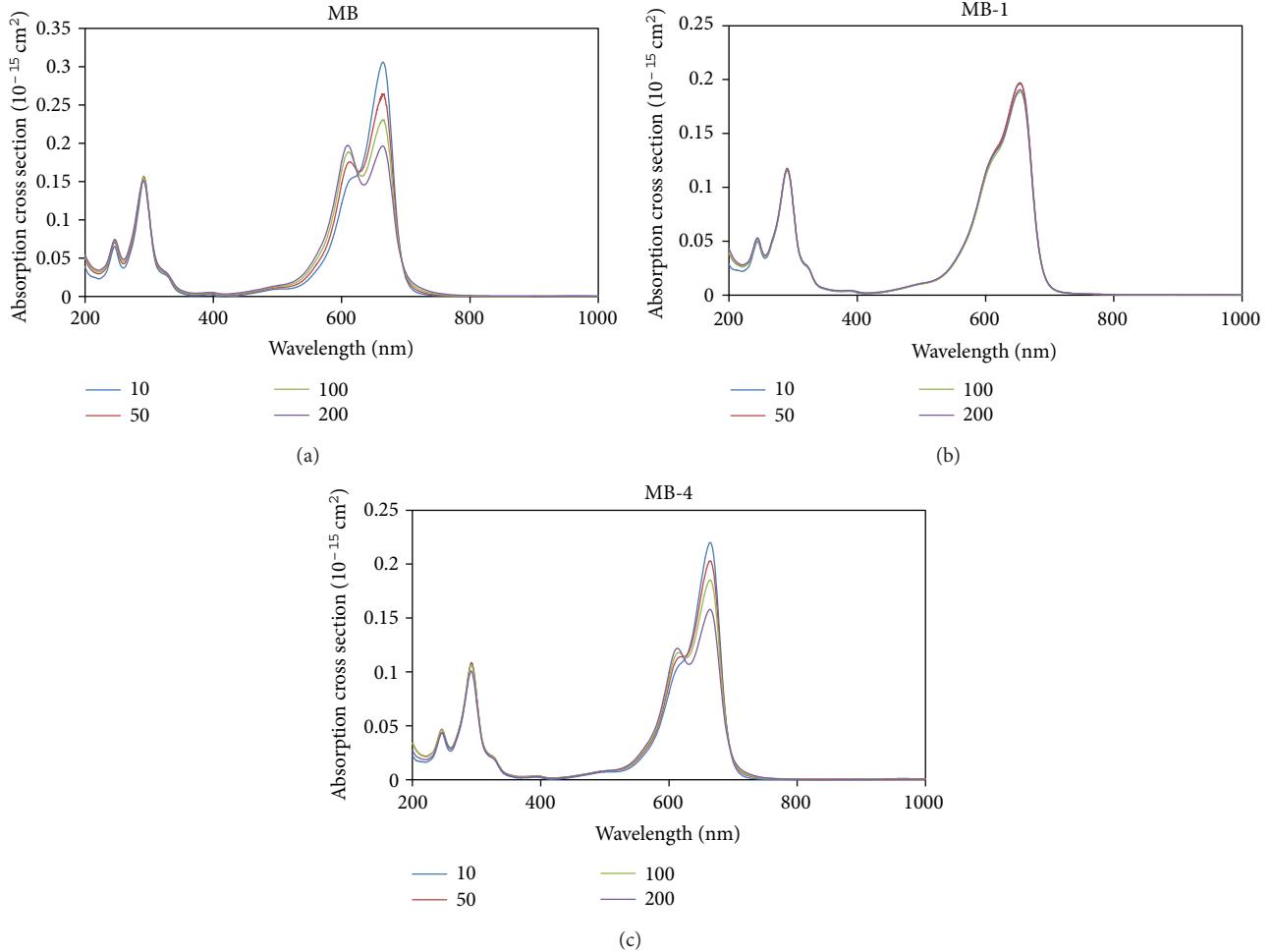


FIGURE 2: Absorption spectra of MB and its derivatives for different concentrations. Absorption spectra of methylene blue and its derivatives MB-1 and MB-4 within a concentration range of 10–200  $\mu\text{M}$  in  $\text{H}_2\text{O}$ ; the measurements show dimerisation for MB, MB-4, and MB-5 in the given concentration range (see supporting information).

**3.2.3. Photostability.** With diluting methylene blue and its derivatives to a final absorption of  $A = 30\%$  at 600 nm the same amount of light energy per time unit is absorbed by each derivative. After irradiation at 600 nm with 180000 laser pulses ( $=3$  min), resulting in an energy of  $E = 16.2 \text{ J}$ , the derivatives MB-1 and MB-2 showed a decrease in their main absorption region and in the UV-range, while MB and the other derivatives showed photostability (Figure 4, Table 1, see supporting information). The value to estimate photostability was given with the ratio of the absorption maxima after irradiation and before irradiation. The photophysical measurements such as time- and spectrally resolved singlet luminescence did not exceed the amount of energy used for the photostability testing.

**3.2.4. Time and Spectrally Resolved Singlet Oxygen Luminescence.** Singlet oxygen luminescence was generated by all derivatives of methylene blue and was detected time and spectrally resolved in an air-saturated solution of  $\text{H}_2\text{O}$  at  $25^\circ\text{C}$ . 20 k laser pulses equals an irradiation time of 20 s.

Each time-resolved luminescence signal showed a rise and decay time, whereas the rise time differed for each derivative but the decay time was around  $3.5 \mu\text{s}$ , confirming the values in literature for the decay of singlet oxygen in aqueous surrounding [43]. The rise and decay times,  $t_R$  and  $t_D$ , are shown in Table 1. The maximum of the singlet oxygen phosphorescence was detected at  $1275 \pm 5 \text{ nm}$  (Figure 5).

**3.2.5. Quantum Yield of Singlet Oxygen Formation.** The quantum yields for singlet oxygen formation of the derivatives of methylene blue have been compared in air saturated  $\text{H}_2\text{O}$  to the quantum yield of methylene blue, since it has been described that the quantum yield can be higher in basic environment [58]. Each photosensitizer absorbed the same amount of energy within the same irradiation time. Furthermore, the same amount of oxygen in the water surrounding of the molecule was given in order to deactivate the excited triplet state of the photosensitizer. Therefore, the singlet oxygen photons give evidence of the effectiveness of

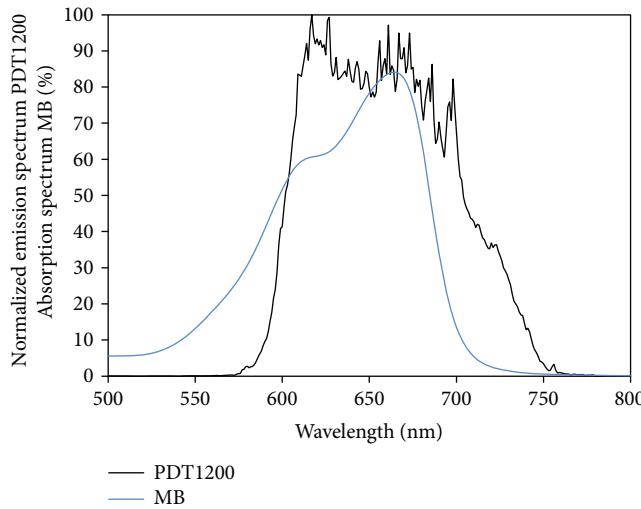


FIGURE 3: Emission spectrum of the PDT1200. Normalized (100%) emission spectrum of the PDT1200 and absorption spectrum of methylene blue at a concentration of  $10 \mu\text{M}$  in  $\text{H}_2\text{O}$  showing the percentaged absorption. An overlap of these two spectra was calculated by folding the values for emission and absorption for each wavelength and summing up these values.

each derivative. In Table 1 the results for the estimation of the quantum yields  $\Phi_{\Delta}$  of singlet oxygen for all derivatives are summarized relative to the literature value of the quantum yield for methylene blue of 0.52 [43]. The quantum yield of MB was then set to 1.00 in this paper, as described by  $\Phi_{\Delta}^{1.00}$ , because it is only needed for reference and comparison purposes. For the values of the quantum yield an error of 10% in regard to the measurement procedure was assumed. Taking into account that the photostability of MB-1 and MB-2 is not given within a range of 180000 laser pulses we consider an irradiation with 20000 laser pulses for the quantum yield measurement as an insignificant change in the absorption spectrum ( $\approx 2\%$ ). The  $\Phi_{\Delta}$  of MB-4 is comparable to the yield of methylene blue, whereas the other yields are smaller (Table 1, see supporting information).

**3.3. Photobiological Activity.** The irradiation of the Gram-positive *S. aureus* and the Gram-negative *E. coli* upon incubation with different concentrations (0–100  $\mu\text{M}$ ) of MB-1, MB-2, MB-3, MB-4, MB-5, and MB-6 caused a decrease in viability of CFU/mL (Figure 6 and supplementary figure) except for MB-1. Light activation of MB-1 achieved only a reduction of viable bacteria numbers of both bacteria strains of  $\approx 1 \log_{10}$  (supplementary figure). Furthermore, MB-2 induced only an antibacterial activity of 99.9% using a concentration of 50  $\mu\text{M}$  upon light activation. MB-3 showed a better killing efficacy as compared to MB-2 upon light activation. However, light-activated MB-3 achieved a killing efficacy of >99.9% at a concentration of 50  $\mu\text{M}$  against both strains, whereas MB-4, MB-5, and MB-6 exhibit the greatest killing rate of >99.999% (5  $\log_{10}$  steps) after irradiation with a concentration >10  $\mu\text{M}$  (Figure 6 and supplementary figure). All bacterial samples

TABLE 2: Overview of the phototoxic efficacy of the MB derivatives on *S. aureus* and *E. coli*; the table shows only the photodynamic treatment with light (effects of dark toxicity can be found in the supporting information). Different concentrations of each photosensitizer were applied and toxic efficacy is described in steps of  $\log_{10}$ -reduction; therefore “<3” means a reduction <3  $\log_{10}$  steps (<99.9%).

$c (\mu\text{M})$	0	1	10	50	100
<i>S.aureus</i>					
MB-1	<3	<3	<3	<3	<3
MB-2	<3	<3	<3	>3	>3
MB-3	<3	<3	<3	>3	>6
MB-4	<3	<3	>7	>7	>7
MB-5	<3	<3	>7	>7	>7
MB-6	<3	<3	>7	>7	>7
<i>E.coli</i>					
MB-1	<3	<3	<3	<3	<3
MB-2	<3	<3	<3	>3	>3
MB-3	<3	<3	>5	>5	>7
MB-4	<3	<3	>5	>7	>7
MB-5	<3	<3	>5	>6	>6
MB-6	<3	<3	<3	>7	>7

that were incubated without photosensitizers exhibited normal growth with or without irradiation, demonstrating that the maximal fluence rate ( $50 \text{ mW cm}^{-2}$ ) at the level of the irradiated samples alone had no antibacterial effects. An overview of the killing rates after irradiation can be found in Table 2. In summary, MB-4, MB-5, and MB-6 killed more efficiently both *S. aureus* and *E. coli* compared to MB-1 and MB-2.

#### 4. Discussion

The synthetic protocol presented allows the preparation of methylene blue derivatives with high yields (up to 70%), matching the highest values found in literature [42].

The photobiological activity of methylene blue for *S. aureus* and *E. coli* was described in terms of minimal lethal concentration for  $10^6$  cells/mL by Wainwright et al. [10, 62]. To achieve this killing efficacy a concentration of 1  $\mu\text{M}$  for *S. aureus* and 100  $\mu\text{M}$  for *E. coli* at an applied energy dose of  $6.3 \text{ J cm}^{-2}$  was necessary. Therefore, the phototoxicity data of the derivatives of MB presented in this report are comparable to the toxicity of MB as described in literature.

The photobiological activities of the derivatives of methylene blue show some dependencies on their photophysical behavior and their chemical properties. The structure-response principle, for example, the influence of the substituents on the phototoxicity, can be derived from the following considerations. We expected lowered dimerization ability in compounds carrying the additional cationic charge (**1**, **2**, **3**, and **6**) due to higher charge repulsion, a higher antimicrobial efficacy of these compounds due to better attachment to the cell wall, and an increased stability of cyclic

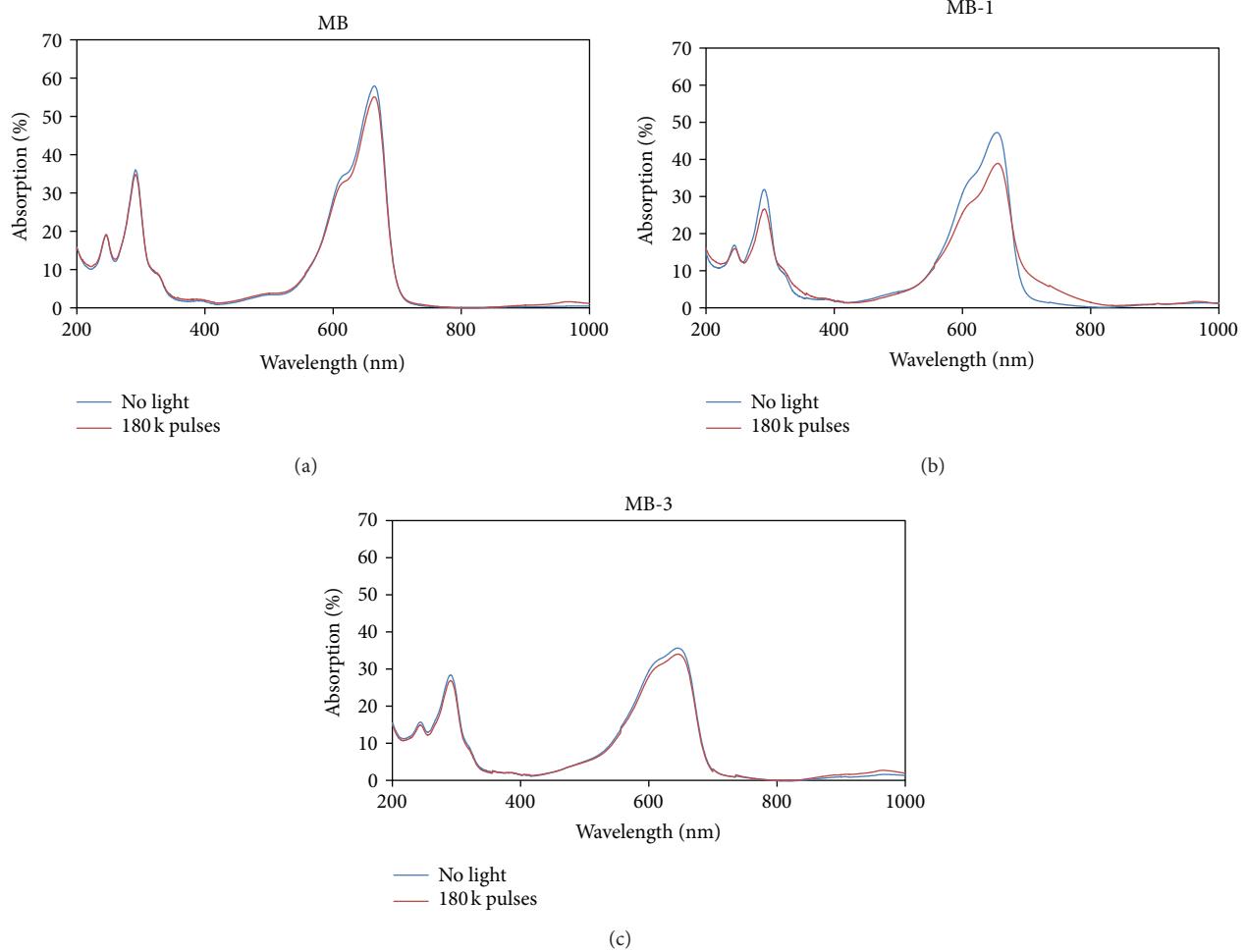


FIGURE 4: Photostability of MB and its derivatives. Photostability measurements in a quartz cuvette with an irradiation at 600 nm with 180000 laser pulses; exemplarily methylene blue and its derivatives MB-1 and MB-3 are shown; only MB-1 and MB-2 show a decrease in the absorption in the visible and in the UV range (see supporting information).

derivatives in comparison to open chain analogs. The last two points were expected to be also true for both compounds carrying the hydrogen bond accepting oxygen substituent (**4** and **5**).

The quantum yield of the photosensitizers gives a value for its efficacy to generate singlet oxygen. But it takes not into account the effective uptake of the light energy. Therefore, the prediction of effective toxicity considering the absorbed light energy by the photosensitizers in combination with their quantum yield is a more realistic value to describe a possible biological killing efficacy. Nevertheless for this study it turned out only for some derivatives to be in line with the measured photobiological activity. Compounds **4** and **1** had a high ranking, predicting effectiveness, whereas (**6**) had a low “Eff.Tox.” value (Table 1). The toxicity data for (**4**) are in line with the calculations but (**1**) and (**6**) show each the opposite behaviour; while (**1**) did not have any toxic effect at all, but was expected to show toxicity upon irradiation comparable to (**4**), (**6**) was expected to show

a low phototoxicity, but inhibited the microorganisms very effectively (up to  $7 \log_{10}$  steps = 99.9999%).

The antimicrobial efficacy of the derivatives that equipped with additional charge in the side chain rises gradually, starting from the open chain substituted compounds **1** and **2**, going to the compounds with cyclic substituent **3** and **6** (for *S. aureus*: **1** < **2** < **3** < **6** and for *E. coli*: **1** < **2** < **3** ~ **6**, Table 2, see also supporting information), and is in good accordance with the calculated “Eff.Tox.” value, except compound **6** (Table 1). Since **1** and **2** show a decrease in their absorption spectrum when being illuminated (see Table 1), the absorbed amount of light energy decreases as well, which might result in a lower generation of singlet oxygen compared to the photostable derivatives (stability: **1** = **2** ≪ **3** = **6**) (Table 1, see also supporting information). Therefore, a lower phototoxicity compared to the stable derivatives might be the result.

The data show, moving from primary to secondary to tertiary ammonium charges, that an efficiency increase can

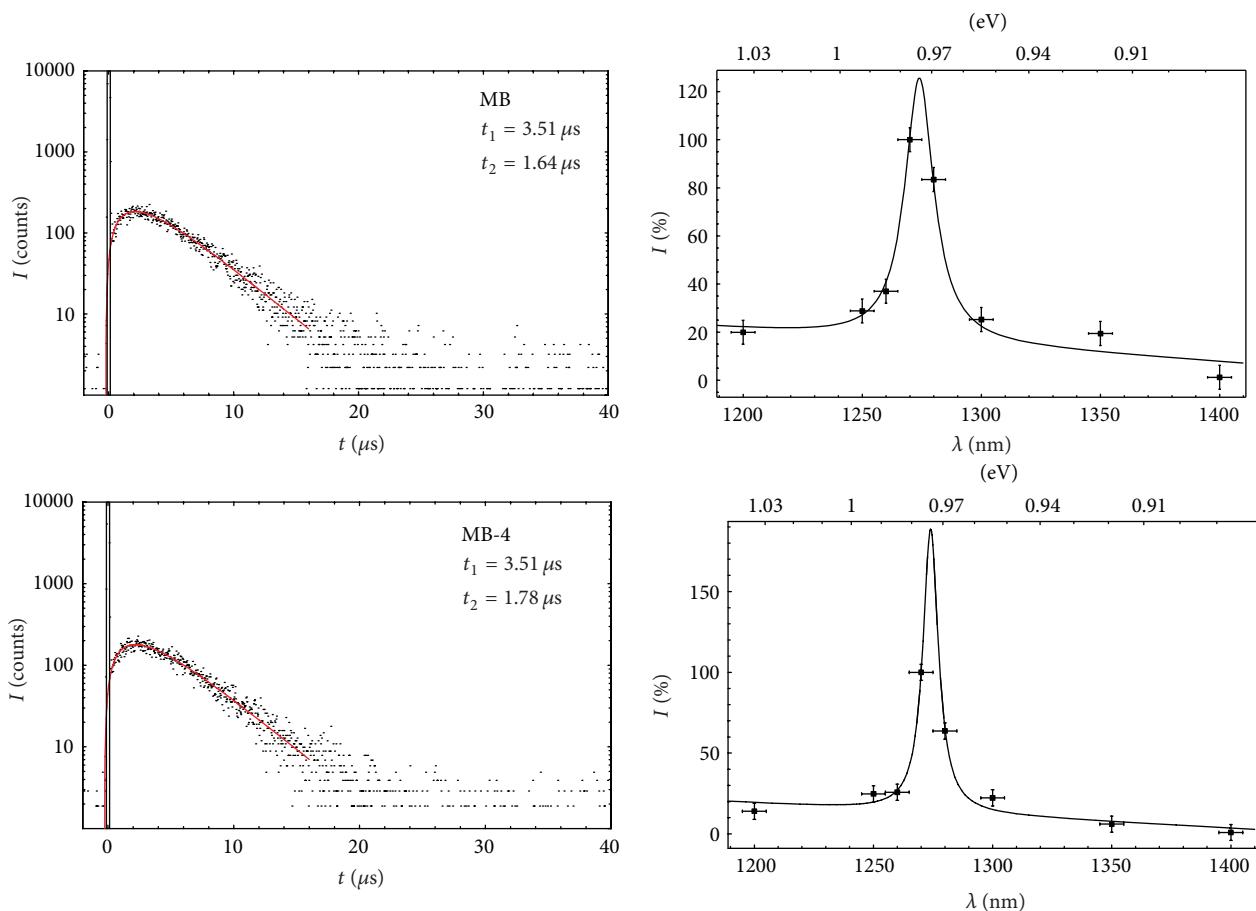


FIGURE 5: Time- and spectrally resolved singlet oxygen luminescence. Time- and spectrally resolved singlet oxygen luminescence of methylene blue and exemplarily its derivative MB-4 in air saturated  $\text{H}_2\text{O}$  at 25°C; singlet oxygen is generated and detected at 1275 nm with a decay time of  $\approx 3.5 \mu\text{s}$  with all derivatives (see supporting information).

be achieved. This may be due to better polarity characteristics facilitating attachment or even uptake. Also, the ease of deprotonation of the groups in aqueous media is assumed to have influence. The pK<sub>s</sub> values of tertiary ammonium groups (**6**) are lower than that of secondary (**2** and **3**), which in turn are again lower than that of the primary groups (**1**). The charges can be seen as more permanent in a pH equilibrium in solution moving from primary to tertiary groups and therefore might have more influence on the attachment to the bacterial cell wall, which is in agreement with the data of the phototoxicity experiments (**6** > **3** > **2**) again. Better cell attachment governed by the additional, more permanent charge might be the main reason for the higher efficacy of compound **6** in comparison to all others, although this was not expected from the calculated value for “Eff.Tox.” (Table 1). Part of the antimicrobial effect can origin from redox chemistry damaging the bacterial cell wall and can be a reason for the higher efficacy of this compound, despite it is only showing a low value for singlet oxygen generation (Table 1).

Free amine bases can be oxidised in solution by singlet oxygen. The more the protonation/deprotonation equilibrium lies on the side of the free base, the more accessible the

compound might be for degradation upon illumination. This can be a reason for the lower photostability of compounds **1** and **2** (Table 1, supporting information).

As expected, compounds **1**, **2**, **3**, and **6** show a lowered ability to dimerize, (Table 1, Figure 2). This can be seen as benefit of the additional positive charge in this class of compounds. It enables the use of these phenothiazinium photosensitizers in a broader concentration range in comparison to methylene blue.

Compounds **4** and **5** are comparable in their stability and ability to dimerize, also matching the values of these parameters of methylene blue (**7**). Both photosensitizers with hydrogen bond accepting moieties show a high activity against *S. aureus* and *E. coli* in the photodynamic inactivation studies (up to  $7 \log_{10}$  steps = 99.99999%, Figure 6, Table 2), whereas the antimicrobial efficacy of **4** is slightly higher than that of **5** against *E. coli* and comparable for *S. aureus*. This is in good agreement in regard to their singlet oxygen quantum yield and the predicted effective toxicity.

In its activity compound **6** can be compared to **4** and **5**; all reach up to  $7 \log_{10}$  steps bacteria inactivation (99.9999%) already below the 50  $\mu\text{molar}$  concentration range. A better linking to the cell wall or even uptake into bacteria is therefore

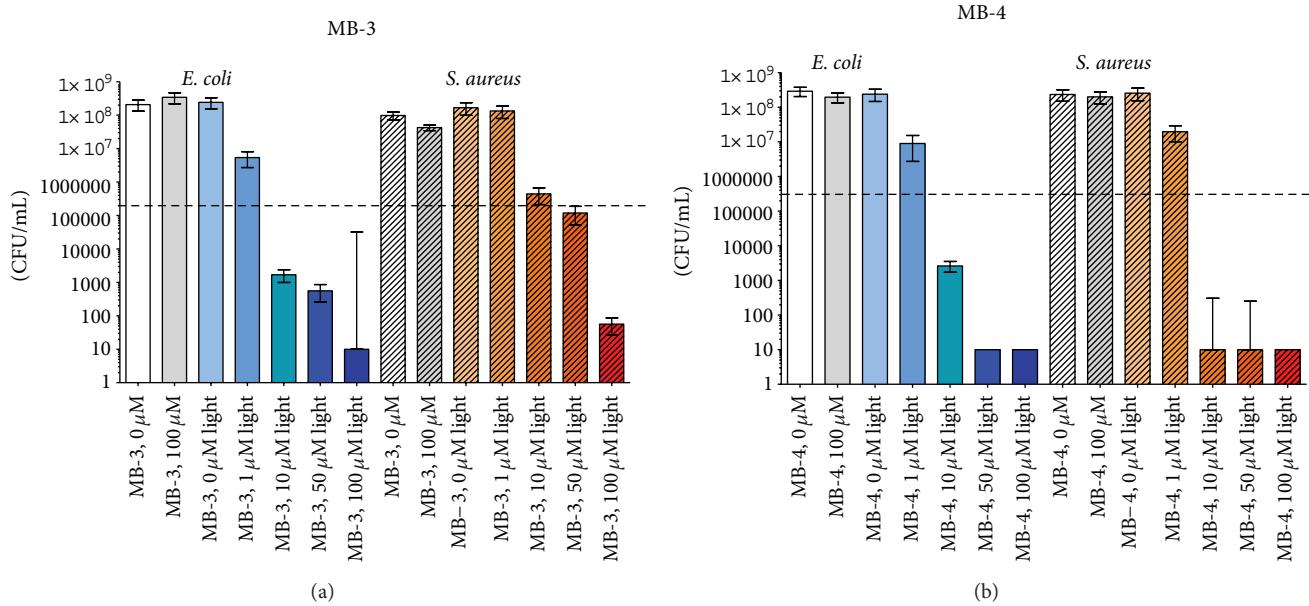


FIGURE 6: Photodynamic inactivation of *E. coli* and *S. aureus* by MB-3 and MB-4. Photodynamic treatment was performed using different concentrations of MB-3 or MB-4 with and without illumination ( $30\text{ J cm}^{-2}$ ). Surviving colonies were counted 24 h later. Black-dashed line corresponds to a reduction of  $3 \log_{10}$  steps in bacteria numbers (99.9% killing efficacy). White and grey column: controls without illumination. Colored columns: MB-3 + light activation, blue: *E. coli*; crosshatched: *S. aureus*. ( $n = 3$  experiments: mean values  $\pm$  standard deviation).

supposed for these derivatives. Therefore, it is interesting to follow the uptake mechanism of each derivative and to investigate the phototoxicity after washing procedures following the incubation process with the photosensitizers in a future study.

## 5. Conclusion

In this study new derivatives of methylene blue derived from modifications of the substituents of methylene blue were described and investigated on their effectiveness for aPDT. One substituent in the methylene blue lead structure (7) was changed, in order to achieve pursuable variations of the compound. We focused on the synthesis and study of derivatives with highly polar and/or hydrophilic groups and prepared the compounds in high purity as chloride salts. For this purpose we successfully revised the literature known syntheses and supplied straight forward protocols for preparation and purification of the photosensitizers.

A structure-response relationship was described from a chemical point of view, based on spectroscopic measurements and on investigations of the photobiological activity against *S. aureus* and *E. coli*. Our results point towards a positive influence on the antimicrobial efficacy by hydrogen acceptor bond moieties and additional tertiary charges in the substituent of methylene blue derivatives achieving  $7 \log_{10}$  steps for *S. aureus* and *E. coli* at concentrations of  $10 \mu\text{M}$  with 10 min of irradiation. This can compete with the best examples of known antimicrobial photodynamic agents like porphyrins. The singlet oxygen quantum yields of some compounds are comparable to the yield of methylene

blue without overtaking it. Dimerisation of such photosensitizers in solution in a broad concentration range can be suppressed by introducing additional positive charges in the side chains.

A simple method of estimating the effective phototoxicity by taking singlet oxygen quantum yield in combination with the absorbed light energy into account was presented for the new derivatives. Not for all derivatives this value for the effective phototoxicity is in line with the data of the killing rates. Some exhibit a high killing rate which is not supported by spectroscopic data and *vice versa*. Therefore, other mechanisms of action have to be assumed and the adhesion to bacteria cell walls and the uptake of the derivatives has to be investigated in a further study, including washing experiments following the incubation period.

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Correspondence should be addressed to Pei-Jen Lou; [pjlou@ntu.edu.tw](mailto:pjlou@ntu.edu.tw)

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We have applied a fluorescent molecule 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC) for tumor targeting and treatment. In this study, we investigated the photo-induced antitumor effect of BMVC. \* OW *Cellline* Studies showed that BMVC significantly killed TC-1 tumor cells at light dose greater than 40 J/cm<sup>2</sup>. The fluorescence of BMVC in the tumor peaked at 3 hours and then gradually decreased to reach the control level after 24 hours. \* OW *Tumor* Treatment studies showed BMVC plus light irradiation (iPDT) significantly inhibited the tumor growth. At day 24 after tumor implantation, tumor volume was measured to be  $\text{nm}^3$ ,  $\text{mm}^3$ ,  $\text{mm}^3$ , and  $\text{nm}^3$  in the iPDT, control, light-only, and BMVC-only groups, respectively. Immunohistochemistry studies showed the microvascular density was significantly lower in the iPDT group. Taken together, our results demonstrated that BMVC may be a potent tumor-specific photosensitizer (PS) for PDT.

#### \* O U S P E V D U J P O

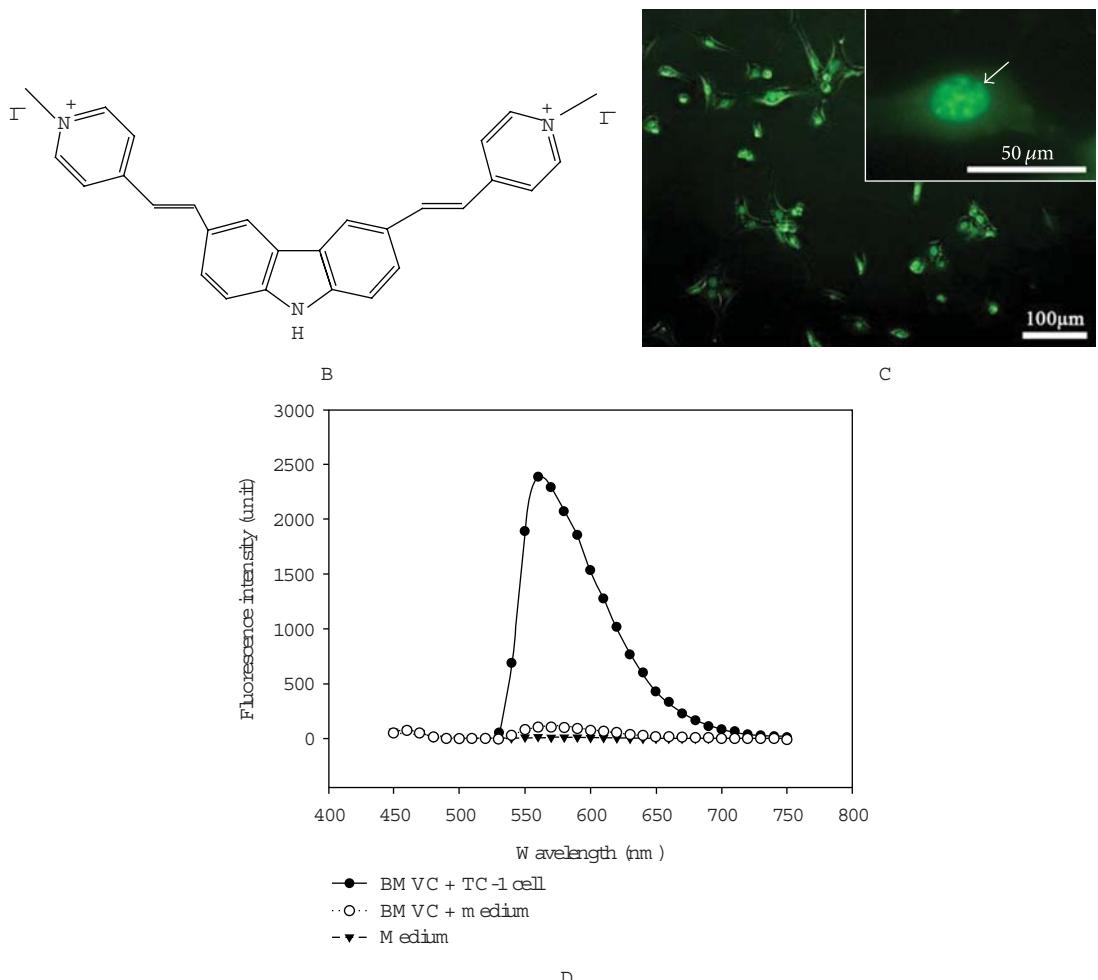
Tumor-targeting therapy has emerged as an effective and attractive treatment for cancer. Among the various cancer-specific targets tested, telomerase has gathered much attention in recent years. Telomerase is detected in about 85 to 90% of cancer cells, but in a low level of normal cells [1]. The maintenance of telomere length by telomerase is required for unlimited proliferation of cancer cells. Telomere has been the target for the development of cancer probes, and telomerase inhibitors have been developed to inhibit telomerase activity and limiting cancer cell growth [2].

In the search for tumor-targeting agents, we have recently developed a fluorescent molecule 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC) for recognizing specific quadruplex structures such as the T AG telomeric repeats and inhibiting the telomerase activity [3–5]. Intriguingly, the fluorescence of BMVC detected in cancer cells was much stronger than that in normal cells, suggesting it

to be a good candidate for a tumor-targeting agent [3]. The maximum absorption of BMVC is shifted from 435 to 460 nm and the fluorescence intensity increases significantly when BMVC interacts with DNA [5]. Because of the ability of telomerase inhibition, BMVC induces accelerated senescence of cancer cells [6].

Photodynamic therapy (PDT) is an effective treatment for cancerous and precancerous lesions [7]. The advantages of PDT are that it can be repeated in the same site if necessary, and it is less destructive than traditional surgery. PDT requires PSs that are activated by specific wavelengths of light. Illumination of tumor results in the destruction of cells due to a photochemical reaction. Reactive oxygen species, including singlet oxygen and free radicals, are generated by the photochemical reaction [8, 9]. This photochemical reaction is capable of inducing cellular apoptosis and necrosis, by evoking oxidative stress [10]. In addition, PDT may cause tumor cell death indirectly by damaging tumor-associated vasculature or activating host immune responses [9, 11].

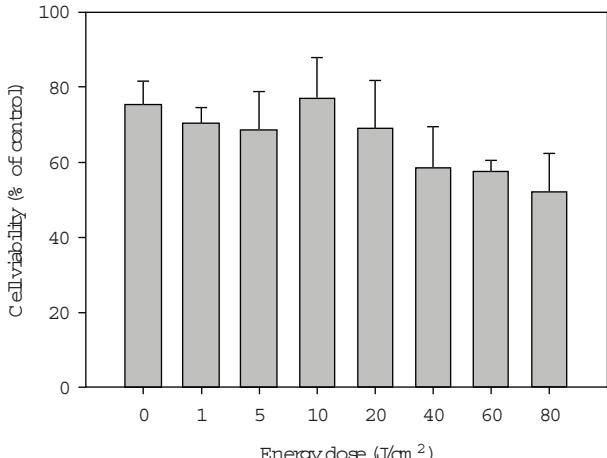
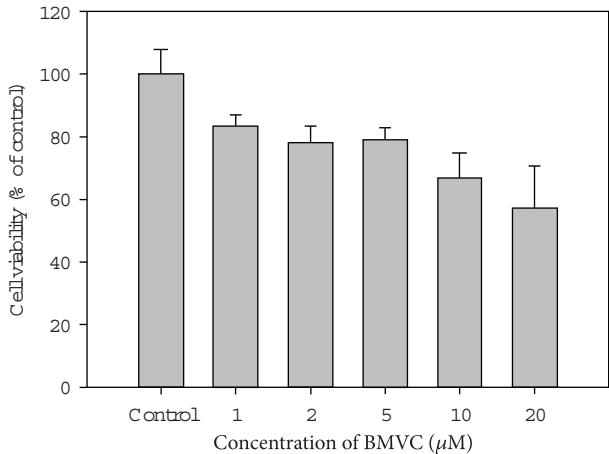
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1 S F W J P V T M Z X F I B W F J O W F T U J H B U F F B O U U I F U V V N P P S F T D F D O D P F S # . T 7 \$ D P N O B O D F F O F S H Z U S B O T G F S ' 3 & 5 C J @ B W F M E H O Z W U P F G M J U H I W X U D T P J @ T W F T T U U J T H P B G U # . 7 \$ D P O K V H B U J O H Q P S Q I Z S J O < > 8 F G P V O E U I B U 1 % 5 F D J F O D Z J T H S F B U F S X I F O F Y D J U F E C Z O N P G M J H I U B T D P N Q B S F E U P O N P G M J H I U \* U J T T V S Q S J T F E U P U E S C F J B M F S 1 Q E F F D U J I F B E Z J T P C T F S W F E V Q P O F Y D J U J O H U I F ' 3 & 5 E P Q P S O U # I F T J [ F E G S P I N F S U I B O U I F B D D F Q U P S Q P S Q I Z S J O E F T D S J C F E S F W J P V T M Z < > F D U D P V M E S F T V M U G S P N B U Z Q F \* Q I P U P E Z O B N J D S F B D U J P O < > C F D B V T F X F E F U F D U F E O F J U I F S U I F D I B S B D U F S J T U J D T Q F D U S B M T J H O P G T J O H M F U P Y Z H F O O T P M O V N U J G P S O P \$ N F M # M - 7 E S N Q P F Y T F \$ # - # M O P S U I F E F D S F B T F P G E J Q I F O Z M J \$ P C F @ B T N G V S B E O J 0 % F 1 \$ # U @ B V I C B H N U Q P B E M J O P S H B O J D T P M W F O U ) F S F X F I B W F F \$ Y B 5 N \$ J O D F E M U M I T F X Q F P H P S P F X Q J J O D 3 J 1 U . 2 N F D I B O J T N P G # . 7 \$ B O E J M M V T U S B Q J F U E I G F U B M Q B M F T O F U S J V B N M U \$ P C F V T 6 F E N - B T B Q I P U P T F O T J U J [ F S 1 4 G P S Q I T U G F Q U Z P N B R J Q U B Q F E S B Q H 1 N 5 ( % F T Q J U F U I F Q P U F O U J B M B E W B O U B H F T J O D M J O J D B M B Q Q M J D B U J P O 1 % 5 I B T T F W F S B M M J N J U B U J P O T U I B U I J M Q F S F T D F U S F M M A F Q F D M U J S P B M E B D D F Q U B O D F " N P O H U I F N T V T U B J # O F B B Q D Q U I F G X B F T F P T F Q T Q F J B F B B O E M P X U V N P S T F M F D U J W J U Z B S F D F M M N V B T Q F Q Q P O F M F S N F T D G M S M U F B F E 1 4 T < > F Q V S Q P T F P G U I J T T U F S F T D P C T D F R J D O S W B Q M B U J H S B F B E F S I F 4 Q F Q I P U P D I F N J D B M F F D U T P G # . 7 \$ % P F Q J U D V F N P 4 S V D O R O M Z M B M F F M M G M V 4 M " B S J U I D Z U P U P Y J D J U Z P G # . 7 \$ X B T F W B M F V R U I F E I M O V S P S F T D F F O M F M J N O F T T J P C

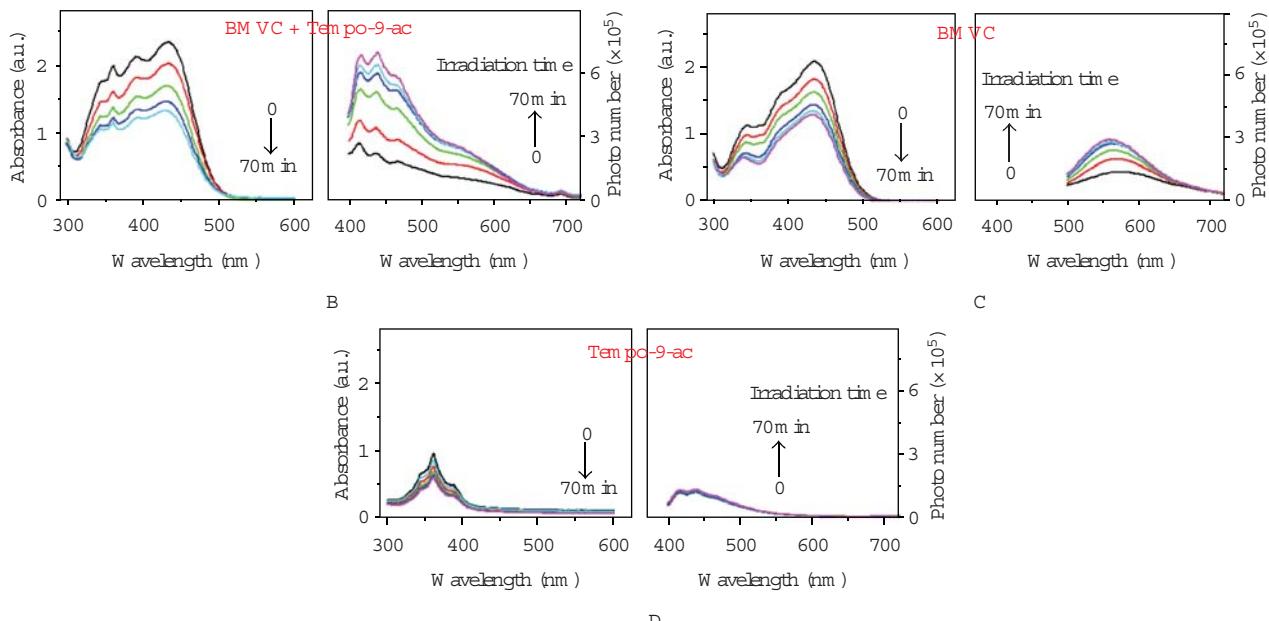
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O ON X F S F S F D P S E F E C Z T D B O O J O H ON X B W F M F O H U I X J O E P X T G P S F B D I T B N Q M F < > P X D Z U P N F U S Z ' " \$ 4 \$ B M J C V S B O E \$ Z U P U P Y J \$ B M N U Z X " F T S T F H Z S P X O J O # J P T D J F E O D F T X F M M Q M B U F T D F M M T X F M M B O E U I F O J O D V C B U F E X J U I # . 7 \$ G P S I P V S T B O E D I B O H F E U P G S F T I D V M U V S F N F E J V N I P X J P U E Z Q V N Q D F D S M Q F E B O E U I F D Z U P U P Y J D J U Z X B T E F U F S N J O F E C Z U I F " M B N B S # M V F B T T B Z < > " M B N B S # M V F P G D V M U V S F N F E J B X B T Q B E F R M T X U F S E Z O D V . C . B U F \$ X U P F B D I U F T U X F M M B O E B O B M Z [ F E T Q F D O B S P L Q O I F P T U T P G N P S G U " S I J F D V S X M B M E Z U X J U F B C T P S C B O D F E J F S F O D F C F U X F F D F M M T X B F G E J N N F E N B U F M Z F Y Q P T F E U P E O N - %. 1 L F C U V O B Y ( F S N F ' S F 3 B E J F F N B J M H ' T T U T B P V S D F G P F F U F S N J Q F E N F O U I P V S T B F S M J H I U U S F P G 1 % 5 F F D U X B T B X I J U F M J H I B G B 8 9 F O P O M B N Q B U O I E B U + D N Q B T T F T U I S P V H I B O O N N J S S P S N P E V M F O B M M Z U I F M J H I U Q P X F S X B P T D U I F T N B N Q M I F N T V S G B D F \* 0 7 J # W . P 4 \$ V E N Z H L H X B T J O K N J D F X I F O U I F T J [ F P G U V N P S S F B D " O J N B " M M B O J N E Z M T U V E J F T X F S B F C P Q H S P E W B F E B C Z S U S I F J O K F D U J P \* O T U J U V U J P O B M " O J N B M \$ B S F \$ P N Q K F D U J P N B D M F X F S F T V F K F D U F E # P J O U N J D F X F J H I J O H O H X F S F T R Q E F T B S J E C F E G F I G P S F O S W F S T J P U Z O C - B C P S B U P S Z " O J N B M \$ F O U F S B O E X Q F T S F E B M F S D X V H B G O S P V T B M B F Q U P U U P I F G P P E B O E X B U E S 5 \$ S D F M M E - T P J G O ) # Q B T T F E U I S P V H I U I F O F F E M F F U J Q P X F S F J O K F D U F E T D J O U P # N J D F C F B U B G P V U D N N P V U T U E F P G U J F O F F E M B N H L H X B T J O K F D U F E T J O H M F E P U P V D J F E U I E B U V N P S U I F T U V N P " T J O H M F X F E U P B S P V O E U F P M F Q H E N U N I E X J F P G I ( F S N B O Z U I F 5 \$ U V N P S N B T T B O E U I F C P E Z X F J H I U P G N J D F B U B O E E B Z T B F S # . 7 \$ J O K F D U J P O X F S F S F D P S E F E 5 V N P S T X F S F N F B T V S F E J O U X P P S U I P H P O B M E J S \* P N V G P I P T O S I P Z D T F H E N U J T P U O S A T S G Y I W P M V N F T X F S F F Q U J < N B U F E > B T M M B . N Y I N D M Q F T T X F S G F P S N Y F M E J X J X H T I H D I B M M F O H F E X J U I 5 \$ D F M M T I B E U I F W Q M N V Q P E T U T B Q M P C M F P T S D 4 V . F N P B S U B U E B Z ' 4 . " Q P T J U J W F D F M M T X B T D P V O U F E J % J T U S J C V U J P Q J F X F S / F \$ T B Q S F H J P O T 6 Q J U D P V O U T X F S F F Y 4 Q . S " F T T B O E I P V S T B Q P T J F U J W F # 7 V \$ P G J U V N F S S I N J N P T O V F B O E U I F U J T T V F T X F S F S F N P W F E I P N P H F O J [ F E B O E B O B M Z [ F E C Z B V P S F T D F O D F N J D S P Q M B U F S F B E F S F 4 V B I U J T U B D F H W E F O U M B B W I E X M B J T T F E U B B O N 5 J T T V F T G S P N N J D F I U P V F S F B Q F O S T F U P H D J E D C K H F F D J W P D G E J U J U J X F S F D P M M F D U F E X B T I F E X J U I 1 # J P T U B U Q E U J D B M G B E B E M Z T J [ T F V E T J C O Z H U I F 4

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# . 7 \$ ' M V P S F T D F D O I F F J 4 D Q F T D U S S V B Y U V M S F & P. G 0 B D \* O W J U S P H F O # . 7 \$ X B T T I P X O J O ' J H V S F B U Y S F T S B F E J D B M P T F N M P M T Y M E M P F O H V M M W F E I U V S F E J O U I F Q S F T F O D F P G # . 7 \$ U I S F T V M P U S G H T J D F V O P D S F T F T D H R P D M F T U V F S C F P C E F U F D U F E N B J O M Z J O U I F D F M M O V D M F J ' J W S V S B F E J C B U . J. P ' C H P O G # S. F T T B E Q F N J T T J P O T Q F D U S B P G # . 7 \$ X F S F J E O D S F J B P T Y F O E T Q ' H D B H M V G S. F P N ' 5 D H V S I F O T D M F B S M Z E F N P O T U S B U F E U I B U U I F V B B O S E F C T D F D P R N d B O S U F E O F V Q J B T B G H # F . E 7 M S J O D S F B T F E G P S B M N P T U P S E F S T P G N B J H V S F U V E D F V Q P T Q F P N P F T S U B Z Q V J D B H X J U I D F M M T

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\* O 7 J U S P \$ Z U P U P Y J D J \$ U Z P B U Q E J 1 D F B U H P D E U W F Y D U S P D U S B O T G F S U I S P V H I U I P G # . 7 \$ U P 5 \$ D F M M T X F S F W C B Z M U V I B T V F Q F S P Y R U E H F G # S B \$ J P O < U S F B U N F O U E J E O P U I B W F B Q Q B S F O U U I P P M M F D U T J U M J U B S S I F F D V M U M T E T " J H V S F X J O U I J H V S F P G D F M M T X F S F T U J M M T W I J P B C C M F X I F O U I F # . 7 \$ D P O D F O U S B U J P O & K F S H C I F F M # P. X 7 \$

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1 I P U P J O E V D F E D Z U P U P Y J D J U Z N J D S P . T 7 D S P X B T F W B M V B U E E B T B F S J G V O D U J P O P G M J H I U E P T F ' J H V S F S F T T P X E T E P I F E Q I P U B U P G F U V N R S D S F J M U M Z T D Z U P U P Y J D J U Z P G # . 7 \$ V Q P O U I F U Q P T J B U J N W F Q U P B G P S # . 7 \$ V P S F T D F O D F

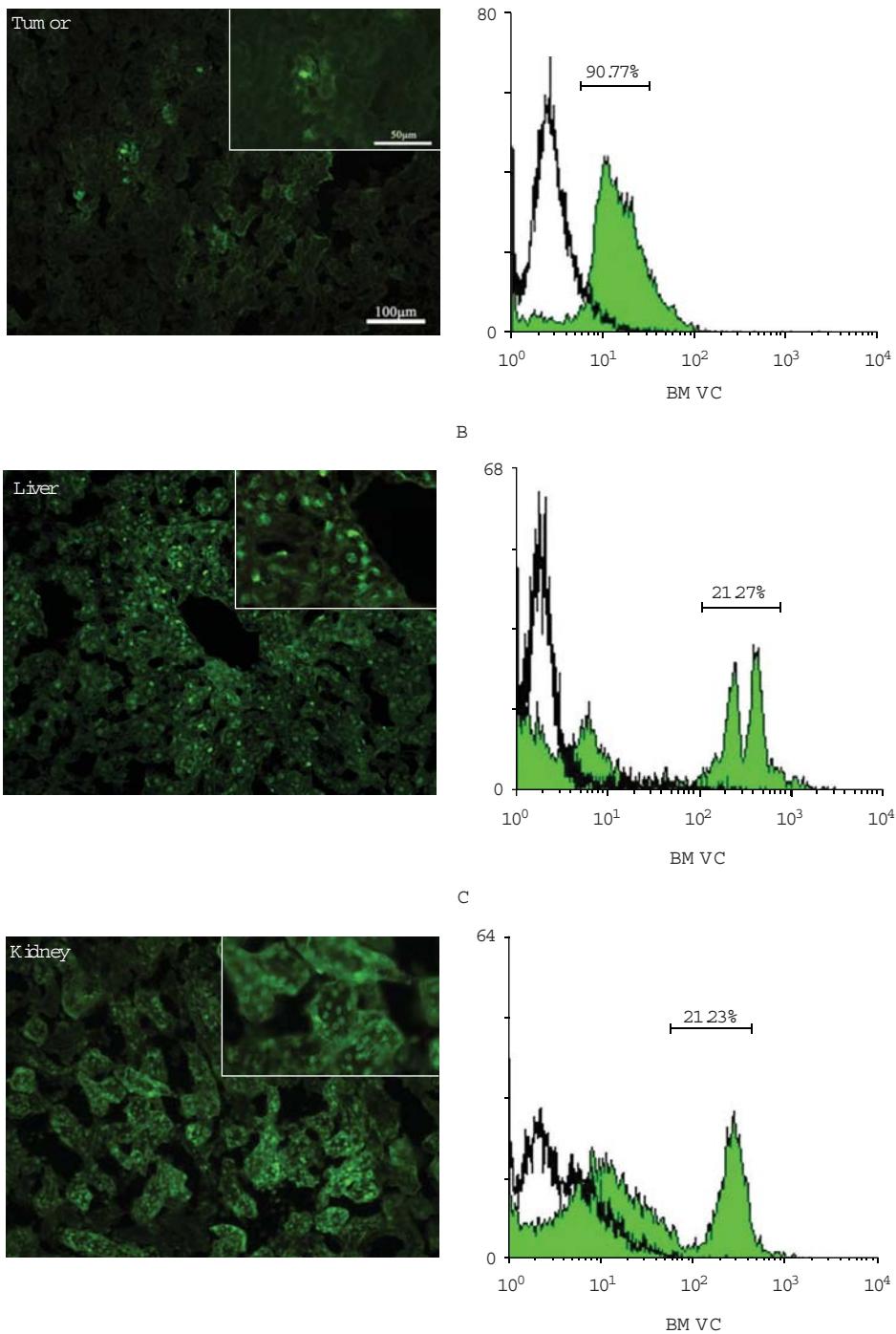
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5 Z Q F \* T F O T J U J [ B U J P O B T T V N F T B J O F F U G I F S H N S B E V B M M Z J O D S F B T F E J O U I F U V N P S U J W J B F M F D U S P O U S B O T G F S F E V D U J W F P I S P P V S T E' B H U V S F B J O W P N M J W M G S M Z

H S B E V B M M Z J O D S F B T F E J O U I F U V N P S U J

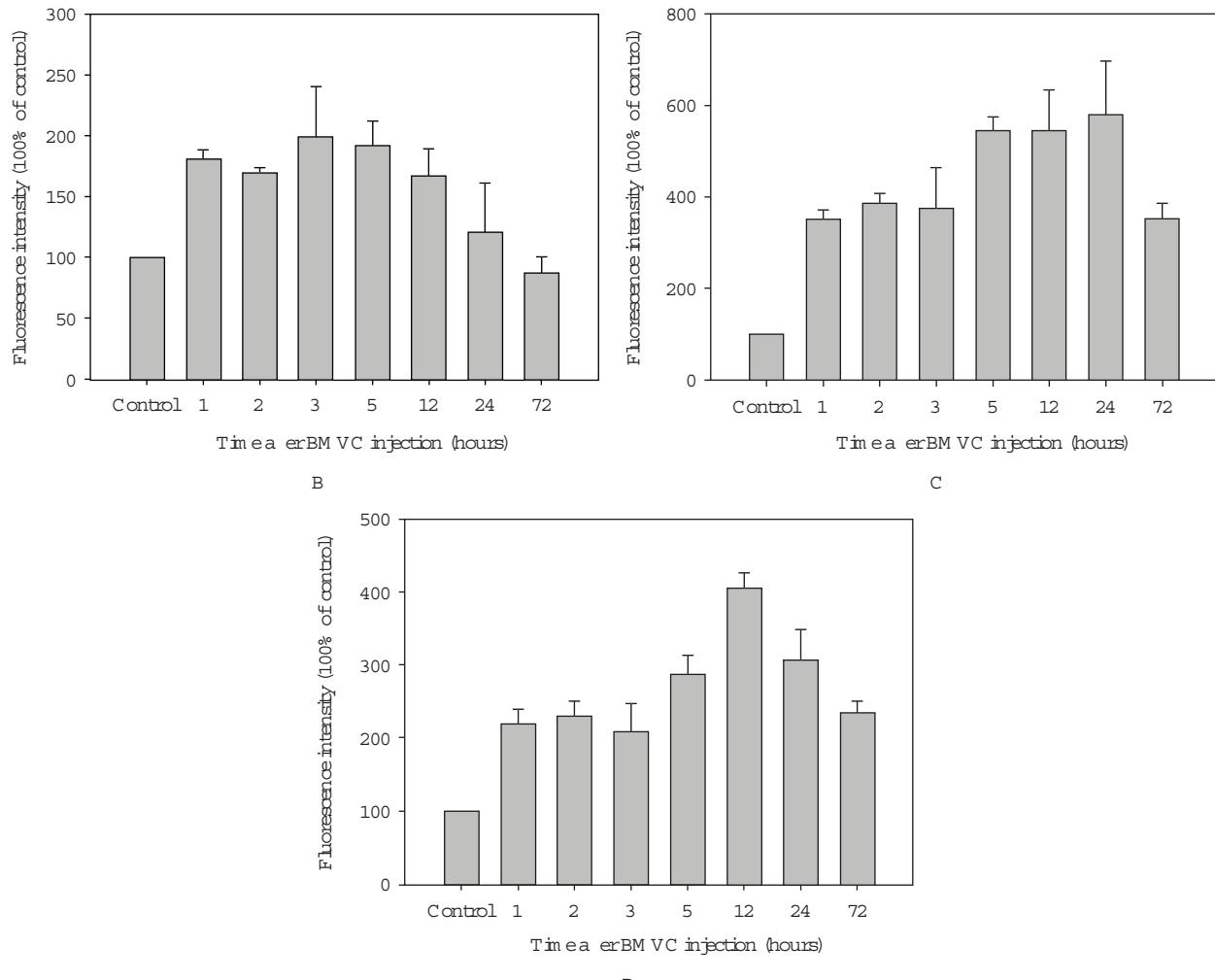
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J O U I F M J W F S B O E L J E O F Z J O D S F B T F E B F\* S O E 7 S W P S V N P S 5 J P D W B M V P R O W H O W E E F E T  
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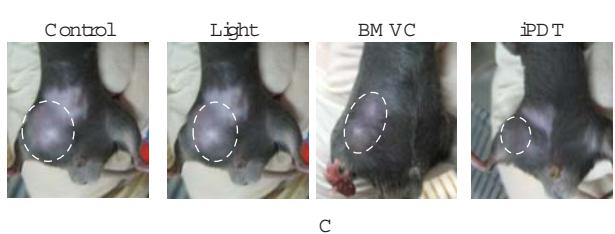
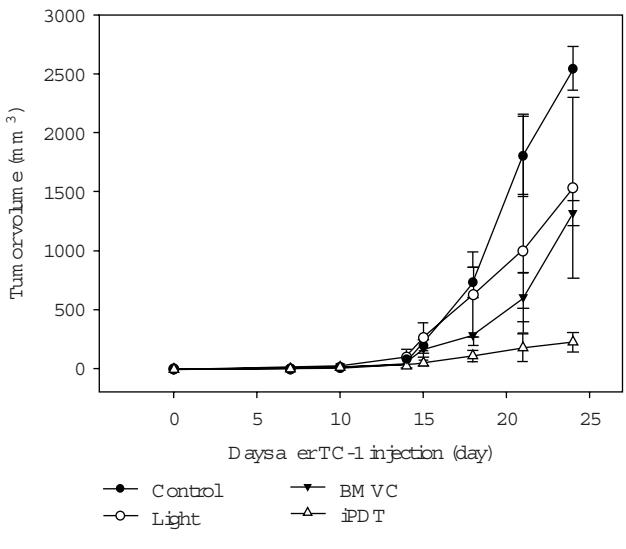


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P G B D V U F U P Y J D J U Z J O U I T J I T P # X F E Q H I F R N B O U ' J J ' H D B S U E Q P D J P U G J W F D F M M T V O H S P X U I D V S W F T P G U V N P S T V O E F S E J I F B O F J O U W U G T F B \* U U N J F T G P V T O E U I B D U M H B S N T I P X F E U I B U # . 7 \$ Q M V T M J H I U J 1 @ T S H O T J D H B O O J U M I Z B M @ X F M E J F O S C J P C U I U U F I E F U I F U V N P S T H O W P W U P I " U E B Z U S E B U F N S F O U T O O U I F P U I F S I B O E # U V N P S J N Q M B O U B U J P O U V N P S W j P M N W N D P S X F B T F N D E B O T U V E N E S J P P W C B D V M B S E

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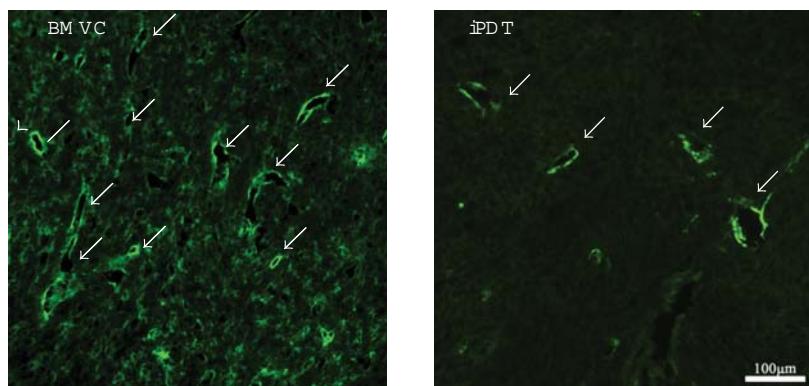
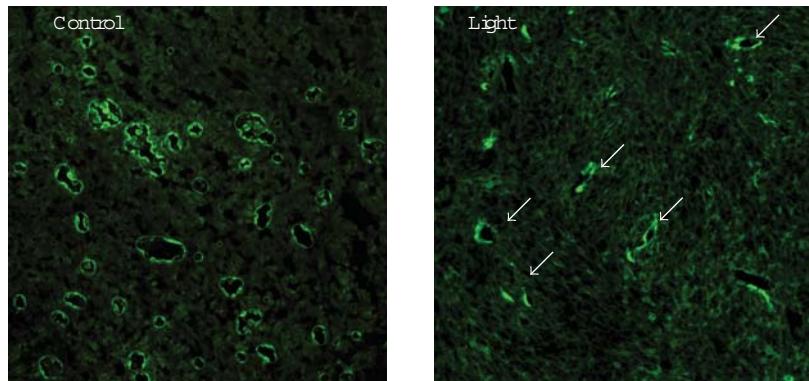
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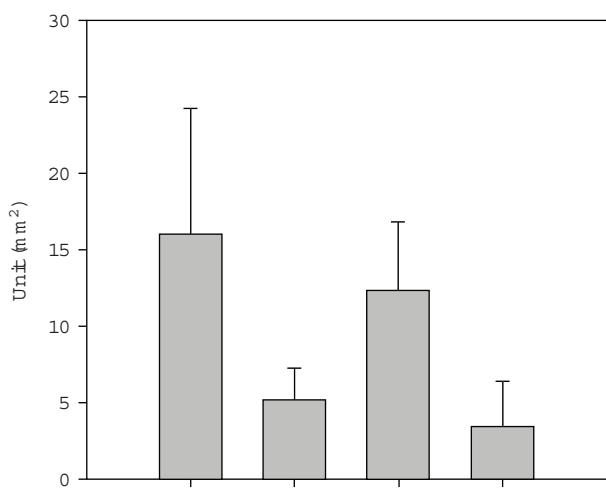
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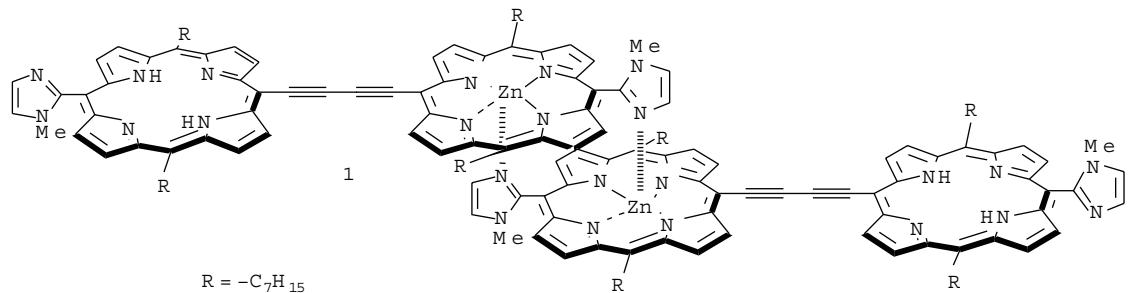
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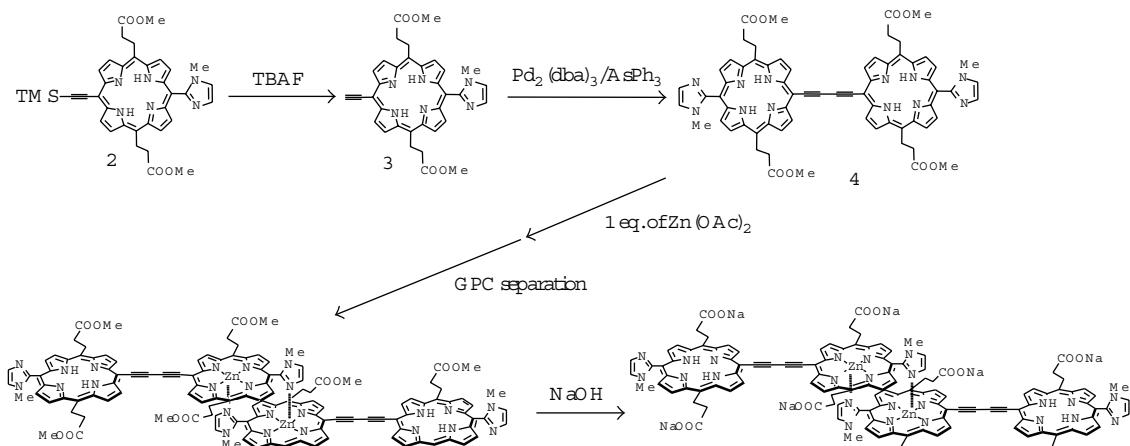
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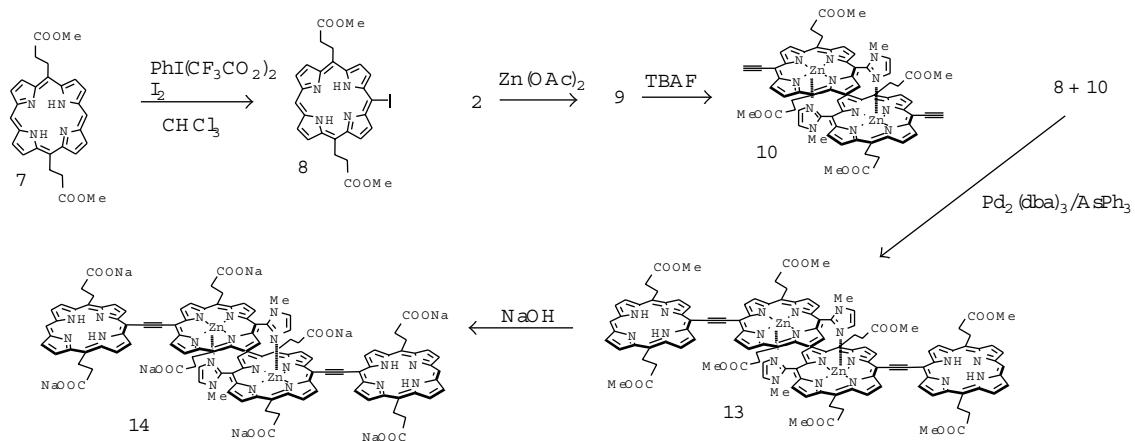


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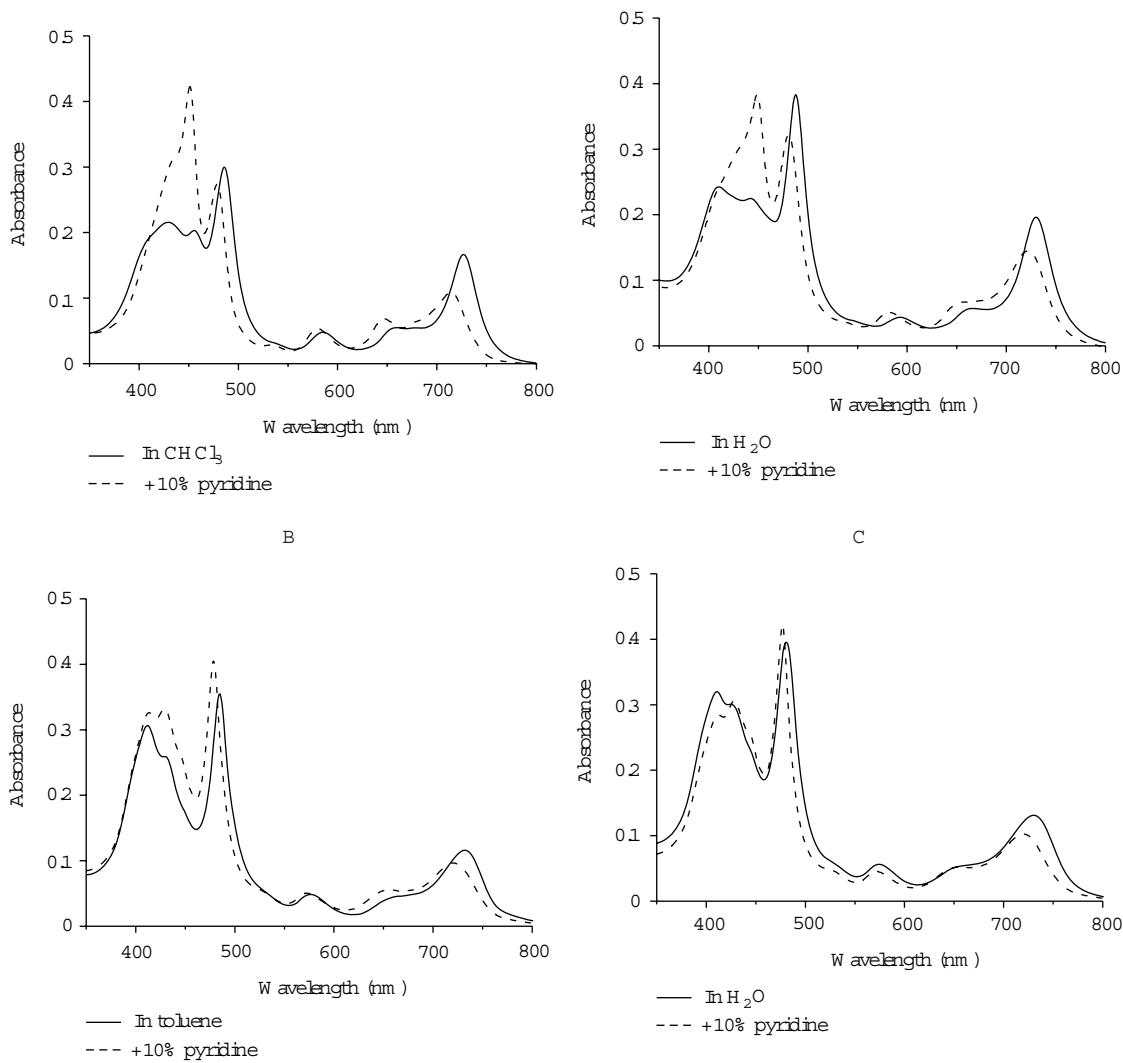
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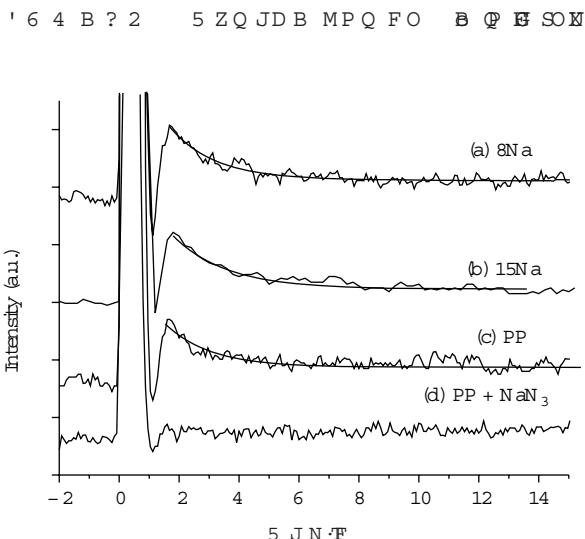
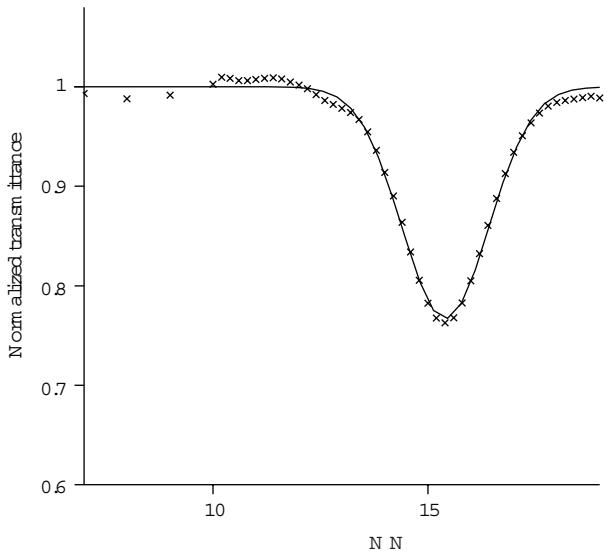


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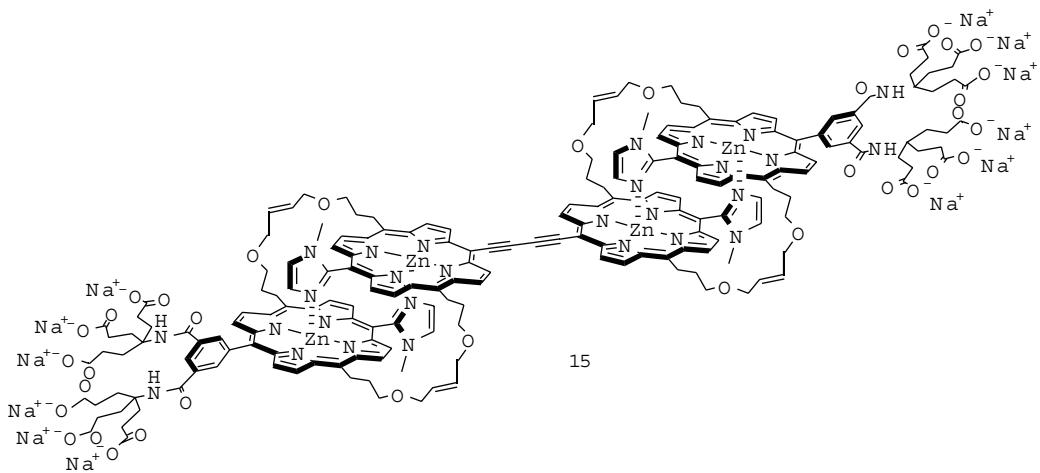
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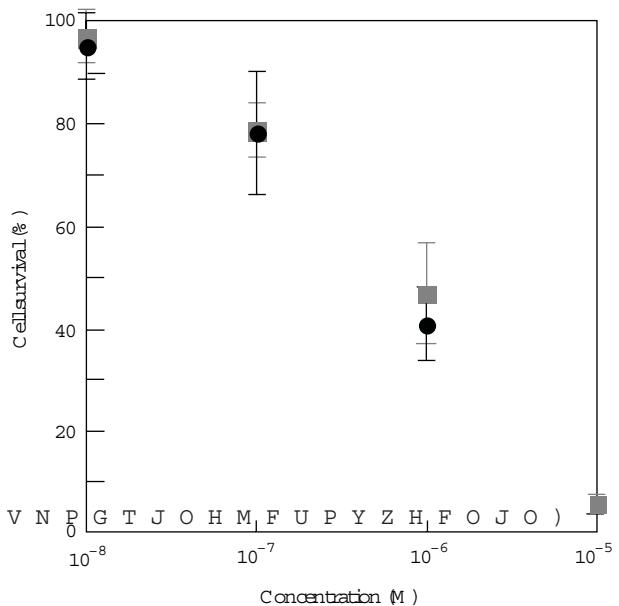
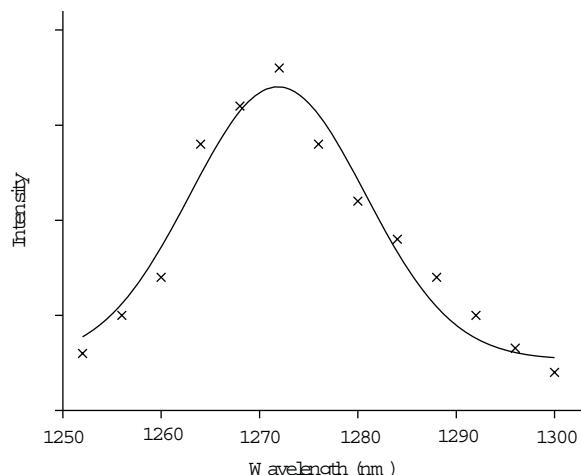
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# J P . F E 3 F T F B S D I \* O U F S O B U J P O B M

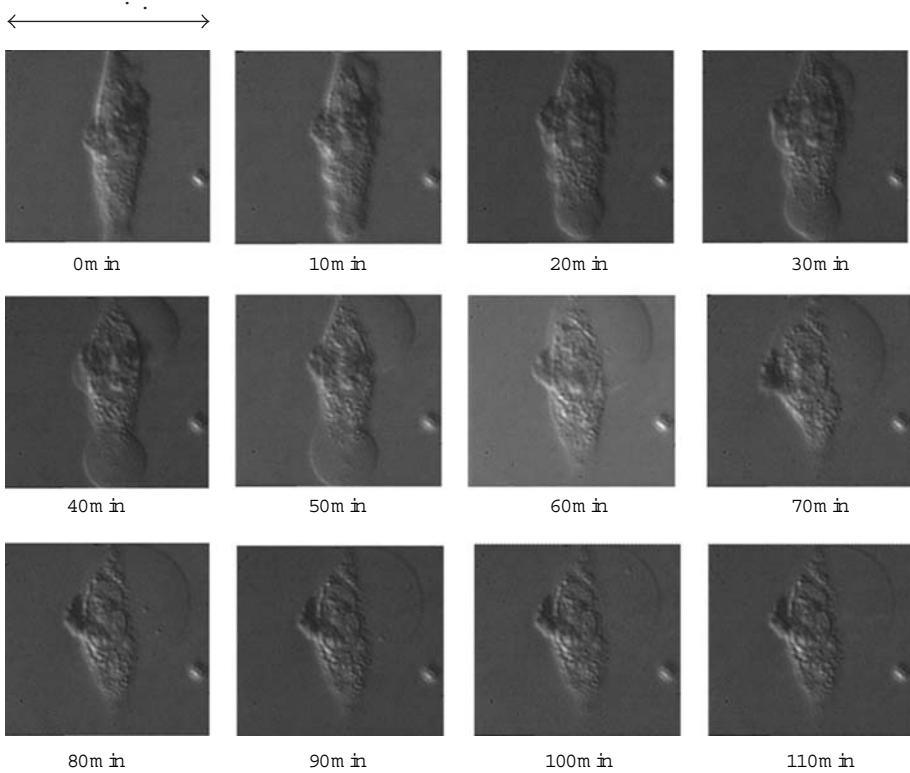


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# J P . F E 3 F T F B S D I \* O U P



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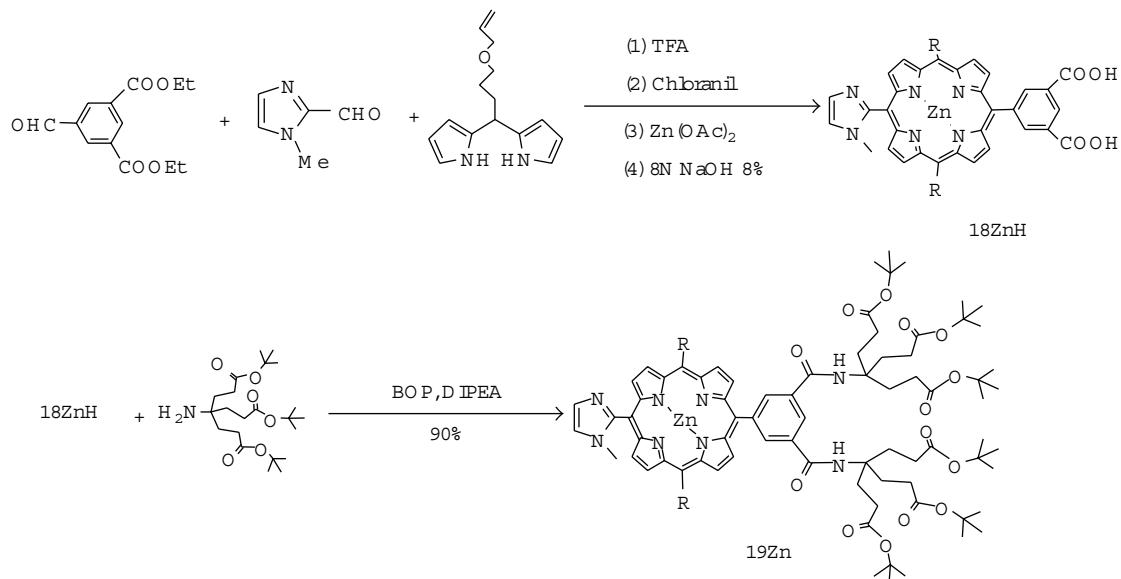
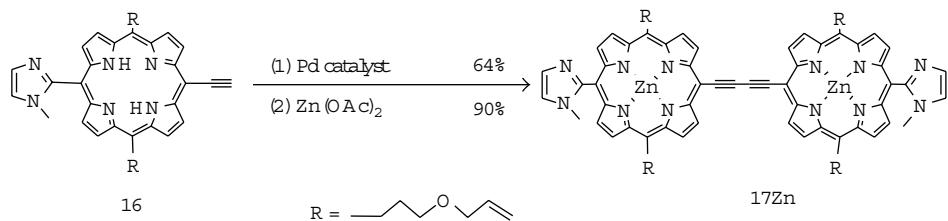
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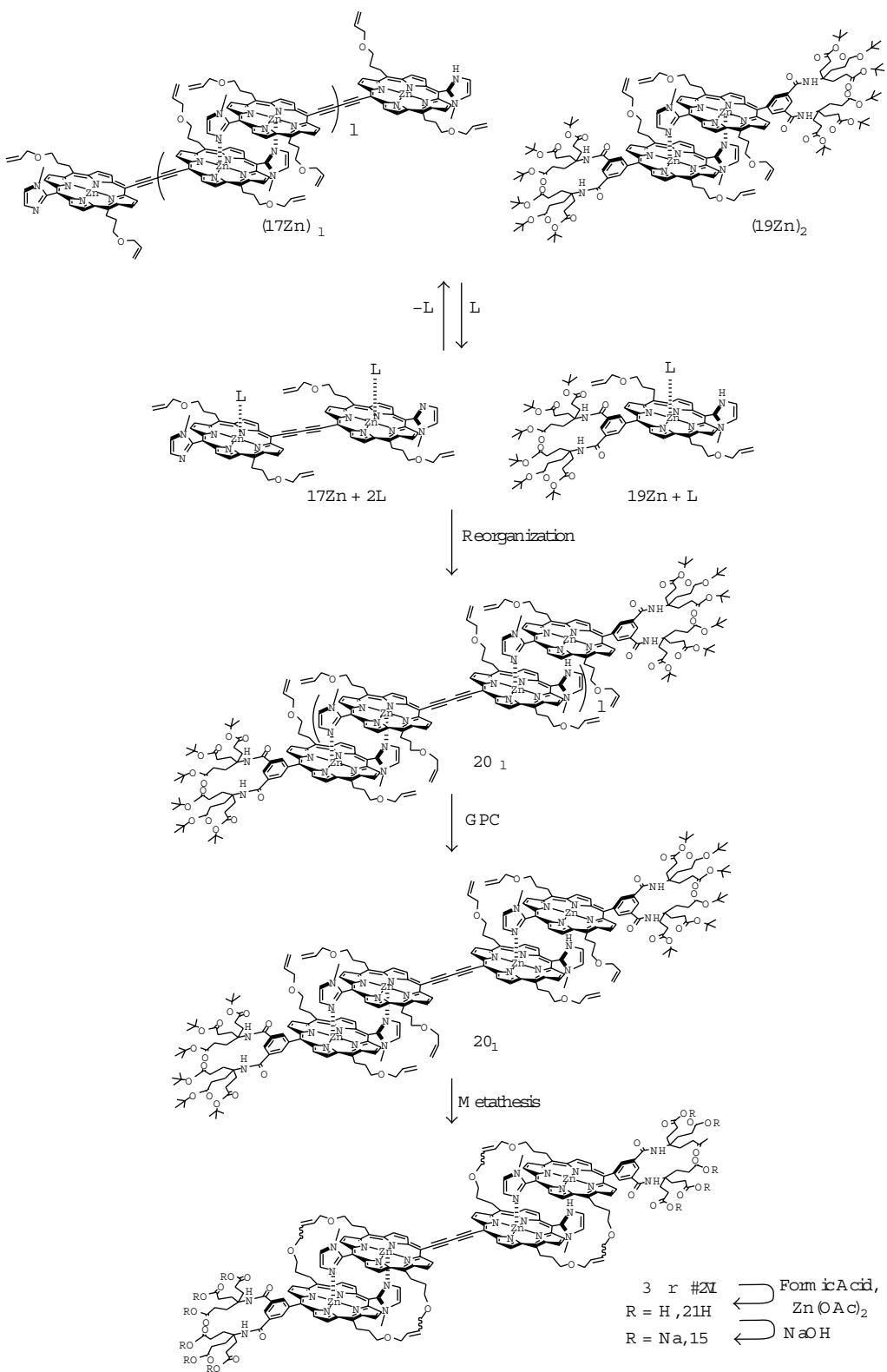
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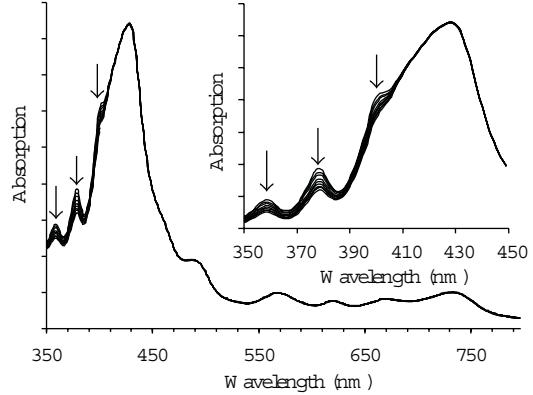
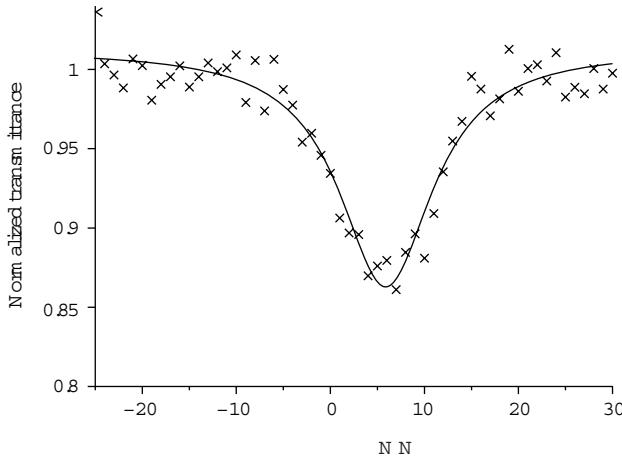
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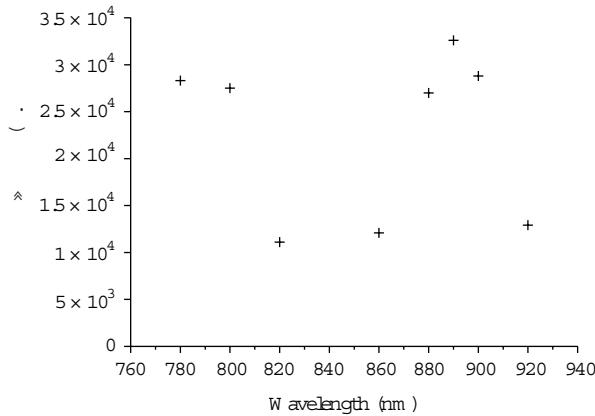
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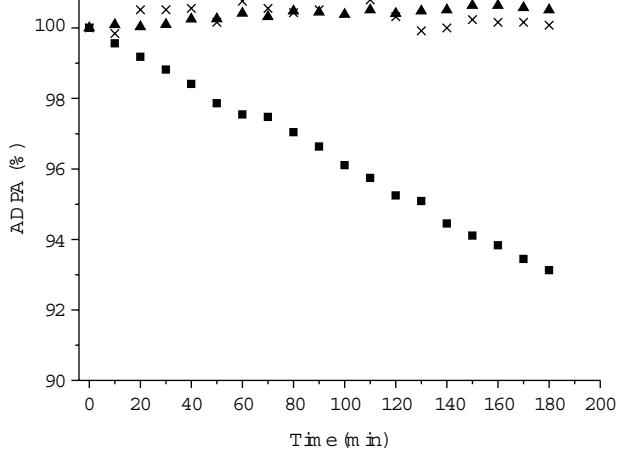


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B C T P S Q U J P O X B T P C T F S W F E J O U F I T F B T N P F M T F S U F T V M O U X T V H H F V J U B Q I P E R U O N P N Q  
F Y Q F S J N F O U X B T D P O E V D U F E C Z V T J P U P U F H G S P B Q F P F O P E Z M O B R N S D B B F S W J P U

U F U S B T V M G P O J D B D J E 5 1 1 4 X I J P F M P I D B F W F U B S Z M F U O H F G U V R P S E F O M D M Z T W  
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T R V B S F J T J O E J D E J U T F B Q P U F B U U F P B N P U P J V O H E P D P O E V D U B D P N B Q E S B U

B H F O U G P S 1 " 1 % 5 F E F U B J M F E F V Y P F Q S F W N F P O V U T B D M P N Q P V E O E U T J B P S T E B F O S E U

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' J O B M M Z U I F 1 % 5 F Y Q F S J N F O U X J O U F Q H S B Q I P U P O J S S B E J B U J P O X B T D P O E V D U F E V T J O H ) F - B D F M M T " ) F - B D F M M J O D V C B U F E

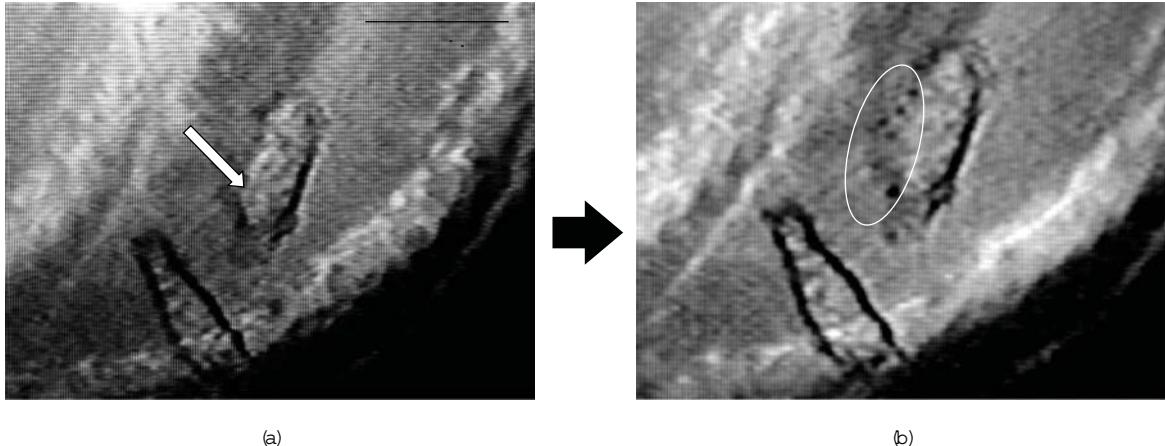
X J U I P O B H M B T T T M J E F X B T J S S B E J B U F E G P S N J O X J U I G T

Q V M T F T B U O N X J U I B O B W F S B D L O P X M F S E P G H N N F O X U I T J D I

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D P O E J U J P O T X F S F E F T D S J C F E J O < G B S G F N F U T H T F X D O P O E O ; J T H D V B S N F B T V S B F N P

B ) F - B D F M M B U U I F V Q Q F S T J U F X B 4 W J T F O M D F B C U E J 5 W F D M O Z M P Y H D Z B W F E E S P P O G F T



(a)

(b)

' 6 4 B ? 2 1 J D U V S F T P G ) F - C F G H F S M F M B J H O O D E V B C B S J F E U X J P Q I I P U P O F Y D J U B U J P O X J U Q P T J U J P O J T N B S L F E C Z B X I J U F B S S P X F E F H S B E B U J P O P G U I F D F M M M N F N C S B O

G P S ) F - B D F M M F Y Q F S J N F O U T B U 5 P L Z & \* P O U P E J Z J O N V I D I U F U P F C S 5 B R Y D E T I U S Z B M P P M H I Z P U B O E B M T P 1 S P G F T T P S 5 ) J S B O P B O Q E P % S Q Q , P O P G P S 1 % 5 F Y Q F S J N F O U T B U ) B N B N B U T V 6 O J W F S T J U O Z H B P B I P P M B P F G B X E E : J D O B J C O B F U B F B V U I P S T U I B O L % S T : \* O B C B J E B [ p M B Z M Q F P H Q B Z S o B T Q M E G + B F T F N E M J F B U / B S B \* O T U J U V U F P G 4 D J F O D F B O E 5 F P S Q O J P P M D S # Z T P D S U J P I O F G T S Q P U F P O S U U B P O U I F T F T U V E J F T U I F S B + Q P V S W B M P G . F E J D J W Q B M \$ I F O N P J T U O

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< > + 5 % Z , 0 H B X B " 4 B U B L F " i 8 B U F S T P M V C M F T F M G B T T F N C M F E C V U B

< + > . P B O B O E 2 1 F O H i " O P V U M J O F E G W B Q P I J F G W U B M U Z X P G Q I P S P O w B J C O T P S C J C 1 I P U P E Z O B N 5 D 1 B U F S S J B Q E & E F 3 P U Z P E M Q B E N D D J U S H I U P N Z S H E Q M Z w O P Q Q \$ I F N J T U S Z \$ B N C S J E H F . B T T 6 4 "

< > 3 8 8 B Z O B O U i - B T & N F D U S J P Q W E D I D B P G E H W B J X Q B + % Z : , P C V L F 4 0 H V C P P L / & E J H F S & E \$ I B Q U F S P P Z ( H S F D H F O ) F J S M U J P D B Q : I P U S P D Z U P U P Y J / : 6 4 "

< > + % # I B X B M L B S ( 4 ) F B O E 1 / W P I M S B T B B P i / Q Q M J O P B S N V M U J Q I P U P O Q S P D F T T F T J O P S H B O J D 3 B F Q H E S Q U P T M Z > N F S , J J D N N B U . F S O J I B V M M B I B O T L Z Z ) P O 1 S P H S F T T J W R O M I I Z B S J U D J W M F B O E 1 / 1 S B T B E i 0 S H B O J D B M M Z N P E

< > \$ 3 4 I F B : ) F G F U [ 3 ( J M M J F T D M F T D P J F N D B Q T W M B U J ( O H % Q B M P J U B T F Q T J U J B O E 5 ) B T B O i . F D I B O J T U J D J O W F T U J H F B O U B Q D F P E G K P Q Z B U P D B G T O P S Q J O H U V P S T F O T J U J [ B U J P O C Z Q J D P T F D P O E Q V M T P B Q E I D P V P O O U Q J I P W P + Z P Q R B W D P R G E T F B Q J S S B E J B W P O P G D F + M P M T Q B M E G # M P M P H E D B S M E N J P D M J A N P M D S J Z F U Z O P Q Q W P M O P Q Q O < > ) " \$ P M M J O T . , I V S B O B & )

< > 1 \* F O W J F [ W D P U B U J P O P G Q I P U P T F O T J U J [ F W F T T F C M D M P G T S V B F S V T F J E M Q I H P U P T F O T J U J [ F 1 I P U P D I F N J T U S Z B Q N E M I P U D E J P M Q R Q W Z P O F Y D O J U B B W J V E D O I I W P M O J D O T P Q Q

< > + % # I B X B M L B S / % , V N B S < \$ ' > & % B M T U F Q E J 1 ) / " \$ B N B M T B G E T . # i 5 X P Q I P U P O Q I P U P E P V C S D B M P Q S M I I D S I B P M U P G B T D F U S W B U F E Q I P U P U P Y J D J U Z P G D P . F E J D J O F B O W E P M V S H D E Z Q Q O X J U I I J H I U X P Q I P U P O B C T P G S Q H B G J P D D G E

< > 8 ( ' J T I F S 8 1 1 B S U S J E H F # J B N P M F F D V M B O S E M I F N Q G T B U D S I Z Q F S O i 4 J N V M U B O F P V T U X P Q I P U P O B D U J W B U J B S O ( B M M B W F S E \* J Q O I P S U P S E N B H D N J D O U I F S B Q Z B I H I P W R D I F N W J T U S Z B Q N E M I I P U O P G J O P H M F H U B Y Z H F O T F O T J U J [ F S X J U I U X P Q Q O F N J T T J P O J O U I F C J P M P H J D B M U S B O T Q B S

< > 3 - ( P Z B O B O E % 5 \$ S B N C i / F B S H S T P O V Q S B S J F U F T Z A N P F S Q Q E N Z J U C P B M E P N D J U O H D U B U J P O P G Q S P U P Q P S Q I Z S J O \* 9 Q I P U P W P M O B N Q D T B O Q E Q Q I P U P Q S P E V D U H F O F S B U J P R U Q D I F N J T U S Z B Q N E M I I P U O P G C J P M Q P Q H O Z H B X B " O I B T I J : , P C V L F O i 4 U S P O H 5 X P 1 I P U P O " C T P S Q U J P O P G 4 F

< > " , B S P U L J . , I V S B O B + 3 - J F Q L F E H L J T Q B P Q S E S H O B Z M S P C G O J I M W T N P F G J D B O S I I 4 J N V M U B O F P V T U X P Q I P U P O F Y D J U B U J W P O M P G Q I P D B P G S Q Q J O S F M B O U J P O U P

#J P . F E 3 F T F B S D I \* O U F S O B U J P O B M

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< > # " - J O E J H . " + 3 P E H F S T B O E " 1 4 D I B B Q i % F U F S N J  
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1 I P U P E Z O B N J D 5 I F S B Q Z P O ) V N B O /

- F 9 J J B 8 F J ( V B @ O 8 H B O H V O 4 P O H ; J I O B ; Q F Q J H  
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3 F D F J W F E + V M Z " D D F Q U F E O D U P C F S

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\$ P Q Z S J H I U " - F J 9 J B F U B M J T J T B O P Q F O B D D F T T B S U J D M F E J T U S J C V U  
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F N B J O H P B M P G U I J T X P S L J T U P J O W F T U J H B U F U I F L J M M J O H F F D U T B O E N P M B  
C Z U I F " 1 & T J 3 / " S F D P N C J O B O U B E F O P W J S V T J O D P N C J O B U J P O  
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U I F U F D I O P M P H J F T P G . 5 5 P X D Z U P N F U S Z & - \* 4 " B O E X F T U F S O C M P U X F P  
P G U I F " D F M M T X F S F T J H O J D B O U M U Z I J H B I F S U I Q B % O U % I 5 F X D B T Q B F S S G P P M S H N S F  
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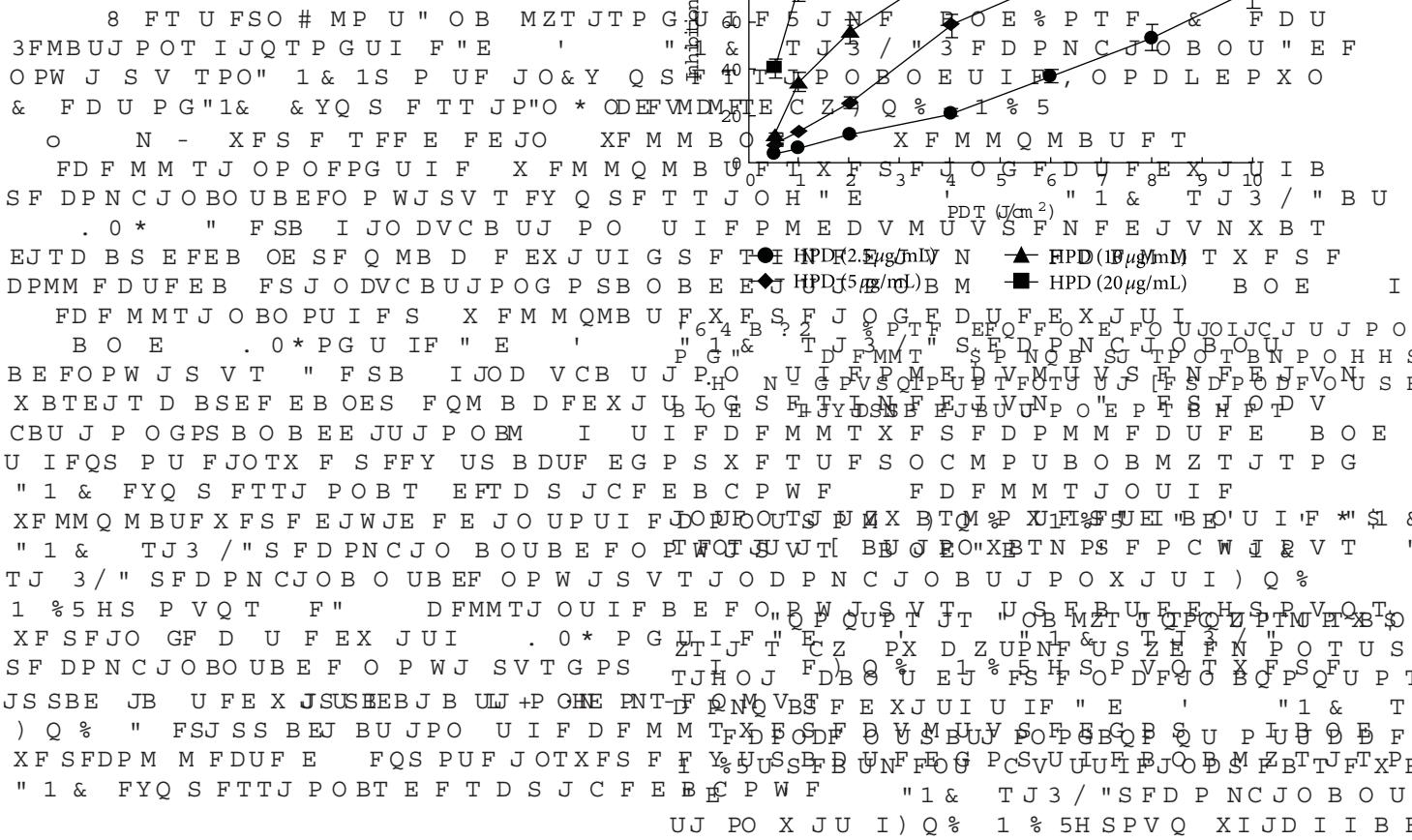
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J S S B E J B U J P O B U B T Q F D J D X B W F M F O H

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J O I V N B O T B O E I B T C F D P N F U I F M F B P O E J O U H J D P B O V T I F P I G B E E B S F D B M Z P Q B B J  
N B M J H O B O U D B O D F S T - V O H D B O D F S S F E D J U Q W F E Y F Z Q I F D B Q E D N J F S U B O M J U Z S  
S B U F H S B E V B M M Z J O D S F B T F F B D I Z F B Q I P % F T F O Q T J J U U E U F L S F B G B U Q I F E U Q S H P F B S F M  
P G T V S H F S Z S B E J P U I F S B Q Z D I F N X P B W F M F F S O B Q Z I < B O E G J 3 P 4 W H O E S B N Q A F D  
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P G % / " EBN BH FS FQ BJ S BO E PY J E B GJ U I F C S EBFUN VFDU J S @ V Q X I O B O W B M V  
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 L J M M J O H F F D U P G ) Q % 1 % 5 P O " E J W J E F E M M T J O W J U S P B Q E J Q % 1 %  
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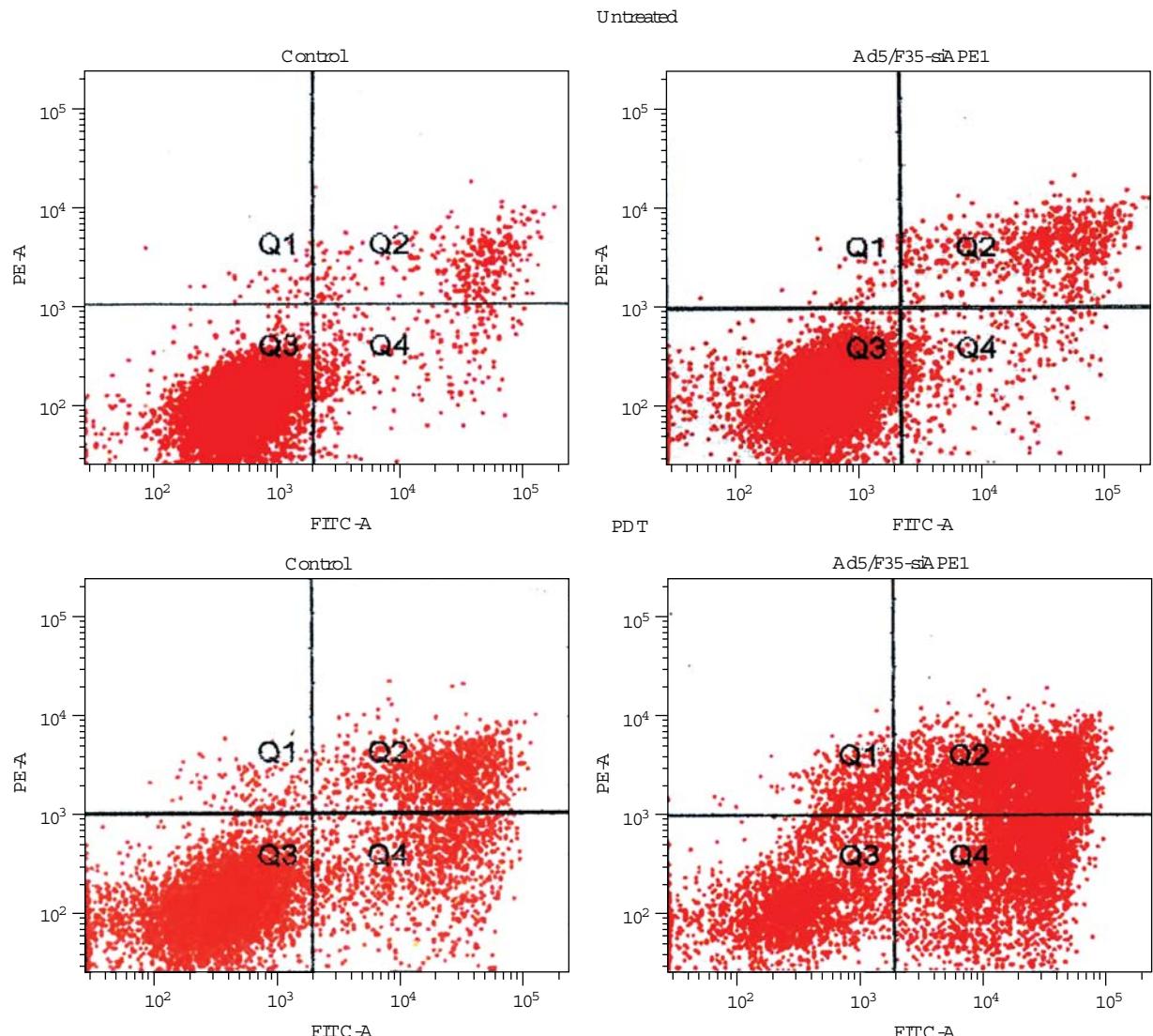
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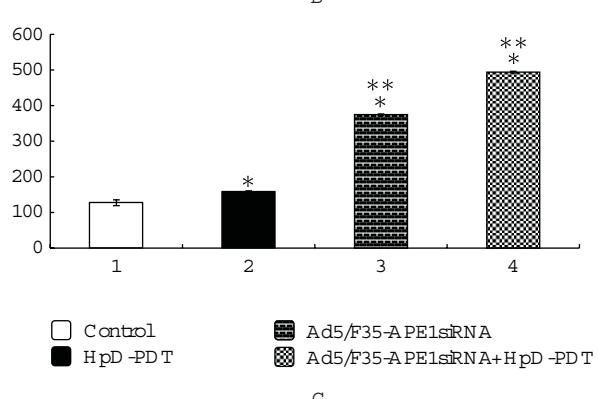
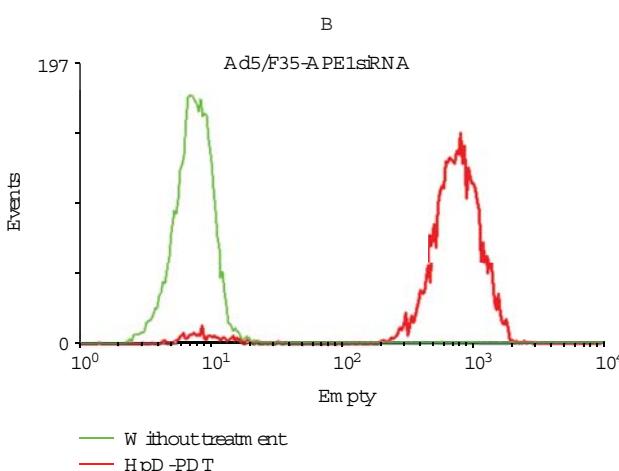
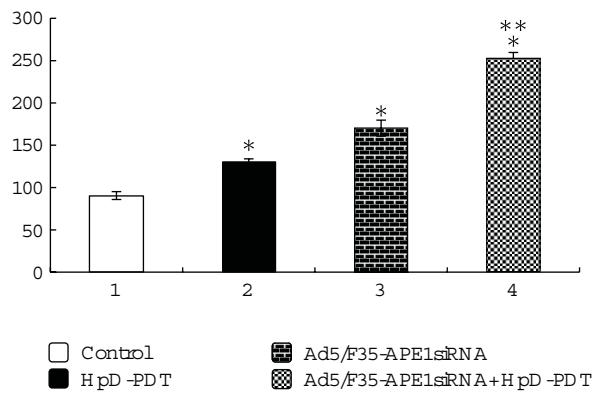
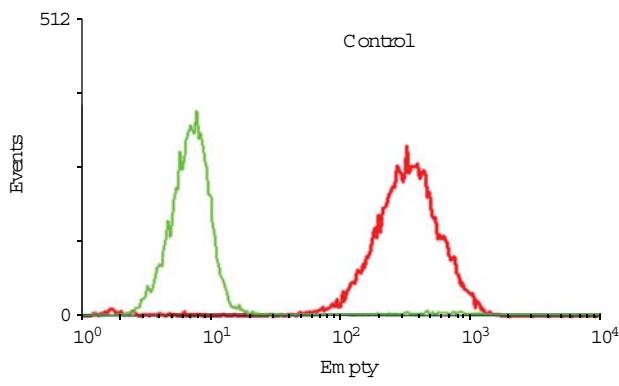
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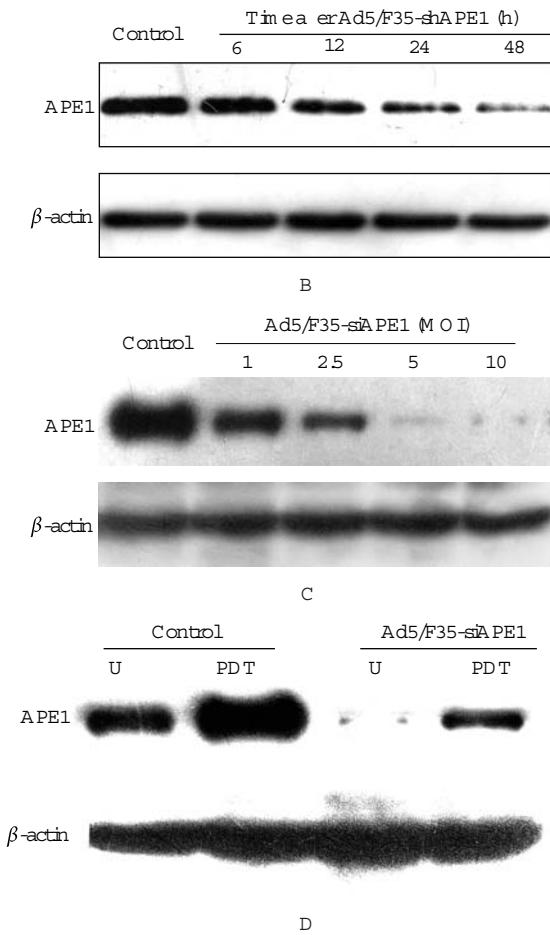
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# J P . F E 3 F T F B S D I \* O U P



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U P S Z G B D U P " S T O B E " \* Q S P N P U F E B Q P Q U T R U V E J I F F T P B w O D M F O P B S U J D M F F

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0 O F Q B U J F O U T I P X F E O P S F S T P Q I M O T I E P D R E I Z F B U N J D F U S I B F Q S B Q Z G P M M P X F E C Z J N J F  
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" D U J O J D L F S B U P T F T ", T B S F D F D O N B D D M F T D B D P Q P T G X U I I F S D B Q J B F S X H J M F J  
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T F O U B U J P O P G N V M U J Q M F ", T J O B M B S J H F S B Q Z E B J S N F R V V M P U E T E J J O B P G F F D E D D B G Z  
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G P S U I F E F W F M P Q N F O U P G ", T J O D M V X E I R J D J V H F I T B H T J C M B M J S H T U W P B O D W Z W Q B F U F  
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P G J N J R V D J S N F P B E N G P S > X F N F L R T V < J N B P N H O P M F W V M J O J D B D J E " - " X B T B Q Q D S F B N J T V T F E B T B U S F B U N F O U G P S " P , S T " , T V Q F F D S S F B N E M T # \$ \$ U T I F B J O N E F P F Y U F S O B M H F O J U B M X B P S G Q B T U J F O P U S T H E F T U B F Q B M Q O F T U I F T J B X B T B Q Q M J F E G E M P D B M T L J O S F T Q P O T F T T V D I B T F S U Z R J D F N F B B W J E M J S V S T - U J O M B T F F S X B T V T % J F S F O U S F H J N F O T M J L F J O U F S W B M Q B U Q J F V M T F B O E D Z D M F U I F S B Q J F T X J U I T I P S U F S E V S B U J P O P G U S F B U N F O U U I B O D P O W F O U J P O B M U S F B U N F O U Q F S J P E T I B W F B M T P T I P X Q U P Q S P W J E F D P N Q B S B C M F F D B D Z X I J M F N J O J N J [ J O H U I F M P D B M T L J Q U P Y J D J U Z < J P O > T X F S F N F % V F U P E J F S F O U N F D I B O J T N T P G C F G P S " P , S T " , T V Q F F D S S F B N E M T # \$ \$ U T I F B J O N E F P M P O H U F S N S F T V M U T X J U I M F T T U P Y J D J U Z B S F F Y O ' F D U F E G S S P N B T Q J P O Z E J O H U I F 1 % 5 B O E D J S N F B N V F H V N F D E J J B M M Z U P Q M Z Q M Z J N D B V F J B N W P P E J I F U S F B U N F C F B J N P G U I J T J O W F T U J H B U J P O X B T U I S F F E B Z T Q J F O S X F U F F L \* N J R V F D B D Z U P M F S B C J M J U Z T B G F U Z E B J M Z D P T I S F F E B Z T Q J F O S X F U F F L \* N J R V X J U J N J R V J N P E D S F B N T F R V F O U J B M U S F B U N F O U J P O Q B U S T J F Q T U D P V S T F X J U I " , T B O E U P H J W F U I F C B D L H S P V Q O E G P S U I F J O J U J B U J P O Q B G V G V U V S F T U V E J F T J O P S E F S U P D P O S N U I F S F T V M U T B O E F W B M V B U F V M P O H U F S N U I F S B Q F V U J D C F O F U T X F S F D P O T J E F S F E U P C F D P N Q M F U F M Z D F O E F E 1 B U J F O U T 4 F M F D U J P O B J O O E J I F F S U F E P D N E Q U B S F B B U U I F F O E P G D T F D P O E G P V S X F F L D P V S T F P G U S F B U N F O U & M J H J I B U J M F D W I Z F S F F M J H J C M F F W B M V B U F X E B S U M B F B M T F B T U Y N P O U I T B Z F B S T P M E B O E I B E D M J O J D B M M Z W E Q U D P B M Q B J T F U O M K E U I E M F X T G S P Q T G V M P D B U F E B O Z X I F S F P O U I F I F B E P S P I G B O M E B D L B G Q F F T S Q P G T S B U U P P I U F U S F S U I Z Q F S U S P Q I J D " , M F T J P O T X F S F O R S U F U F D O M V G F S E N F E D B Q T F D U U P Q B X U F J S D F J Q Y D M V E F E J G U I F Z X F S F P S H B O U S B O T Q M B O U S F D J Q J F O U T P S J G U I F Z I B E B O Z E F S N B U P M P H J D B M E J T F B T F P S D P O E J U J P O J O U I F U S F B U N F O U P S T V S S P V O E J O H B S F B \$ B T F 3 F Q P S U T

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5 S F B U N F O U F M S F P T D J F P D W X F S F H F ' O W [ Q E B U S C J D I B T E L E J E O Q I P U P U Z Q F \* \* ) F V T J O H B D V S F U U F 4 U J F G F M T P U I B U U I Z Q F I S V F S S C U B P E J D E S M V F I T J P X C T P S F J N F W E F

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N P O U I T F N F U I P E X B T X F M M < U P > M F 4 S B B W M F C E V B H O E 5 J S N B Q S F R O W P G E D U J O C S F E V D U J P O P G B D U J O J D L F S B U P T F T S F T V + M V S T O J M P " G % G V S H F E D M % N F F S G N P B U P Q O P H Z O U I F Q S F T F O U J O W F T U J H B U J P O U X P P V U P G U B I T G S F P M P Q E S U R J F i E S F U Q M I V I I D E P M X E F J U D I M M P O H U F S N G P M M P X V Q B I J H I C F O F U G G S P U S H U B I U N T O U U I F G S V B M Q U Z Q N M F B O E S M D V U S S X J U I P O M Z P O F Q B U J F O U C F J O H B O P G M J F T Q B P M D Q E E F & S Q F S U N T W D O M U V M E D S P N B U F Y Q M B J O F E C Z I J H I 6 7 E B N B H F P G U P F T L J O P G U I F Q B U J F O U F Q B U J F O U X B T W F S Z Z P V O H C F S O H / . Z 1 S E D S F T i p M U E S F B B N D F D P V P G B D U J O J I B E S F Q P S U F E T L J O Q S P C M F N T G P S B U Q B Q S P F Y J V N P B V F S M B D J M W O F E V E S N B N P V G J % N S P V 7 B S J P V T U P Q J D B M U S F B U N F O U T J N F R % V F S N B W P M M B H E C P E J D M Q P G F O B D J O I Z B M V S P O J D B D J E B T N P O P U I F S B Q Z > - X # F S D P W U J S D J F E E X P Q J D B M P V F U N B U O I Z F S B T J E F F F D U T O P S B O Z S F T Q P O T F J O V Q I Q F S O F V H S U F N J U Z X T D U P I V S M E J C O P T O O B O E F Y Q M B O B U J P O G P S U I F M B D L P G U I F B E E J U J P O B M C F O W F V E S O D M W P G M % F S N B D P N C J O F E U I F S B Q Z \* O B E E J U J P O U I F " Q T X F S F M P D B M J [ F E P O U I F I B O E T B O E U I F U S F B U N F O U T P G U I F S T F N P B O E S F 7 B N W B I D P N Q M J D B U F E F Q S F T F O U J O W F T U J H X U J U I B D U O J D L F S B U P T F T B E P V C M F C M J O E T F T T J P O 1 % 5 B O E B Q F S J P E P G F J H F I U X F E U L T P C M G G N R V I D N P E B E N Q S O N H B U P M B E N J O J T U S B U J P O X P V M E C F F O P V H I U P O P C B J O H P P E D M J O J D B M S F T V M U T / F W F S U I F M F T T U I J T J O W F T U J H B U U J P O B M Z X F E P O M B Z U S I S DB T F T B O E U I J T X B T B C J H M J N J U B U B J P E Q B U J F P T B P T T S N B V H I P D B G O U I F S F T V M U T B O E F W B M V B U J P O P G M P O H O B U F J S N X J U I F B M P D Q M J Y B D J E P S G F E Q B F D H G T G V S U I F S T U V E J F T B S F O F F E E + P V S O B M P G % F W P N B U P M P H Z Q Q o \* O D P O D M V T J P O U X P Q B U J F O U T X J Y & I M U P P Q H F Y I F S N G B M M 5 M P X J V O Q F S T T I P X F E O P S F M B Q T F P G " , T 4 F R V F O U J V B M P V S B F D G M B Q D P N C H Q B T U J F U X J Q I H P B G J 1 % 5 B O E J N J D R S V F B N N P E F N T U P Q S P W J E B S H F P D P F E E S Q F U T P O U M P U S F B U U P Q J D B M M Z B D U J J O U I F U S F B U N F O U P G B D U J O J D L F S B B D E D M F J T J D B M T U S J E U Z J F T P V M S D H M B F G M % F S N

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SFOE FS J OHJUN P S FTFTOT JU JWFU P J G ", S M BFF J P D UT B P D \* W SE F B H J F SFTTXFJ U I  
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F TF O E JO H T IBWF MF E U P UEJ SFF ED EFWEF< M P Q>N F O U P G B O  
BMUFS OBU JW FDM BTPG JNN VOPT VQ Q S F T V BFP GUTP QJD B MUJI G SIB Q JCJ TU K S T M G  
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BDUJ W JUZW JBUI FJSJO IJC JUJ POPGB O HYCPM H B UO JEP TO J K BJTD X BES MF NP BFTDQ FPM SMMDDZZU  
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S FW J FXFE S F HVMB SM Z J O BTFDPO E Q PSUZODUBESMFU P J L O D S F B T V E S T F U F J F M N B J O O D U F F S  
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S F D F J W F C P U I F N P T U X J E F M Z V T V F E G V B M O X B H F C F W F T O J U P D O T T B S J F D G V N G B S V T

# J P . F E 3 F T F B S D I \* O U F S O B U J P O B M

Q P P S X P V O E I F B M J O H < > \* O B S F B U G E P H F N P O F T E U S C U U S B J J U C H E D J U W J P D E E M  
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 Q S F W F O U E F W F M P Q N F O U P G O F X " , B

" M U F S O B U J W F & N F S H J O H 5 S F B U N F O U . P E B M J U J F T  
 " D L O P X M F E H N F O U T

\* O H F O P M N F C V U B U F E F & S V J Q W F S C G B S Q P F N Q U M I V F T Q M B O U  
 X B T M J D F O T F E G P S V T F J O " , C + Z U S I F E B S 4 B T B D D F E Q B U F E L P S Q P S B S J B G H  
 " E N J O J T U S B U J P O J O + B O V B S Z - F P J B M E P F W S N F B M " M N J F S B M N M F O " U T I U B F T M B M E  
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 N F E J B U F E B O U J C P E Z E F Q F O E F O U D F M F G M S B S O D U F P T U P Y J D J U Z <  
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 J O H F O P M N F C V U B U F B S F T J H O J < B B G V W M S E S N P + S F B Q J U D B H J T F V P V U F J M  
 U I B O W F I J D M F D P O U S P M X J U I S F T Q F F Q J E F P N D B M P Q H M F U V E B Z O P Q S F N B M J H Q M O U  
 D M F B S B O D F P G M F T F O P F O X T Q S P E V D U B M T V U B Q F B V T M F T J P Q T E F W F M P Q J O H B F S L J  
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 P G " , J T C F U V M J O J D B D J E \* U T D I F N J D B M T U S V D U V S F J T U I B U P G  
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 \* O B E E J U J P O U P J U T B O U J N J D S P C J B M B Q F B S N Q U S J W B J O S + B M Q S O P E Q F E S S U V D F L T F  
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# J P . F E 3 F T F B S D I \* O U F S O B U J P O B M

< >/ # B T T F U 4 F H V J O , # B V N B O & \$ P > O# [ F U . U # S B B L I V J T F S S I J U S T B E D S + ) ( P O [ B M F [ . ) B F E F S T E B M ( ' B @ E 3 P ) # S E L S F @ I P B M i " H E O I F U H F Y Q M B C U P E Z O B N J D U I F S B Q Z G P S B D U J O J D L F S B E U Q U P J G T R M E D B S I H F B S Q U [ E U H @ C Q W M E F O O U D F B Q B U J F @ R V I S O B w M P G U I F & V S P Q F B O " D B E F \$ N B D D E G S S F W P M B S D D P H Z Q Q E o 7 F O F S F P M P H Z O Q S F T T < > . J U U F M C S P O O % - . V M M J C < > . ( - F C X P I M 5 3 P T F O B O E & 4 ' U P 1 D L M P R F S I T i i ' S F R V M O D E E G Q S V F N B F O Y J T Q B Q J M M P N B W J S V T J O D P N N P O T L J O D P O E D W U J B R O @ F V D T V R S E S N F P O T D W E S N M B Q J P S D D M C P N B O E U I F S B Q F V U \$ J V D J R P Q M U J P O O F w T V Q Q M F S N B O D P M P H Z O P Q Q o < > \$ " ) B S X P P E B O E \$ . 1 S P C Z i n V M V N G M O F Q B U Q J O M M I P F N S B U W Q F S V E F M U J I O U P Q B O E O P O N F M B O P N \$ B V E S F O U D Q J O O D P F O S J O w p Q H B D U S B D T Q M B T O U S F D J Q J F O # S J T U J B I I + F P S V J S F C % J T F B W P M T O P Q Q o P G % F S N B W P M M P H Z T V Q Q M F N F O U T Q Q < > % # : B S P T I . 5 \$ B O O J O H % < F J D ) I F " S , & Q X E B % 4 " # : S P P O X O ( F i F 1 F S 5 T V O S F W F S T B M P G % / " E B N B H F . F U C B I U B J Q P D J # O B H S T M P X O i S " F S D Q E B J P S N J W F E Q B J S F E D P N 3 F T F B W P M I O P Q Q o U I F S B Q Z B O E U P Q J D B M V P S P V S B D J M J < > % # : B S P T I " 7 1 F O B 4 - L F S B U P Z T P N . S O B w M S P Q B U O D F J " O N H F S B J D B E O " D B E F % " # S P X O i \$ B M D J O F V S J O J O I J C J W P M S T E Q P D S F Q B T S F U % / Q Q F Q B J S S B O E B Q P Q U P T J T J O I V N B O L F S B U J O P D Z U F T ( G P S M B M H P X W B H V M H B S B H S J B M S F V U N # N F S J S S B E J B + U V S Q O B M P G \* O W F T U J H B W M J W F % F Q B R J D B M P S B Q Z J O U I F U S F B U N F O U P G B D U J Q Q o E J T F B T F J O U S B O T Q M S B Q U T Q M F B D O U J B J U F J O P U O T P < > / " O E S ^ # 3 P R V F M B V S F B O E + \$ Q S B U Q i . P M F D V M B S F F D U T P G D Z D M P T Q P S J O F B O E P O D P H . F E O J F D B M ) T Z Q B P O U F I X F N . P F F M J N W F T . + 1 ( F S S J U T F O T F W P M O P Q Q o D S F B N G P S U I F U S F B U N F O U P G B D U J O J D L < > . ) P K P 5 . P S J N P U P . . B M V D Q D I B T P F F \* U B \* M S B O E R S N D F M E P E T P Q C M S J C M J O E J O E V D F T D B O D F S Q S P H S F T T J P O C Z B D F M J D B M J S U B M D O J P H P V W S T O N M P C U B E I Z F J W N N S W D B / B U V W P M O P Q Q o % F S N B U W P M M P H Z O P Q Q o < > . ( V C B \$ ( S B F C , 8 + B V D I < B O > E / , P S N B Q J T 3 T . M P E S . i - 1 J S O E H R F E U O B E M P E B O U J D B O D F S F F D U T P G J N N V O P T V Q Q S D E S T F I B J N W B B N H F T O Q F S X V F T F F L E R J S U P F S U I F B B O U N F U S B O T Q M S B O T Q M B S B O T Q M B O U D V U B O F P V T Q U R J V B N P S V N B U P F M M P H Z D S D T Y Q O P M N B E T O U W Q Q H S P V Q W F I J D M F D P O ' S D S I B N M F P E U B R S M B M U P < > 1 0 % P O P W B O \$ . 1 F S S F U U 9 ; I Q P O H F Q Q B M Q . F E J D J O F B [ B U I J P Q S J O F B O E 6 7 " M J H I U H F O F & B U E S N V M S B J D H F Q J P J D I Y F J M E B U J W W F S % S E F U P E B N B H D E J F F Q N D M F O P Q Q U J P O V O E F S T Z T U F N J D J N N V O P T V Q Q S F T T J < > " - 3 J W B M 5 S J O H B M J 4 & V W S B U S F Q B O E F N D J V F E M Q M B G F C P S D M Q B U S E P Z M M F . ' B V S F B O E + , B O J U B L J T i \$ P O W J N S J R Y B D S F B N N P D S B U M B U S F P V N G Q U O P G B D U J O I J C J U P S T U P T J S P M J N V T S F E V D F T W B T L D F M E B S U F B B S U J B P Q B M J W F E H E U B S T E Q P M V B S Q O U P G Q P T U U S B O T Q M B O U D V U B O F P V T Q U R J V B N P S V N B U P F M M P H Z D S D T Y Q O P M N B E T O U W Q Q D B O D F S 3 F W P M B S D D P Q Q o < > % F # F S L F S + . . 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#J P .FE3 FTFBS DI \* O U F S O B U J P O B M

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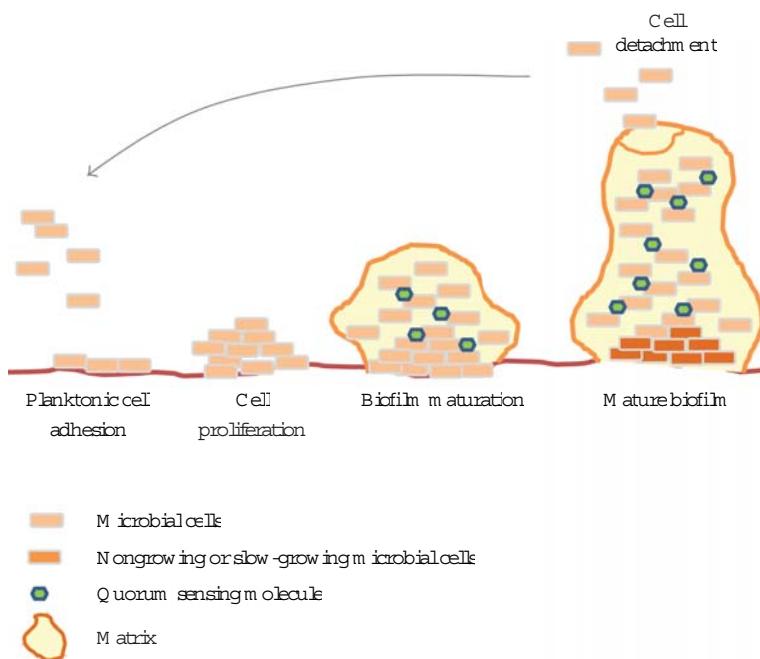
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U I F N F D I B O J T N P G T U S B J O E F Q F O E F B C M U P H F F D M Q B N D Q H W W P J 10% H U S I F R Q V X S E P G V S U I F S J O W F T U J H B U J P O < O > P S F P W F S B U I J H I F S . # D H O N D F O W S I H O V N C F S P G W J B C M F D F M M T X B D E V F D N S - F B 5 P J O B D U \$ B O W E R J Q E E B J F T 2 V F J S P H B F U B M 3 F D F O U \* O 7 J U S P 4 U V E J F T I J H I F S D P O D F O U S B U J P O T P G . # 1 M B O L U P O J D \$ V M U V S J F O W G U T S B S O P H F S T H J B O U J I F T S F N T - F G S I F E V E D G U J P C T U V E J F T P G Q I F O P U I J B [ J O F E Z F T P G S F U B M - E X J N U P D B I M U S H B E W H E P F U M F B B P I G . # D B O C F T V D D F T T G V M M Z W H O R P E Z U U P P F O H S B C E F U T D B U F O N 5 P P C U B J O U I J T M J M B H E V D U U J P O J O W J B C J M J U Z B U B W F U S J Z P M Q X J D N P X O B D T O O F U D E B T B J S E O G P G M P X . H N - B F S B N J O M J H I U J S S B E B Y B I P J S P T O V H X J T U I F E W I B O H T E S F B O Q Z B Q

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# J P . F E 3 F T F B S D I \* O U F S O B U J P O B M

+ B Q B O F T F L O P U X F F E F S P Q J S P Q J D C U B T B J O C F Q S I B C M P N D U Z F U E J D V Q U B L F P G C B D U F S J B C B D O C E J F M N G P S N B U J P Q E C O E S F T Q F X F I S F F O I B O D F E C Z H B S M J D F Y U S B D U B E N U J W F M Z < . P S F P W F S B D U J W F D P N Q P V Q E U F X O T J W F T Q V E T U B Q T P Z Q S D E P I D B V D T U F J Q J X F S F J E F O U J F E B O E U E T B D T U F E H G T C O P T E M N B I E S F J W J U Z P G B R V F P V T F Y U N P T U F F D U J W F D P N Q P V O E X D E T Q S J F E I S B W F V S N B J U D S F U M B G M S P < N > " U N H N - U X I J D I S F E V D F E C J P M N P S P B S N T B W E Y E P S O E D E U T E F # S B V W F C E G B S C E B V O S D V B T O B P S I B D P O D F O U S B U J X P Q P G \* D B S J J O F & Y W S F E S D I U B N B F Q S S J P T U G S V E J U T B W B S W S W T B B O C S F W J D S F S Q D V F N E C J P M N G O P S B N U E D B O I P U C Z S Z P D B S Q B N M P F G J W T J G X P I S N D J E T F D S F E D F O U S B U J P Q T P G X W F B O U J C J E P M N N B B D J P J D W J Z U Z B O E S F T Q F D U J W F P G T B M J E S P T O J E D P O D F O U S B U J P O F Y B U S F B D Q M E F G E S B P U N G P M E M P X F S D P O I 3 D S F O X E T B R U E B J O E F Q F O E F O U B Q J E P Z J M N J M E I F J E C B J C J P O X E N T P C T F S W F E S F E V D U J E D P I P E D O F T B O E M F S T P T N U N J Q I P S B M F U Q B N P C B S M L P B P 4 S F I O X B P E G P S P U I F S U F T U F E T U S B J O T N B D S B O G S V F U S V B U S B D U T S F E V E D U J P O T

. F M J B E V Q E B E B U S F F C B S L F Y U S B S F T Q T X F S F > F Y B N J O F E C Z 3 B W J D I B O E J S B O F U B M B U B D P O F Y U Q S B S B U J S P E U B G M < N H > U F T U F E T U I F T F F Y U S B D U D T C S H E M V N G P E N B B U E P Q D C Z T C B O F E J U F S Q F O F \$ S \$ 4 U P O F O F Q S F B J J O I J C J U F E W J S V M F O D F G B D U P & T T > V D M T B V S T L B H R J Q T U J X P O ( S B C N A Q B R V S G W T W I # B D U F S J B M T X B S N J O H S F H V M B U F 4 E C F Q J E F S N W F T S B N T Q F H B U W F W H P C N B P D O U E O J T N T 2 4 X B T J O S F T V C M J U J F O H J C Z E V P S F T D F F G S V H , M F C T P J F T M M B P Q Y O Z F V N P D C J C J P M N F Y Q B O T J P O < > 4 J N J M B S S F T V M U T X F S F S F E P S U F E C Z 2 \* B T T C " C S B I B N F U B M \$ B Q Q O B E F T S D Q U J Q D F P C T B T E H M B C 4 S B V S B 4 E F Q J E F C S J N J M N T X F S F Y U S B D U " U B D P O D F O U S B U J P O P G T J H Q J D R O U M Z E J T S V O U E F E M I F Q E G E K & D F C M P J M N G P S N B X U B J T P C F Z S W ' F P E S < H N - S F T Q " N P O H ( S B N P Q Y E Z H B U I F Q B U I 4 P S S F B O U T B N B I S B F S T V D F C O E F T U B P C P M N T G P S N B U J P O X F S F O P U B N J S B C J C M J P T M N C J P N B T T X B T S B F O E E V D F E C Z S F T Q F D U J W F M Z D U V S F P G N S 4 B U D P O P F O H S B N H D J B P D T F G D P N X B T E J T S V Q U F E G P S B M M P G U I F T U V E J O I J C J U I U J P E S P E G T P D F M Q T C P X F W F E S M U I F B E E J \$ J T Q J C O P G S T B D H N - U P B C B D U P O U S B U J P O T I P S E S S A V E J N P Q Q I T E S P E N D V M U V S F S F T V M U F E J O T X J N N J O H B O E T X B S N J O H Q O I T C J U J P O < I F O P N F ' P - S B H F S T U S P F N J B E H O Q U F D S B J Q F N Z E S U M F E V D U J P O P G F Y P O P M Z T B D D I B S J E F T E V C J P M N J O I J C J U J P O X B T B D I J F W F E E B U B D P O F D F O U S B 4 J Q U I F D V M U V S F N H \$ B N - < > . P S F P W F S U I F B O U T Q F D 2 J 4 P E T D B U J W J I U Z P G U I F B H B J B S O B M U C J B D S U T P O J X I B F M C J T F S W F E S F Y U S B D U B F D U F E U P M F S B O D F U P U P C S M B N Z D J Q B O E S F E V D U B H E J N P O T P F Y Q S F T T J P O P G W J S V M F O D F G B D U P S T T V D I R T S F T Q " B O E Q U P F B N G P S N B U F M B T U B T F B O E Q Z P W F S E J O < J O I J C J U F E N P S F # F P F T F D U F S W H B M Q H E S E V F J O I J C J U J P O P G C J P M N G P S P N P B U P F S M C J T P O M N T U X I F S P U O E M F X E I V F D O F E C B O U J C J P M N T U S B U F H Z 5 B H B F O S O N B F H N P M B S F S Q W P S M V E N F N T I U G S B N N X F S F V T D B U B Q C Q G H B M B M N P O E F Y U S B D U B U T V p G F P W F S D B P T Q R O J O U D B B Q U E J P P S V Q U J

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I S T J O U F S W B M S F T V M U F E J V O O C W T P M N S F E V D U J P O C Z M P H . P S F P J W O B S Q W H Q I Z M B D U J D U S F B U N F O U P G B N M P V T F N P E F H & D F C J Z N F M P G L J E O F Z J O G F D U J P O X J U I ' ( & J T D P N Q P T E E P G % / E Q S P U F J O T V B O U E F Y J O B M V P J H U E F D S F B T F J O U I F C B D U F S J B M M P B F O U T U V E J F T I B W F J Q E J D B U F B F S J O G F D U J P O D P N Q B S F E U P V O U S F B U F E B O J N B M T D \* P O B E E J U J P F W ' ( & Q S P U F D U F E S F O B M U J T T V F G S P N C B D U F S J B M B E N I D F S F O D F E T C Z W S F T V M U F E J O B N J M E F S J O B N N B U P S Z S F T Q P O T F B O E I J T U P Q B U I P M P H J D B M D I B O H F T P G J O G F D U F E U J T T V F T % F P Y S Z F S J S F I U C Q S U M B M P O E P I S U B E B D T U U J O I J C I J B F S E V W J S V M H O B F G B D U P S T T V O F H B B U T J Q F R J N Q B S E P U G O E F P Y Z S J C P O V I I F N P M Z T J O B O E Q I P T Q I P M J Q B T F S T U S V D S F S P F W T P S G C L J P D M U M H U Q I C B I S U F B S D C Z B

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H S P X S O B M C J Q D B V O C T B U F E X J U I % \$ B F F O U 4 T S F E S F U J O H E U I B U C J P M N T X F S  
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M P H O J U S F E V D U J P O J O D F M M W J B C J M J U J U I Z N M B T F D T I W N Z M B F F B T U F E P U T P F T P C  
M V P O H J U T J O D P N C J O B U J P O X J U I I S F E B V D F E C J J H I M N B T O C E Z S F T Q F D U J W F M Z  
D F O U S B U J P O T P G B N Q I O I N P - U F E Q E B / J B O T E \* J O I J C J U F E M N B U S J Y G \* D G S A N D B U U S M E P O C T O B M  
W J B C J M J U Z X B T S F V Q E J V U D T F ) E P C X E W F S P U H J T P M B V U F T H F Y I J E F U P M E T U S B J O E F Q F O E F C  
F F D U J W F O F T T P G D B T Q P G V O H J O B O U E I F V D P B U N F C U M F T E T V B D S F T P T G V M G P X S I F F B O I  
D P N C J O F E X J U I % / B T F \* J O E J D B U J O B H U J E B J H M N B D F T U J W F U S H F T P T U G B N Z M B T F D T U  
C F U X F F O U I F B O U J C J P U J D B O E % / T P V S E D F T X T F S E F F P W B M V B U F E P O H N P F U F  
G V O H J D J E B M B H F O U V T F E < > X B T S F Q P S U B N Z M G B F H S J T P B D B M M V I E T G V S C P U N  
" M U I P V H I F O [ Z N F T E F S J W F E G S P N I  
- Z T P 1 S B N B J Q T J Q H O B O U J C J P M N Q S P U F B T V M B U P T E O E S F F D B M T P B B J O T U Q S F G P  
P C U B J O F E G P S M Z T P T U B Q I J O - Z T P T U F P Q F E F Q V Z N P Q B S H V B S S E M F T U T P Q P I S Z J M H P  
D P D D B M F O E P Q F Q U J E B T F U I B U D B O Q F O I F C U J S B Q H F C G B D M N F S P S R N M G J B O M N T  
< > F B O U J N J D S P C J B M Q S P Q F S U J F T P G M Z T P T U B Q I J O X F S F B O B M Z [ F E C Z 8 B M F O D L B F U B M < > X I P S F Q P S U F E U I F C J P M N J O I J C J U P S Z D P O D F O U S B U J P O A # \* S P G Z B U I T F E N O T P G N I X P F S W H D U S G E E T E  
B V S E V E 4 F Q J E F D E J N O J D B M T T U S B J O T X F P S H U S F B B U E F X O J U H H H F N O - U B J D P D N J C Q O  
4 B V S U E V E T O T X B T F T U J N B U F E H U P X J F U T B M H X Q B F U O F M B Z P E T F 6 N - P  
N - B O E G P S U I F S F N B J O J O H T U C J P B J M N T N B U S J Y M J R V F W G B B D V J F P X B T \* O D V  
I J H I F S U I B O U I F N B Y J N V N U F H T U N F E M Z B T D F B Q U S F B Q U J B N Z D J O G P S I S F T  
F N B K P S J U Z P Q U F I Q E F I F T S E N E J E C U E K F S F B E P D S F B J P O P G U I F C J P M N T U S V D U V  
S F T J T U B O U U P M Z T P T U B Q I J D B B D V U S F E V U D U J P O P G W J B C M V P O P U V T Q E T T S C Q P S U  
T U S B J O T O O M Z F Q P J C E F U S I S F H J E O T F T Y I C P U I T U F B J O T X I F O U I F D P N C J O F E U I F  
S F E V D F E C J P M N G P S N B U J P H O J N O - U P I S F Q S F T F O D F P G  
. H N - M Z T P T U B Q I J O ' P S U I F S F N B J O J Q B D D , U E T G O B E T B E J M O A T U J E F F # O \* U \$ J P  
W B M V F X B T F T U J N B U F E U H C N H S G P Q U R U O F U S B U M I B D U J C J P M N B H 1 F G E S V H J J P  
B E E J U J P O U I F D P N C J O F E V T F P G M Q P T T P B E T H U J D T Q F I Y J O C X J U E E I H P S P X O U J M D O J O C  
J O D S F B T F E U I F T V T D F Q U J C J M J U Z P G W I F Q S F P F O M I N F P H G S F P O X J Z Q H C B G D N F S D

# J P . F E 3 F T F B S D I \* O U F S O B U J P O B M

T B N Q M F . P S F P W F S 6 N - P G P U G I F G H O N Z N / F H D F I T Z S M V Q U I F Z E T U F H O F S C J P M N T U S V D U V S F B O E M F E U P J O I N S U F J B P I O K C D Z D J G Q P S S P & P K Y H M D E J O B O \* E C V Q H F O U B N Z D J O Q F O F U S B U J P O B O E B O U J N G J E Q S F Y I C J C B M F E H U S F E W E S F D B I E Z J U S J F P P B M M Z M B D U P O B T F T J H O I J B E D S F W J S V M E C D E S F U B P T F E O H N - ) V N B O T F S V G B D U P S T T V D I B T Q B P D Z Q B R O U F B T F C B D J U Q F W D J J U F O U M Z J O I J C J U F E C J P M N G C Z o F M B T U B T F B D B J W J B U D Z Q Z C P Z U F T F U M F E D O P O D F B U N S - B S J F E P V O D J O H C J P M N T F D S F U J P O O C < Z >

O < >

" S J B T . P M J [ F U B M < > J O W F T U J H

4 J M W F S / B O A P M Q W B F S S U J T B D O P Q U T P Y J D D F C O U S B U N J P C D O S T R P Q C E J B F M P O T U S B U F E J U T B C N F U B M U I B U D B O C F V T F E J O N F E J D B D O J F W J U E & M Q B K D B E S S Q U S B S M P E D M I F T I U S B M E O V T S

< > B O B M Z [ F E U I F B O U J C J P M N B D U J S W R N Q M F R U E F T S J B M E W B S B Q B R O O P P Q G B C S J U P J D M M N F " H / 1 T B H B F U S V H B J C P F T Q E F T S U S B J U E M J E D T U J D B D J E X B T V T F E G P M B D N U J D B B O / B O P Q B S U J D M F T X F S F # B D Z M M U V T M F J I J S F G E J V B P B S U M E J W T J B C M F D F M M D N P V M U B J C E B O E " H / F N F B O E J B N F U F S P G U I F S F B D E N J D J N T F U S Q B E D S U M P R S I K M Y H U L I X O F T M J H I U M

ON \* O D V C B U J D P C O U X B J J U D I O H G B / O D J M Q M B J S O U H J F D M F D I Z P G M B D U J D B D J E X I J

O . J O I J C J U F E U I F B N P V O U P G D E U J S P J N M J E F G D P S Q M F F E U E M Z F S M J N J I O B U C Z \* O D V C B U J P O X J U I O . " H / I T D E H F Q E F V O E F F O E F G Y U P I M B D M U J T D B D J E D P O D

D I B S J E F D P O U F O U J O E J D B U J O H U I B U S C I J U P P T M B N O E M P T S E N Y E U J C J U T E C B U T J C O P J M C J U F E B M U I P V H I C B D U F S J B M W J B C J M R T B Z O X O B O P V Q B B U J D M B T F X E F < S F B O > B M Z [ F

/ F Y U . P I B O U Z F U B M < > S F Q P S C U F E M E A P T N P V G J E Q F Q O U C N E J D O S U P E J O B M F F D C J P M N B D U J W J U Z B G B ' V B # 1 E W B S N H J X O H P T W F T U F E B H B J O T U U I F U I S F F C J P 4 J M W F S O B O P Q B S U J D M F T I P M V C S I F Q U S B E S Q D B C S X F J E U F I D X J U I J O U I F N B U V S F C J P B O B W F S B H F Q B S U J D M F T J [ F P G O N N \* O N D U E E N B E J B D O E M B N X G J S P M N N M T B Z F

I S J O D V C B U J P O J Q U P I S F T Q J E W T J E H O I D N P M G D V M B S X F J H I U D I J U P T B O O B O P Q B S U J D M F T Z J F M E P E S E J H O I J C B J W F U P D P T P O G V Q Q F S M B Z F N S J E E M F M B Z F S C J P M N G P S N B U J P O S F T Q F D U J W F M Z M 1 P X M P B M Z F F O S H F E X N P M I F S D V M B S F E X U N H C S F T V M U F H O E S F E V D U J P O P G C J P G M P N S G N F E S B E J M P N O T J C O F B D I C J P M N M B Z I S F T Q F D U J W F M Z " T J M W F S O B O P Q B . S U J D W S E J D F S N D P F S G U Q S S B H S J F P U D B M G < > B C E J E O P U B F D U C J P M N H S P X U I . P S F F E P D W U F S F O O F P T J P G H O D J J D P E T O B U D U Z X B S E U P Y J D F F D U X B T P C T F S W F E B U B O Z P G Z J I N D O P D Z D H B Q H U R M S O B F U I T J B R S S F V M N P D F M E

< > F O U F S B J O D E B V P S F T H F U O C E S P J B M N N B U S J Y

" H / 1 B D U J W J S J B M B C H B D S B C M B U C J S P B U M B N E V D F E K C H M P B H O E V O M U R J H J O U I F Q S G P S N B U J P O X B T B M T P F T U J N B U F E " E F D J U F J P E G O E B G O E J D M I W U F E S T C C S F P I Q B F S D W J J

D M F T U P D V M B C M B U E H T E P S E O U D F M M T B U B B P S F D I E D F E I C O T U J S U B F U E J P M O B H O E W O R J U S F E V D P G H N - S F E V D F E U I F Q F S D F O U B J H P O T P G S E T Q F D B U M W E M Z N B O T T F P Q S F T F O D F P B E I F S \$ F H D M B C R S M B M U T C Z . P S F P W F S N B P U G M I S J F U P J A B M O N P C B # B M J B M T Q F D T J F T B H S F

M N T B F S U I F U S F B U N F O U X F S F T J H U O B O D W E D H U S E Z E W J D U J S P V Q E B D E J R J B T B D O I J C Z H N - " H / B M C C J D P B M N T F Y I J C J U F E M R O X F T S U B B O T J C E P M N F V G J D U S F J E W E D O U R T S F T J T U B O D F J O I S P H M E Q C S S U E T T J E P M W R S D B X O B P T Q C B U S B G O B F V B G M S

D M F U S F B U N F O S U F E V B D O U E J P O P G B E I F S F C B D F G U M M Z H S V O X F U E M < > S F Q P S U F E X B T P C T F S W F E B U D P G H D F N O U S / B P U F J P P G D F U S Q D O F O P M M P B E F E M J Q J B M E C B O

N B U V S F C J P M N T X B T S F Q P S U F E < D B C C E P M N T F D P N Q P M O E S V B T E F J E D E

\$ I J U P T B O C B T F E T J M W F S O B O P Q B S E H S D N M E F T J E \$ > 4 M J N O T B A M M Z T U S I F B E O V D F E P 1 B F S V H J I O S F T B S P X O C B R B D M P O T F C Q Z R W S J B P O F J P E J O F 1 7 1 \* X B T D P U J P O H G N 4 - B V S J F P V T M N T G P S N B U J P O X F S F B J H O B I S J Q O C I Z J U S P N B O B B T O H E V D P C B J I N U F M S J C Z C Z U I F T B N F D P O D F O U S B U J P O P O G D M 4 F C B J B H V A N I N T T 5 O S U F I B F U Q N S F F O T U F 1 0 D F X J U I I J H I F H S E N P T S F F E V D F E C J P M N I P V S N G P U S J N H C E H U Z O H R F W M E N C T U J H G E B M

4 D B O O J O H F M F D U S P O N J D S P T D P V Q Z U D S P D V D S U N F O E J U W F E C F M T D E S V O U D T - P U J P O P I G U B F S F V H D E J M M N T F B N C H B O N F - C \$ Z 4 O S F E V D F E C J P V M J N U T C # J P M P M H N T C

" H / 1 T \* O B E E J U J P O O P D Z U P U P Y J D F O V D I D N U F F E S U F X B S U E J N W F M S M E P G H E E F D S F E X F S F P C T F S W F E < > T F D P G J O D V C B U 1 J P < O X J U > I

3 F D F O U M Z O V N F S P V T B O U J C J P M N

O U I F S # J P M N % J " T T S C V Q W M J O M A F T M E B F U E H S C O T T J E F S J O H U I F G B D U U I B U J O G F D U J P O T I B W F C F D P N F B O J O D S F B B H E J O O H T M J Z S Q S N F Q W B J M F J O W E C Q B S D F C S M B N J C D P O U F N Q P S B S Z N F E J D J O F G B D U P S G T V O I H B J U K E S J F T B S O V Q Z U [ G E P B O N E N E J U S F E B O

U V S F P S F Y I J C J U B O U J C J P M N B D U J W N B U U Z S B F W J F P C M F N D S B H J F T F U G J K P O D S B G

J O U F O T F J O U F S F T U N B U J P O X B T B T T F T T F E J U J T E J D V M M

F B D U J W J U J F T P G U I S F F U I F S B Q U F I V F Q S F D T M C U M E S F V T M V M U T T B W P N E F G E Q F B

F W B M V B U F E B D E J P J M T N G P S N B U J P O " W D P P Q F D W F F O S Z Q S B W J N E T D J D H " N P O H E F T D

GS FTI HB S MJ DT I P XFE U I F I JHIFT UNBBOEUFJ VC JT WBO MNJB BM CD EPBOWS W JC VUB EDOUTFU P  
 SJ BM QSP QFSU1J F TS H "EMT D T BQ B O F T QBL S E SJ E QIFTB O E I FMQ F E ESB U I F Q B  
 1 DV T Q J EYD SJ VINT F TH P PEF D BD Z J O US J D D E MD BBUEN FV OCTU UPBGO U J B M D P O U  
 1 BD CJP T M NG P S N BU J P O 8 I B UT F UFJNPTO PIG E X FF Q E QSF SHD E C H U JI D UF S Q S P  
 NPTUJOU FS F TUJ OHJT UI F BCJM J UZU P JTOW B M W HEGJPO SETS B O F G H J H QHJQF SBFO EFSDP  
 CF U XF FO E J FS F O U BQ Q S P BDI FT JF NYQP IS UBOMUJJO UFF EMC FZ DU UI VF BBMD DU RJ OP UO FP OG U  
 CJP MN E JTS VQ U JO HF O [ Z NFTJOD P N C J O B U J P O X J U I B O U J C J P U J D T  
 4 B V SC JFPV T MN XBT DPN QM F UFMZ EJ T S V Q U E X C Z M E Z H N T U B U Q T J O  
 XJ UIOB G D J M BME V H DOP P TM C ZBD P N C J O B U J P O  
 PGM ZB TFX JUI H F O U B N Z D J O B O E % J T K E S IXXB Ut H & Q PISPU F E C S Z J DBJO O U O  
 # F FD UJ WF M\$Z \$MFCEJCD BFOEMN \$ I J UG S TP /OB U PEO BM 4 D J F O D F \$ F O U S F B O E (D  
 DIJUPTB O C B T FETJ MW FSO BOP Q BSU J D M F T D P # O F 3 B T J M Z SPNTU S F /OB U N P U  
 CJP M N BPGS V H B Q PNTVB BOET PVM E Q S FWF M F Q NF O U J O 1 P M B O E  
 QFOFU SBU J P O P G C J P M NT USV DUVS F T C Z B O U J N J D S P C J B M T J T E B U  
 TVHH FT U F E U I BU "1 % 5 F O [ ZNFT Q M B O U F Y U S B D U T B O E P U I F S  
 D P N Q P V O ET D BOC FV TFEJ OWB S J P V T D P N C J C F T B U J P O T B D U J O H  
 BTH PPEBO U J C J P MN BOEB OUJ NJ D S C J B M B H E P O B U F i 1 F P Q S U F T F B O Q U Z F E G S  
 J OOPW BUJWF T USBUFH JF TN B Z Q PUFO U J B M U M F T U H S P O B M A M 1 F E V M Q B Q U P P T M U D M B N F  
 DBMU SFBUNFO UTBO E DBV T FBO JOD S F B T F O B G U I F J S F F D U J W F O F T T

< > 3 3 P F M B O E U T i FI JT UPS Z P G Q I P U  
 \$ P O D M V T J P O T V O E F S U I + P V S O B M P G U I F " N F S J D B O " D  
 U P M P W P M O P Q Q O

\* O FOW JSPONFO UTU I B U J ODMVEFU I F< I V & B\$ D T CUPF S & PON3J D7 S EIC J B4M B P W M J  
 GP S NBXF MM PSHBOJ [ FETU S VDUV S F U F& S M F E B I C F B Q Q M N D B U J P E F G W C F J M P  
 P QN F OUPGTU S B U F H J F T U P D P N C B U C B D P Q U S S P M P G D I S B O X D G B D H E + S P C E C B M P O G S F M J  
 J T BDI BMMFOHJ OHUB TL U I F TFC B D U F \* O W F T U J B W B M I S F N V D O I N P S F S Q F T J T U B O U  
 U PDMB T T J DB MB O U J N J D S P C J B M U I F S B > Q + J F B T O U E S U F Q D I 1 B C H 4 P H X P S U F E Q B d  
 NBUF S J B M N P S F F B T J M Z V T V O E F S U C J P Q M S F T T P V P S N N P Q D B V T B G Q D R J S T D M F U J F  
 BOUJC JPUJD S F T J T U B O UD M P O F T B S F T F M F D Q P E " O Q J N J D S O P C J B M Q I P  
 E Z O B N J D U I F S B Q Z B Q Q F B S T U P C F B W F S P Z Q B P N J U P F V C D H U F F S P B M Q F S V U / J F  
 PQU J P O U P F F D U J W F M Z D P O U S P M U I F H S . B U X J D L P i G Y N U D S B D E M M J V M B S C & P S F M R N T  
 ) P X F W F S B T X J U I P U I F S B O U J N J D S P C J N B U A J P E O N E M F S B Q J O P T " 1 % Q J T  
 H FOF SB M M Z M F T T F F D U J W F B H B J O T U N J D S P J F Z S B Q K P S T Q T H M S U P X . J (O J H W F  
 CJP M N T U I B O B H B J O T U Q M B O L U P O J D D i F " Q M U M G J P U Y F S C F B J F U B Q P F P D G F S Q B U C F O S F P  
 U P E F W F M P Q B U I F S B Q F V U J D B Q Q S P B D B M U P G " O U J N P D S W P M J B J M Q H O D S Q F B T F  
 U I F T F O T J U J W J U Z P G U I F N J D S P P S H B < O J T N P U S P T B J M S S F O B E Z O P O U S E D V E U M U P O T B M H  
 N F U I P E T F H B O U J C J P U J D U I F S B Q J F P T G " Q U J N W B S P M W B M P M " O H Q H U O U F T Q S V D U V  
 P G U I F C J P M N P S E J T U V S C J O H U I F D > B N # N K B S C O T J D B U J P O C F P T F X F F O + B ' J  
 Q P Q V M B U J P O P G N J D S P P S H B O J T N T J O B F S V H C J Q P P T B M N P P M T J O J U I F S F N Q C S D U F P  
 T F W F S B M N P E F T P G N J D S P C J D J E B M B D U J P B U F G E E B D W I S J D H W W P M P T O Z P O M P H J Q U J  
 F F D U " O F Y B N Q M F P G U I F S T U B Q Q S P B D I J T U P V T F F O [ Z N F T  
 U I B U B F D U U I F C J P M N X I J M F U I < F T > D P O J E B Q Q S J P B V D P D 5 V M H E B C S P O T I  
 B D I J F W F E C Z D P N C J O J O H " 1 % 5 X J U I F V T F P G F O [ Z N F T O P . P S F Q P W F S Q J G X F D P N C J O F  
 " 1 % 5 X J U I U I F V T F P G F O [ Z N F T B < U B S > F T Q B F K D J ' D G T P S B N J ' D S [ P C [ J B B M E  
 T U S V D U V S F T U I F T F M F D U J W J U Z P G U I F E P Q M N S E B S N J I O M Q S P Q F S U J F T P G D M S J P B T H M  
 B T J U Q P U F O U J B M M Z X J M M Q F S N J U U I F V P V S P V P M S P R M P G C \* Q O F F P D U P P O J O F W F M U M P  
 D P O D F O U S B U J P O T O O F E J T B E W B O U B H F P G " O 1 % 5 J T U I F M J N J U F E  
 B N P V O U P G E B U B C B T F E P O B O J N < B M N P # E 8 F M P N T F P X i F . W P B S H F N U F P U P G Q V M N  
 H S P X J O H O J V O N T U V E P F G W F S J G Z J O H X J U S D Z C B J D F E S P F X T & J O T H M B O E + P V S P O M B M P G  
 W B S J P V T Q I P U P T F O T J U J [ F S T J T F O D P V S B H J O H B O E X Q M M E F U F S N J O F  
 U I F E J S F D U J P O P G G V S U I F S S F T F B S D K 5 ' \$ . B I B O E ( " 0 5 P P M F F  
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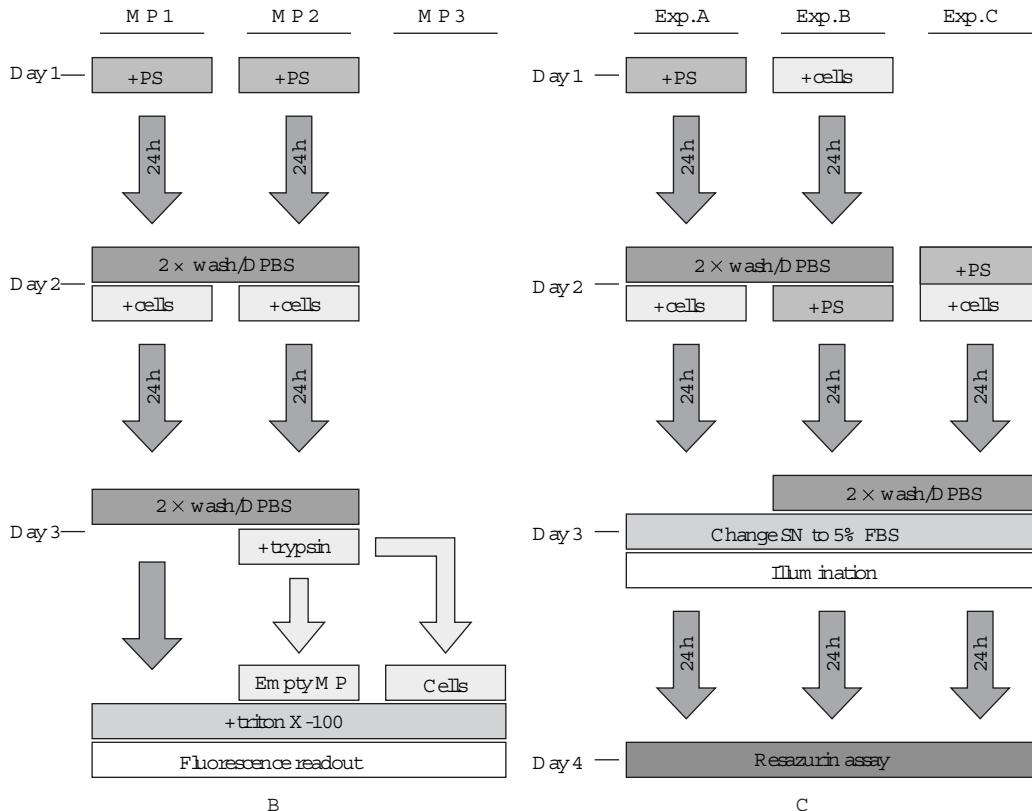
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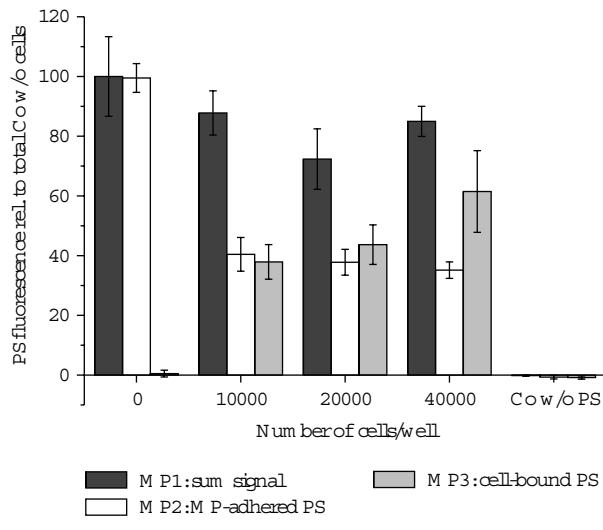
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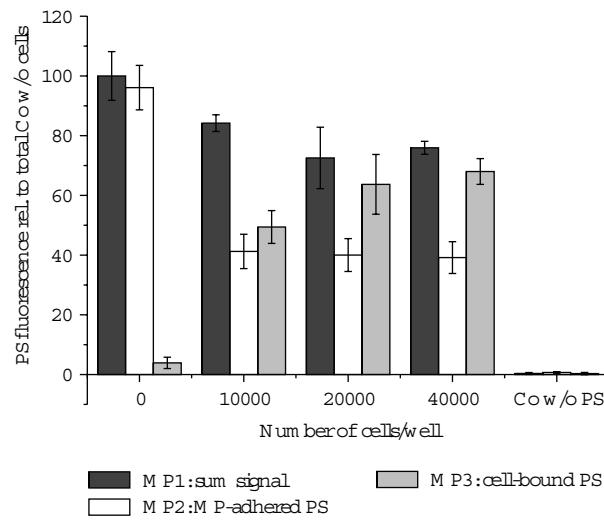
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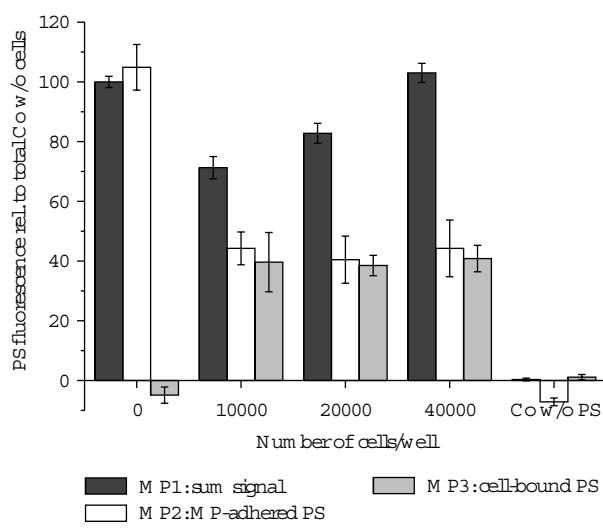
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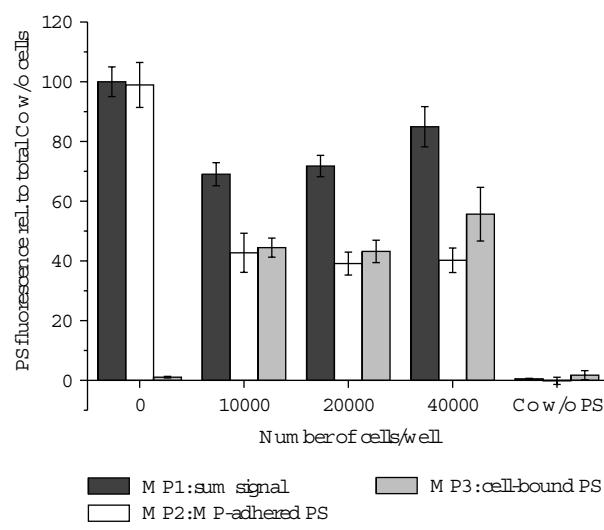
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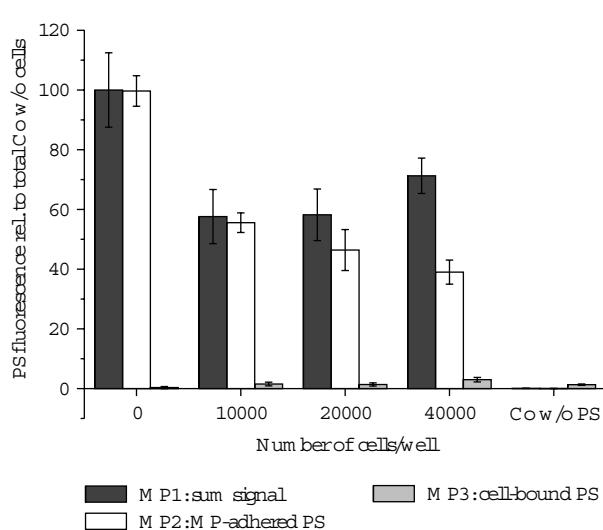
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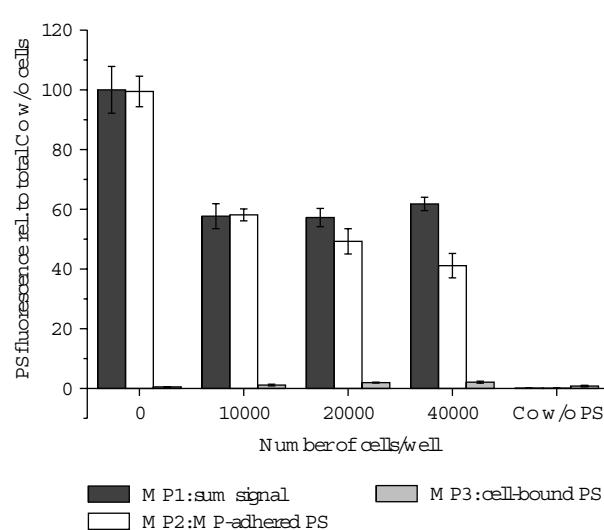
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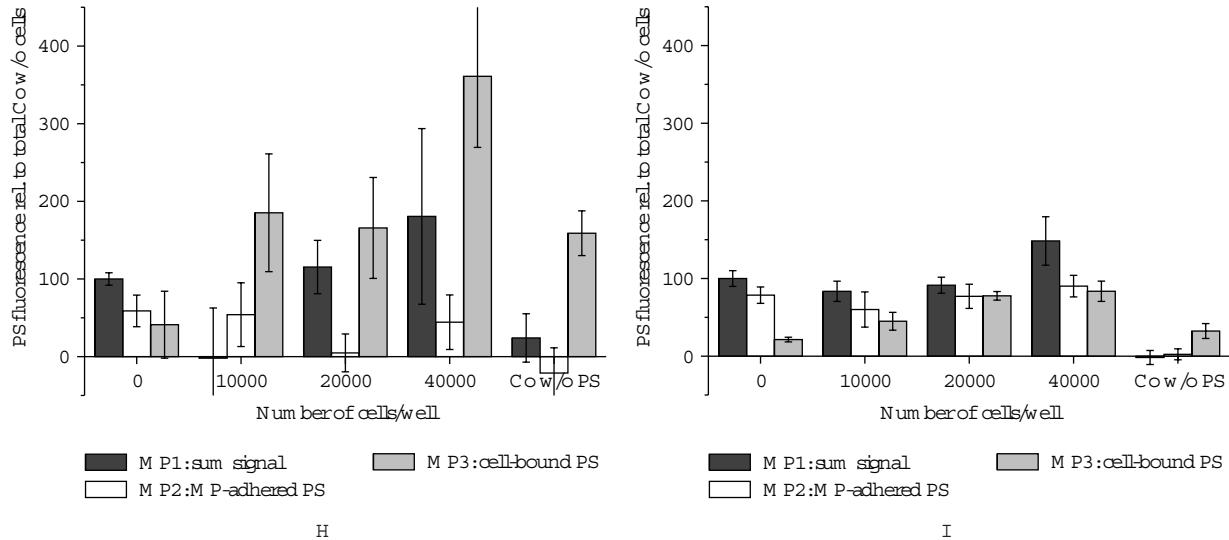


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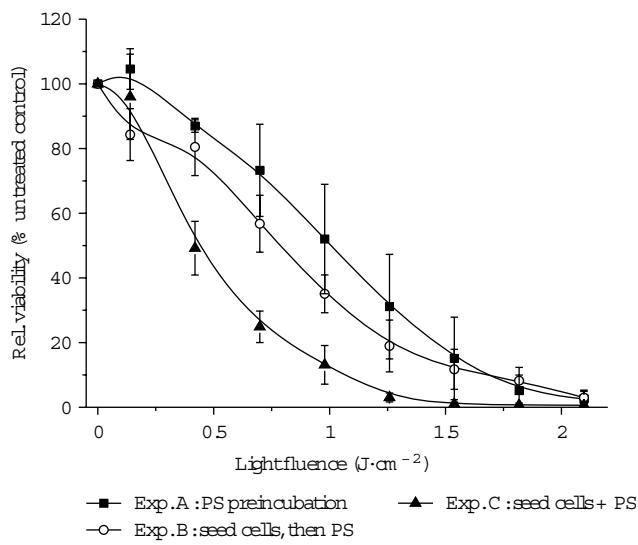
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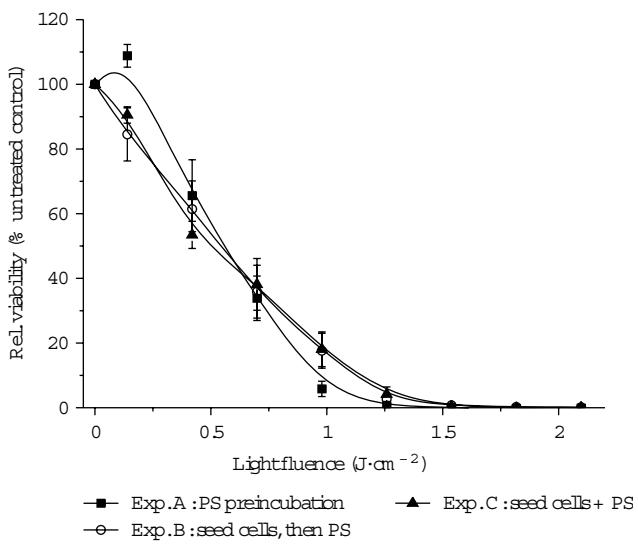
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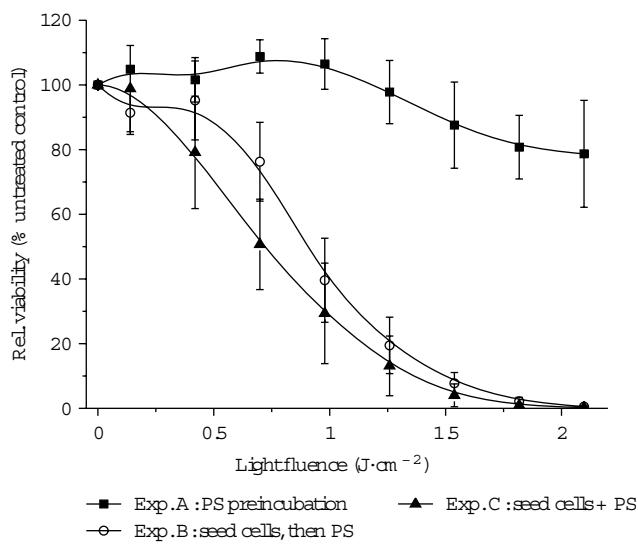
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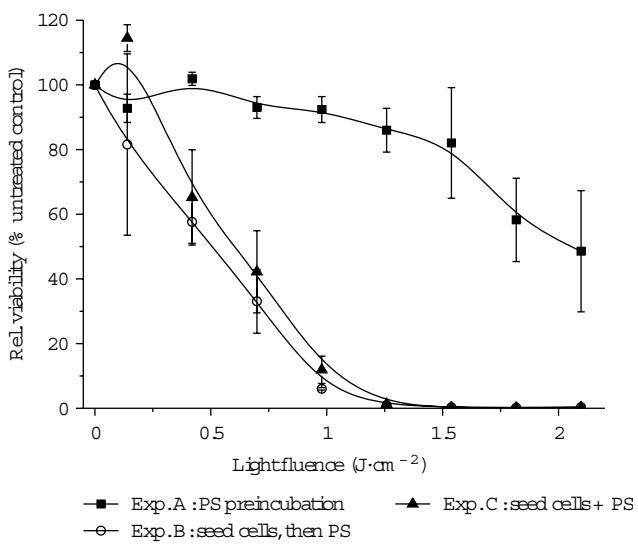
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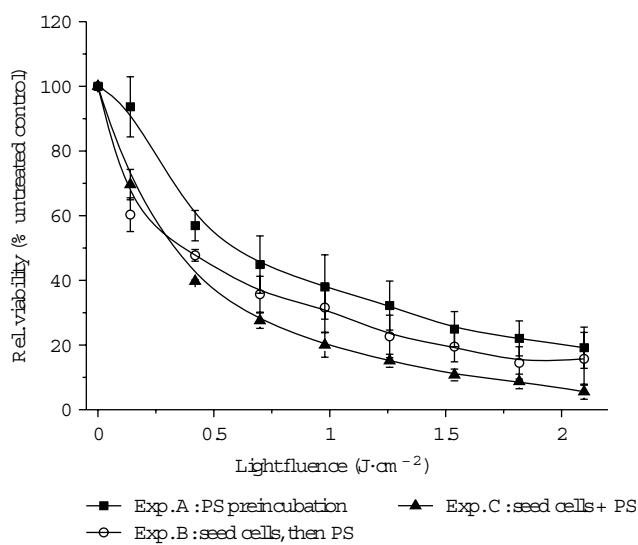
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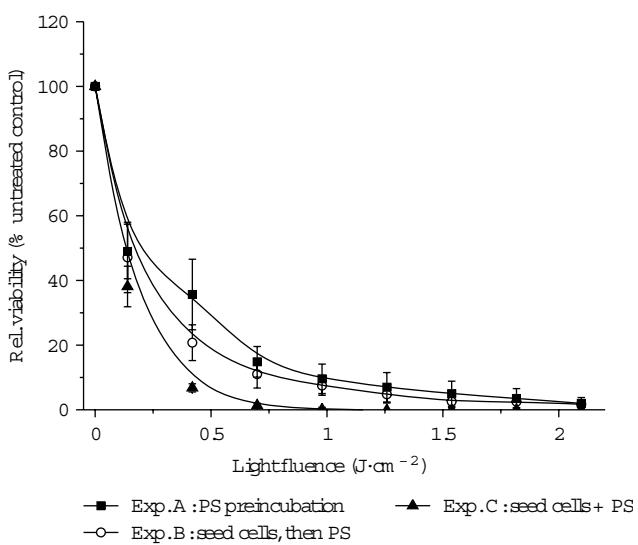
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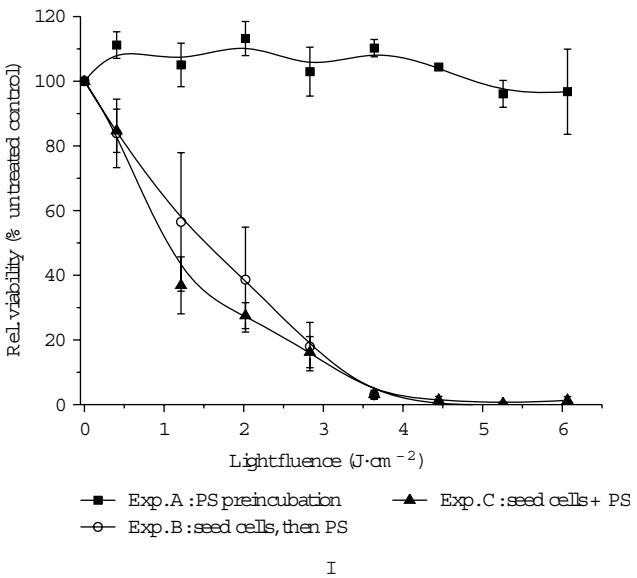
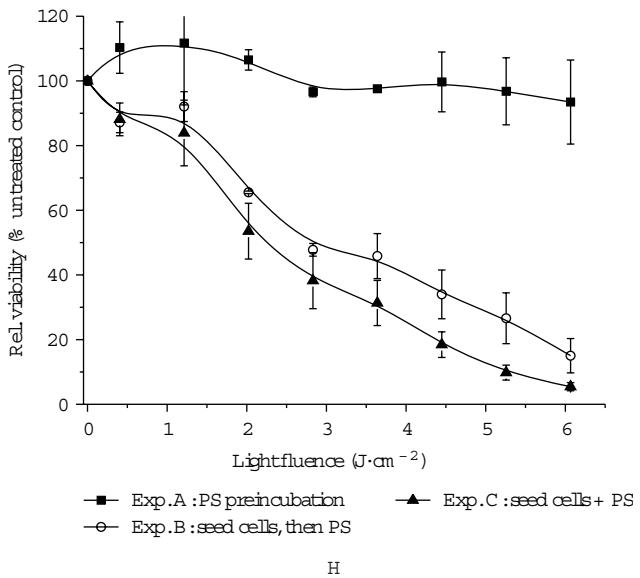
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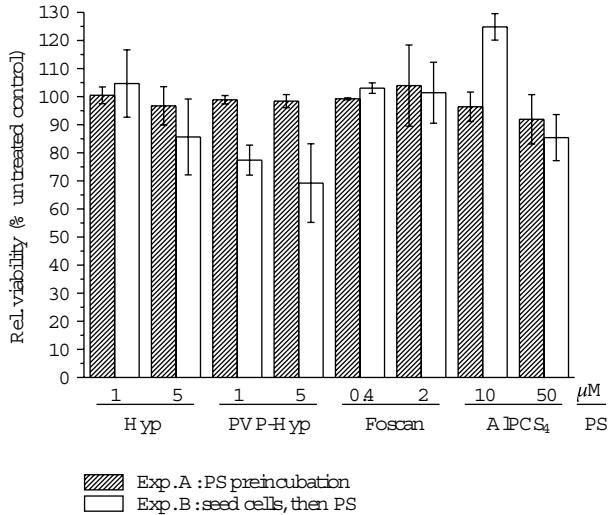


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# J P . F E 3 F T F B S D I \* O U F S O B U J P O B M



" F S E J S F D U J O D V C B U J H O P M G I U \$ L P F S D F F M  
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B D U F S J [ F B 1 4 J O U F S N T P G Q I P U P U P Y J D  
E B S L U I F V Q U B L F C F I B W J P S J O U P D F M M  
P G D F M M E F B U I P S Q B U I X B Z T B O E G B D U P  
E F T U S V D U J P O P G U I F U B S H F U D F M M T # B T J  
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J O D P S Q P S B U J P O J O U I F D F M M T M J Q P Q I B W M F J I D F Q F U S F V O D U U B M U S P B U M F B T V U Q H S B U T J U B I  
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#J P . F E 3 F T F B S D I \* O U F S O B U J P O B M

U I F 4 U J V O H T V O E ' P F S E F S V O H T H F T F M M I T H D T M B O H P G U , I F M B O F U [ W F E S S T J # U Z  
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< > 7 & O H F M I B S E U 5 , J F T T M J D I + # F S M B O E B 4 ) P B V F S # , S B N  
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# J P . F E 3 F T F B S D I \* O U F

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OFHB UJWFCBD UF SJB JODMV EJOHBOUJCJPUJD SFTJTUBK OUTUSBJOT \$  
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" OP UIFSJO UF SF T U JOHUF DIOJ Review PG 14 B JWB UJPOUIBU  
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UPIFBUQSP EVDU JPO XIFSFBT M P X G S F R V F O D Z U S F B O T G F S S F E J O U P C S B J Q O T  
DBWJU B U J P O & YQP TVSFP GCJPM PH J D B M U J T V F T U P V M U S B T P V O E D B  
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.J LB O E3 J F T [ CFM J FWFUI B U C J P M P S H N J D B M F F D U T S O N P O E 4 % E J M B V Z U C F E  
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N F N C S B O F S V Q U V S F B O E G S F F F S E B B U J B D M F O F M V F < # 4 J H N B " M E S  
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J O H T D I F N F B E S V H V O E F S H P F T Q Z S N F N C Z S T B J F T O B M J M E S P D P M M B U B Q P O T J Q H  
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G P S NJOHGSFFS BEJD BM JOUFSNFEJ B U F T X P J B S D F B D V U X J S B M E J B T T P M W F  
P Y Z H F O U P G P S N Q F S P Y Z M S B E J D B M T B O E U I F M B U U F S B U U B D L D F M M V M B  
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N J O F T D F O D F U I B U J T M J H I U B T I F Q V S C E B V F D E E E S V N J J Q O J H O U I F E D B S P L V S 7 T J F  
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U I J T U F D I O J R V F I B T O F W F S C F F O E J Q O T M W F S B G M P F D H J S B B M J E D M B V W J P P D G B C  
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# J P . F E 3 F T F B S D I \* O U F S O B U J P O B M

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# ) N F E J V N N J Y F E X J U I T P M V U J P O T P G . # J O H M B T T U F T U U V C F T  
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3 F T V M U T B O E % J T D V T T J P B D U F S J B X J U I P V U T P O J D B U J P O J O U I F B C T F O D F P G 3 #

5 X P U Z Q F T P G D F M M T X F S F D I P T F O Q B S J Q V T D R O B D S F Q J S B Y J M P Q B P U G J U P I Q F G D U  
U I F T V J U B C J M J U Z P G 4 % 5 G P S E S D P E U J P D C E U F S P O E B R V C F B P D V U T F S B M V U J P O T P G  
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T P O P E Z O B N J D B O U J D B O D F S B D U J W T P Q E P Z G O B N J D B T C D P B E N Q S E B P Q G M F F E J  
J O T F W F S B M X P S L T < > F T P Q E H Y S B E O N E P D B V S B S W M B D E J H G V S F  
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T F W F S B M B V U I P S T C F M J F W F U I B U Q I P Q F P S T F E O O I T J J Q J U I F E S C F 3 B # S V P F F S D B F D S I B U M F T  
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> 3 # B O E . # I B W F H P P E T P M V C J M 4 P Q J D J B O U X J B P Q P F G S U I B P D E V U M I U J V S G F B T D J U O U  
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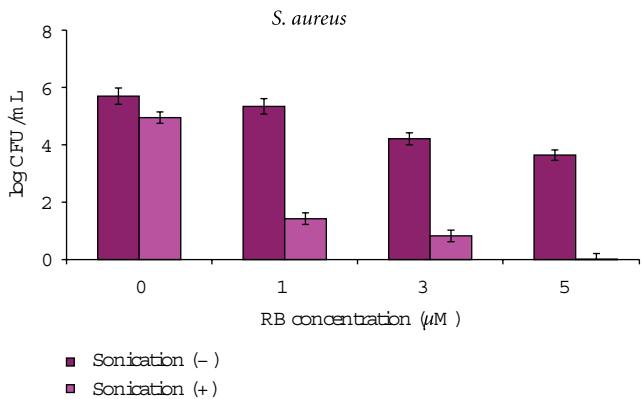
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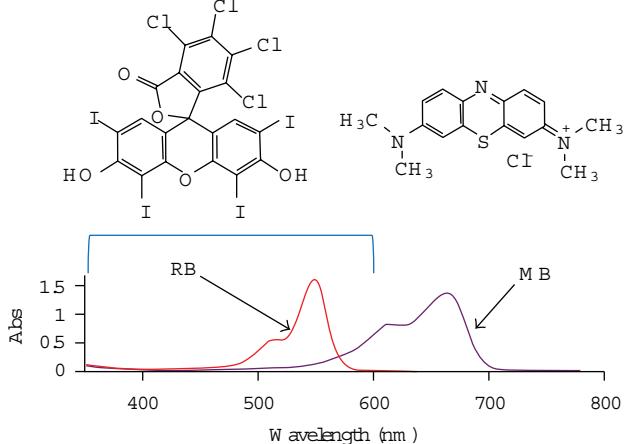
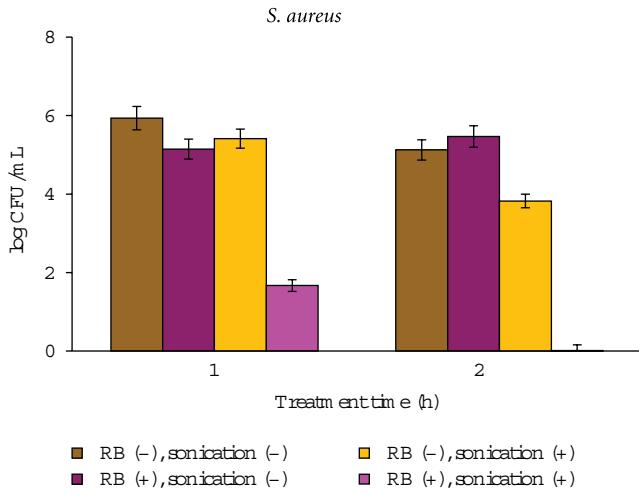
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1 M V T E F T J H O B U F T 4 " \$ 5 U S H E B T W H E B I B T N D Q M U S T P E M O T E N S J C H U B J M D F O M M U D U P J O N B F K G T U S H U J P O

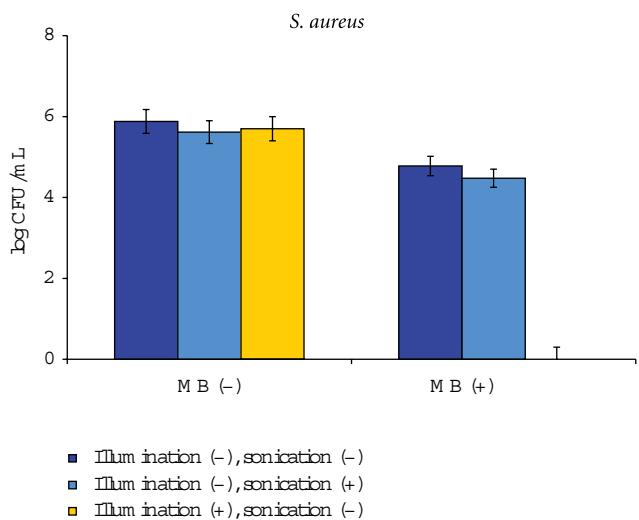


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# J P . F E 3 F T F B S D I \* O U F S O B U J P O B M



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B U B V F O D F S B C U F Q Q G M J F E G N P 8 S D N M G E  
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# J P . F E 3 F T F B S D I \* O U P

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B O E T V S H J D B M B D D F T T P S J F T D P V M E C < F Q S P Q P T F P E S E B " O T J O N & M F L B M P E J B U O J E B  
P G T P O P T F O T J U J [ F S T G P S F Y B N Q M F P Q G I P # P E U P B U N J D F U B F R S B F Z P O V T F Q S I F B U T N F F J O C  
U I F V M U S B T P O J D C B U I X J M M T J H O J D B O I U M U Z B J N B Q H S P T W J F T B O E E I J W Q M B F Z B O T P B F N U I  
F F D U J W F O F T T P G U I F T U F S J M J [ B U J P O Q S P D F T T  
< > 1 . F J T F M B O E 5 , P D I F S i 1 I P U P E Z C  
U B M E J T F B T F T T U + B P W S D S M P G F I B P S U P D W I F N  
1 I P U P C J W M M P H Z # O P Q Q O  
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< 5 > % B J : : ) V B O H B O E . 3 ) B N C M J  
F Q S F T F O U X P S L T I P X T G P S U I F S T B U Q Y J P N M F P D B M J [ B F U F C P G Q P F E I P O P E H T H U B H U T  
B D U J W B U J P O P G 3 # D B V & T D B M B E S J E D V B B W F E S J P I Q P B F G E Z O B N M J D Q P S B Q Z Q Q  
J O U I F E B S L F E F T D S J C F E O P W F M 4 " \$ 5 B U W B D F T O S J R V F I S B T W H M P I B E .  
Q S P T Q F D U T G P S C F D P N J O H B O F F D U J W D F S H C B J S M Q F I U P F E Z B R M D G I F S B D F N G P B E J  
J O H J O U F S O B M J O G F D U J P O T B O E G P S D P M J M J T W Z B G E Q M U F d B J U M E P W O F M E Q N F O B P D S B  
J O T U S V N F O U T . B S J O F W E M V H T O P Q Q O  
" D L O P X M F E H N F O U T  
< > - # S B O D B M F P O B O E ) . P T F M F Z i -  
T P V S D F T G P S Q I P U P E - Z B O B N S U J D O U F I E J S D B B M  
W P M O P Q Q O  
J T S F T F B S D I X B T T V Q Q P S U F E J O Q < B S U F S J C L J U T F 3 F T S 1 M B F U V Q T S S # C  
J U Z P G U I F " S J F M 6 O J W F S T J U Z \$ F O U C B S T P E P O M H N I B S F J N B U U J O H E J P E F M T B Q P T  
U I F \$ I F S O B . P T L P X J U [ ' P V O E B U J B Q P D P S W P M O U J S P O B 6 M " J H B U O T E P S Z H F S Q I  
3 B Q Q B Q P S U ' P V O E B U J P O G P S . F E J D B M E J T B D M S B P T F C S J W B Q M R H Z O B P U J B P S Q \* M B O O  
6 O J W F S T J U Z \* T S B F M U P : / J U [ B O > 0 \$ F C V S L P W B O E ) ( P M M O J D L i  
E F S N B U P & M E H Q F B W O + P V S O B M W P P C % F S N O B P U F Q Q O  
3 F G F S F O D F T  
< > 3 - B Q U F W . / J T O F W J U D I ( 4 J C P C  
< > 5 . B J T D I i " O U J N J D S P C J B M Q I P U P E Z O U S B D N F M M V M B S D I F N Z J M V N J O F T D F G B F P  
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3 F W J F X T J O . F E J D J W P O M B M S O I P F N J Q T Q U S Z U F O D I O J R V F G P S F D J F O U F S B E J D B U J P O

#J P .FE3 FTFBS DI \* O U F S O B U J P O B M

1 IPU PDI F N JTUSZB QWEM1 I P UOPPC J PQMQP H Z > . 4 D I B G F S \$ 4 D I N J U [ 3 ' B D J V T P  
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< > ' / B L P O F D I O Z . / J T O FWJUD I MJH I P P O C B D U F O S J B M O D F M M T D P N J Q B \$ J S T P O C  
i / F X U F D I O J R V F T J O B O U J N J D S P C J B M Q B P E U T P O H M G B N P Y Z H U F I P Q S D P E W J D I U S Z H B Q O F E W  
P G B Q Q M J D B U J P O B O E P W F S D P N J O H E S V R H S A P T M J T U B Q Q D F J O O O P T P D P N J B M  
J O G F D U J E D O J T F O D W F J " H B J O T U . J D S P C J B M 1 B U I P / H F Q E D I F W P B N N V O S J 5 F J D I F S U B  
D B U J O H \$ V S S F O U 3 F T F B S D I B O E . 5 I F S D C O P P M G P U H I F D B M I " Z E M F D P C D M F V T F B O E U P M V J E J O F  
P H Z # P P L 4 F S J F T O P ' P S N B U F Y 3 F T F F B D S B D Z B \$ B Q O U F H S B 4 N Q B P T Q J O U J W F B O E H S  
< > 4 \$ B S Q F O U F S . + ' F I S ( " , S B J V I N T - B E W F E S A T J O 4 1 V E H U F S S J E B M O E i \$ F E O P N D J T O  
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N P M F D V M B S E L S P I D M E H J I C H T R G U I F / B U J P Q . B M J " T D F W F J U N D Z P G / B L P O F D I O Z B O  
4 D J F O D F T P G U I F 6 O J U F W E P 4 M U B U F O T P P G B O N U Q O D S B P C J B M D I F N P U I F S B Q Z C Z M J Q P T  
o  
< > \* 3 P T F O U I B M + ; 4 P T U B S J D B O E J T U S B E M F T [ O P 4 P Q Q E Z O B D N J D U I F S  
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T P V O E M U S B T P O J D T 4 P W D P M D I F N O P T U S Q Z Q E F M J W P S Z P G Q I P U P T F O T T J U J [ F T G P O N E J C W B F D U H  
O J M O 5 F D I 3 J K F L B \$ S P B U J B  
< > 1 8 B O H - 9 J B P 9 8 B O H 9 < J B 8 O E 2 J S B F Y B i ) 4 B Q B E Z O B N J D , F S J  
F F D U T P G Q S P U P Q P S Q I Z S J O \* 9 E J T P E J Y N Q B M Y T P P O C I F S U M F D O I T B P T P E Z O J B D N J D  
U V N P S D E G M M U S E T E W D M J D T O P Q Q F D U X J U I Q I P U P T F O T J U J [ F S T P O G S F F S  
< > ; I B P 2 - J V 8 5 B O H F U B M i % B J M B U J D F H E B W F P D U D T P F W Q M P D U P M D U P M P T S U S Q Z Q  
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& IS M J D I B T D J U F D B 6 M S U S E T C P D J N E T D H W M M J T I R N 4 J T U S B Z U B O B L B ) . J U P N F , : B T V  
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< > : ) F % 9 J O H ( : B O B O E , \* \$ P V E B D B M P G J - I F D N F S J D E M D M S N I F O N F J D P B M 4  
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< > 4 \* 6 N F N V S B / : V N J U B , 6 N F Z N V G E D B B O E 3 / J T I T J H B L J  
i 4 P O P E Z O B N J D B M M Z J O E V D F E F F D U P G S P T F C P O T H B M B Q J T C P M B U + F E J M M  
T B S D P N B \$ E D O F D M F M S I F W N P U I F S B Q Z B O E 1 T B S N B D P M P H V M J Q J E M J Q P T P N F T C  
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J O E V D F E C Z ; J O D \* \* Q I U I B M P D Z B O J O F U P N J D S P U V C V M F T  
B M Q I B B D U J O J O B O E L F S B I U P U P D P G N F F B S Z M H P G Q I P T Q I P M J Q J E M J Q P # T P D N I E T N J U D B E C U B # D J U P F C  
1 I P U P C J W P M P H Z O P Q Q o  
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P G Q S P N F U I B [ J O F I Z E S P D I M P S J E F C Z N V M U J T O F D U S P T D P O J Q D U F D U I B S H E  
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< > / : V N J U B , 4 B T B L J 4 \* 6 N F N V S B " : V L B X B B O E 3 / J T I T J  
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< > 7 . J L B O E 1 3 J F T [ i ' S F F S B E J D B B M J P O J O O S F O B S B U J P O J G T U P F P M H E Z J N  
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< > 5 : V ; 8 B O H B O E 5 + . B T P O i " + S F W J 3 Z X P G ) S F T F B S D I V Q F U B M  
U I F V T F T P G M P X M F W F M V M U S B T G M P U S E T E P J O Q D B D T O D F T P S O B Q B Z U J D M F T G P S U I F S E  
4 P O P D I F N E J M T U S Z O P Q Q o D B O D F S U I F E S W B Q D F E % S V H % F M % O Q S F S T Z 3 F W  
< > / : V N J U B 3 / J T I T J H B L J , 6 N F N V S B " B O S E B A O L T N F N V S B O H i B 4 O E J  
F S H J T U J D F F D U P G V M U S B T P V O E B O E I F N B V U O P U I F S O B I Q D I F O D E S W E M T F B S D P N O B P  
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< > 9 . B ) 1 B O ( 8 V ; : B O H B O E F . : " J ' J S F M Q S B F D J V O D U N B S H F U J O H  
C F F Y Q M P J U F E G P S U I F U S F B U N F O R E J P E N M P D G S P U J B M U E P B J M O V G T S L P V M S V C T Q H E J D S R B O F T T J T  
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" Q Q M J F E B O E & O W J S P O N F W O N B M . O B S P I C 4 P Q R B P Z N J D B M M Z J O E V D F E B O U U V U N P  
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