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Lipophilic rather than hydrophilic photosensitizers show strong adherence to standard cell culture microplates under cell-free conditions

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ABSTRACT

Analysis of photosensitizer (PS) uptake kinetics into tumor cells is a standard cell culture experiment in photodynamic therapy (PDT) – usually performed in plastic microplates or petri dishes. Organic substances such as PS can potentially interact with the plastic surfaces. In this study, we provide a qualitative comparison of three lipophilic PS (hypericin, Foscan® and Photofrin®) and two rather hydrophilic PS formulations (PVP-hypericin and aluminum (III) phthalocyanine tetrasulfonate chloride) regarding their adherence to the surfaces of 96-well microplates obtained from four different manufacturers. For estimation of the relevance of PS adherence for cellular uptake studies we compared the fluorescence signal of the respective PS in microplates containing A431 human epithelial carcinoma cells with microplates incubated with the respective PS under cell-free conditions. We demonstrate that lipophilic PS substances show a strong adherence to microplates – in case of direct lysis and fluorescence measurement resulting in 50% up to 90% of the overall signal to be caused by adherence of the substances to the plastic materials in a cellular uptake experiment. For the hydrophilic compounds, adherence is negligible. Interestingly, adherence of PS agents to microplates takes place in a time-dependent and thus kinetic-like manner, requiring up to several hours to reach a plateau of the fluorescence signal. Furthermore, PS adherence is a function of the PS concentration applied and no saturation effect was observed for the concentrations used in this study. Taken together, this study provides a systematic analysis under which conditions PS adherence to cell culture plates may contribute to the overall fluorescence signal in – for example – PS uptake experiments.

1. Introduction

Fluorescence detection (FD) and photodynamic therapy (PDT) utilize the interaction of a photosensitizing agent (photosensitizer, PS) and visible light to detect or destroy harmful cells [1,2]. In addition to the treatment of tumors [3–5], PDT is established for treatment of age-related macular degeneration [6]. Recently, inactivation of microorganisms using photodynamic procedures gained attention due to the fact that even bacterial strains resistant against conventional antibiotics are susceptible towards this treatment [7–11]. For all modalities, (semi-) selective uptake of the photosensitizer into target cells and its accumulation therein is central to effective killing of these cells whilst sparing healthy tissue [12]. Up to date, a broad range of photosensitizers with different physicochemical properties (e.g. water solubility) does exist, ranging from naturally occurring substances like hypericin or protoporphyrin IX to synthesized compounds (e.g. phthalocynanines) [13] and still new drugs with improved properties for the application in the frame of PDT are reported. Initial testing of new substances frequently includes experiments on uptake kinetics into the target cells, usually by measuring the fluorescence signal of the photosensitizers. PDT is a multi-parametric procedure affected by, for example, the concentration and incubation period of the PS as well as the light intensity and -fluence. Therefore, in vitro investigations on cellular mechanisms of PDT or for establishment of basic data for subsequent in vivo protocols may involve testing of various conditions regarding incubation time, concentration, cell density, etc. The use of multi-well microplates is convenient due to the small amount of cells and substances required for parameter optimization. Additionally, microplates allow for high throughput analysis as numerous samples can be tested simultaneously. Growth of adherent cells requires a special surface coating which

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varies among the manufacturers and is a corporate secret. Although cell culture plastic has several advantages over glassware including certified sterility and single use, organic substances may interact with the chemical surface of the cell culture vessel. For biological experiments, these interactions between the substances of interest and the cell culture plastic materials have to be minimized where possible and potential influence on the results have to be considered. To correct for e.g. cellular autofluorescence, standard control samples in cellular PS uptake studies are cells incubated with incubation medium without the photosensitizer (for examples, see [14–17]). Similarly, several studies considered the effect of PS binding to plastic culture dishes including appropriate control samples for fluorescence background correction [18–20]. However, up to date, no systematic analysis, including different types of PS, incubation conditions and products obtained from different manufacturers was reported.

Therefore, in this study we qualitatively compared both lipophilic and hydrophilic PS for their interaction with tissue culture microplates obtained from different manufacturers using a standard procedure for performing uptake kinetics into cells. A detailed analysis of the fluorescence background signal caused by the PS adherence to the microplates provided information on the time- and concentration-dependent characteristics of this effect. Comparison of the fluorescence signals read in microplates without cells to the signals from wells containing different cell numbers allowed estimation about the quantitative contribution of this background fluorescence to the overall signal measured.

2. Material and methods

2.1. Microplates

For investigation of PS adherence to microplates 96-well types were used from five different manufacturers (for details see Table 1). For better readability, the plates will be referred to as Falcon, Costar, Nunc and Greiner throughout the manuscript. One type of microplate (from Greiner) was representatively tested with both, coated and non-coated surface to examine the effect of surface coating (data not shown).

2.2. Photosensitizers

Foscan® (meso-tetrahydroxyphenyl chlorine, mTHPC, temoporfin) was provided by Biolitec AG (Jena, Germany). Stock solution of Foscan® was used as provided by the manufacturer (i.e. dissolved in ethanol–propylene at a stock concentration of 5.87 mM). Hypericin was obtained from Planta (Vienna, Austria) and dissolved in dimethyl sulfoxide (DMSO) at 1.98 mM for the stock solution. Hypericin bound to polyvinylpyrrolidone (PVP-hypericin) was synthesized as described by Kubin et al. [21] to improve water solubility. Stock solutions of PVP-hypericin were prepared in DPBS (2.0 mM). Aluminum (III) phthalocyanine tetrasulfonate chloride ($AlPcS_4$) was obtained from Porphyrin Products (Logan, USA). For stock solutions, $AlPcS_4$ was prepared in DPBS (1.0 mM). Photofrin® (Porfimer sodium) from QLT Pharmaceuticals (Vancouver, Canada) was dissolved in DPBS (16.67 mM).

Working solutions of the respective PS were prepared in medium without serum (diluted 1:10 from stock solutions) and stored at 4 °C in the dark.

2.3. Cell-free PS adherence

Every photosensitizer was tested for adherence in media with and without FBS under cell-free conditions. The PS solutions (medium volume 100 μl per well) were incubated in microplates for 0, 1, 4, 8, 12 and 24 h in the dark (37 °C, humidified atmosphere, 5% CO_2). Microplates were then washed twice with DPBS and treated with 50 μl Triton X-100 (1% v/v) for 10 min at room temperature prior to fluorescence measurement. Fluorescence was read with the parameters given in Table 2 using a Infinite M-200 microplate reader (Tecan, Groedig, Austria).

2.4. Cell culture

A431 human epidermoid carcinoma cells (ATCC-Nr. CRL-1555) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM HEPES, 5% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine, 1 mM Na-pyruvate, 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin at 37 °C and 5% CO_2. All experiments were done using cells of passage number 5 to 20. All media and supplements were obtained from PAA (Pasching, Austria).

2.5. Cell density-dependent adherence

Cells (human epidermoid carcinoma cell line, A431) were seeded in microplates in 100 μl medium containing FBS at nine different cell numbers ranging from 702 to 15,000 cells per well. After 24 h incubation at 37 °C and 5% CO_2 in a humidified atmosphere, microplates were washed twice with DPBS and 100 μl fresh medium (without FBS) containing the respective PS were added. According to standard concentrations applied in our lab [22] Foscan was investigated at a final concentration of 400 nM, PVP-hypericin and hypericin at 1 μM, $AlPcS_4$ at 10 μM and Photofrin® at a final concentration of 2 μM. After 24 h incubation at 37 °C and 5% CO_2, fluorescence signal from cells and plates was measured by direct lysis in the microplates as described above. For each PS, the fluorescence signal under cell-free conditions was read using the same instrument's gain which was used for quantification of the PS in the cell dilution series.

Table 2
Fluorescence measurement parameters.

Photosensitizer	Excitation wavelength (nm)	Emission wavelength (nm)	Gain (a.u.)
Foscan®	426	658	100–119
hypericin	340	604	140–170
PVP-hypericin	340	604	140–170
$AlPcS_4$	368	684	110–175
Photofrin®	410	630	140–158

Table 1
Microplate description and manufacturers.

Manufacturer	Product description as provided from manufacturer	Catalog number
BD Biosciences, Heidelberg, Germany	Falcon® 96-well microplate, clear, tissue-culture treated, flat-bottom	353072
Corning B.V. Life Sciences, Amsterdam, Netherlands	Costar® 96 well clear flat bottom polystyrene tc-treated microplates	3598
Thermo Fisher Scientific (Nunc GmbH & Co. KG), Langenselbold, Germany	Nunc MicroWell™ plates, cell culture, clear	167008
Greiner Bio-One, Kremsmuenster, Austria	cell culture plate, 96 well, PS, f-bottom (chimney well), crystal-clear	655180
Greiner Bio-One, Kremsmuenster, Austria	microplate, 96 well, PS, f-bottom, crystal-clear	655101