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Compromising the plasma membrane as a secondary target in photodynamic therapy-induced necrosis

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ABSTRACT

Photodynamic therapy (PDT) is a non-invasive treatment widely applied to different cancers. The goal of PDT is the photo-induced destruction of cancer cells by the activation of different cell death mechanisms, including apoptosis and/or necrosis. Recent efforts focusing on understanding the mechanisms of cell death activated by PDT find that it depends on the type of photosensitizer (PS), targeted organelles, and nature of the light used. It is generally accepted that very short incubation times are required to direct the PS to the plasma membrane (PM), while longer periods result in the accumulation of the PS in internal compartments such as the endoplasmic reticulum or mitochondria. Glycosylation of the PS targets cancer via saccharide receptors on the cell surface, and is generally assumed that these compounds rapidly internalize and accumulate, e.g. in the endoplasmic reticulum. Herein we demonstrate that a minor fraction of a glycosylated chlorin compound residing at the PM of cancer cells can activate necrosis upon illumination by compromising the PM independently of the length of the incubation period. The results presented here show that the PM can also be targeted by glycosylated PS designed to accumulate in internal organelles. PS activation to induce necrosis by compromising the plasma membrane has the benefits of fast cell death and shorter irradiation times. The findings described here expand our understanding of the cellular damage induced by phototherapies, presenting the possibility of activating another cell death mechanism based on the incubation time and type of light used.

1. Introduction

As most widely practiced, anticancer PDT employs a dye capable of photosensitizing the formation of reactive oxygen species (ROS) such as singlet oxygen upon visible light illumination of the cells or tissues containing the dye.[1] The formation of reactive singlet oxygen species promotes damage to different cellular organelles, ultimately leading to cell death.[1,2] Research using a variety of cell lines and methodologies shows that the cell death resulting from the organelle damage can be either mediated by apoptosis, necrosis and/or by autophagy or by the combination of these processes.[1–6] In general, the primary location of cellular damage corresponds to the compartment in which the PS is mainly localized inside the cells.[2,6,7] A PS that localizes in the endoplasmic reticulum (ER) and/or mitochondria generally activates apoptosis under photodynamic treatment,[8,9] while a PS confined to the PM or endosomes/lysosomes induces necrosis.[2,10,11]

Importantly, a PS may be more efficient at inducing cell death in a specific cellular compartment. For example, Zhang et al.[11] recently reported that a PS linked to a peptide, first binds selectively with the PM of the cancer cells and then localizes only in the ER after an extended incubation time. Activation of this PS conjugate by light is more efficient to induce cell death when located at the PM than when concentrated at the ER. These findings are in agreement with prior literature showing that the incubation protocol determinates the cell death pathway activated in PDT.[12] These studies accept the paradigm that targeting the PM requires a short PS incubation period, while longer incubation protocols promote intracellular PS localization at the expense of the PM population.[3,10,11,13–16] Since most PS exhibits some fluorescence, fluorescence microscopy can be used to follow the partitioning and localization of the PS within the cell.

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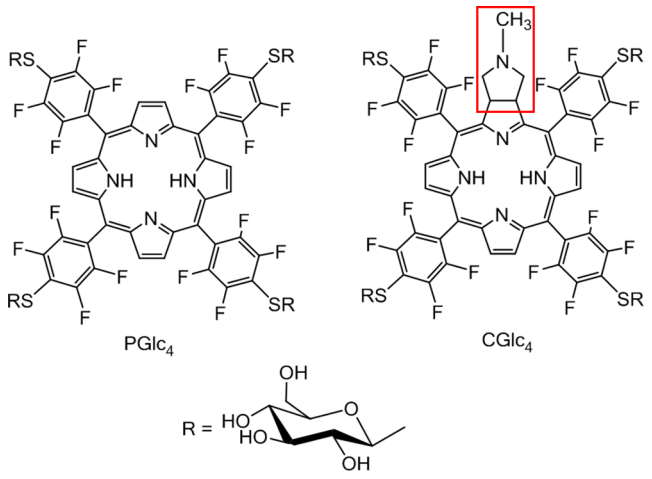

Scheme 1. Structure of $PGlc_4$ and $CGlc_4$; the red box highlights the reduced double bond on the pyrrole stabilized by the *N*-methylpyrrolidine.

Many porphyrins conjugated to sugar moieties to target cancer are reported as potential next-generation PS for PDT.[17–19] We previously reported that simple click chemistry allowed the formation of a tetra-thioglycosylated porphyrin ($PGlc_4$) and other tetra-thioglycosylated derivatives such as the chlorin ($CGlc_4$), isobacteriochlorin ($IGlc_4$), and bacteriochlorin ($BGlc_4$) wherein the sulfur linking group resists hydrolysis by acid/base and enzymes.[4,20,21] These compounds were shown to be effective PDT agents in a variety of cell lines, especially those expressing glucose transporters on the membrane. Upon extended incubation of about one doubling time, these compounds localize mainly in the ER and upon white light irradiation promote apoptosis mediated by ER damage.[4]

Even though the glycosylated compounds $PGlc_4$ and $CGlc_4$ (Scheme 1) initially target membrane receptors these dyes eventually partition mainly to the ER. However, we report here that the small fraction remaining on the PM even after extended incubation times is sufficient to induce necrosis upon light irradiation. This observation indicates that short uptake time protocols meant to exploit the PS while transiently in the PM are not an absolute requirement for photo-induced damage to the PM. PDT with nonhydrolyzable glycosylated porphyrinoids can be used to damage the PM independent of the incubation time. We demonstrate that the PS population at the PM, either static or transient, is able to induce necrosis by compromising the PM, even though the majority of PS is localized in intracellular compartments. These findings expand our understanding of cell death mediated by PDT by showing that there are multiple locations of action occurring concomitantly thereby overpowering the diverse cell repair mechanism. The description of this mechanism suggests development of new activation protocols in PDT that take advantage of PM damage-mediated necrosis can significantly improve both efficiency and efficacy.

2. Experimental procedures

2.1. Cell culture and treatments

Chinese Hamster Ovary Cells (CHO) were maintained in F12 medium, 5% Fetal bovine serum Cell medium was supplemented w/v 1% antimycotic, at 37 °C and 5% CO_2 atmosphere. Typically, $\sim 2 \times 10^4$ cells mL^{-1} were seeded in cell culture plates and allowed to grow for 24 h. For experiments where endocytosis was inhibited, culture plates containing cells were placed on ice for at least half an hour and all the buffers were maintained at 4 °C. $CGlc_4$ was dissolved in DMSO and added to the cell culture medium depending on the protocol used. The final concentration of DMSO in cell culture was never higher than 0.1%. The cultures were rinsed 2–3 times with fresh medium M2 to remove any unbound PS before proceeding to the various assays. Cell fixation was performed using 4% paraformaldehyde. The stripped-membrane protocol involved five washes with PBS containing 5% of KI to assure the membrane proteins, inducing PS, are removed from the PM.

2.2. Fluorescence microscopy

To detect necrosis, ethidium homodimer (EthD-1) from the LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen) was used. The dye was added to cells in M2 medium for five minutes. The fluorescence emission of EthD-1 increases 5-fold when intercalated into DNA, and is extensively used to detect necrosis when the plasma membrane integrity is compromised.

Fluorescence microscopy was performed using on an inverted Leica DM IL instrument, exposure times were 100 ms for imaging and 30 s for PDT treatment, a 'Cy5′ band pass (excitation 580 nm–640 nm and emission 650 nm–720 nm) was used for experiments involving $CGlc_4$. Bright field images were taken on the same microscope. A4 band pass (Excitation 455 nm to 495 nm–Emission 515 nm to 535 nm) band pass emission filter for dextrans-fluorescein. Filter TRITC (Excitation 500 nm to 550 nm–Emission 560 nm to 610 nm) for CellMask and EthD-1 experiments. All the dyes were purchased from Thermo Fisher Scientific. In general, the activation of the dye used focused light from the microscope using the aforementioned band pass filters such that the power was 6 mW.

2.3. $CGlc_4$ synthesis and purification

The details of the synthesis were previously reported.[20] Briefly, to a solution of the chlorin (32 mg, 30 μmol) and 2,3,4,6-tetra-*O*-acetyl-glucosylthioacetate (52 mg, 135 μmol, 4.5 equiv.) in DMF (3.0 mL) diethyl amine (0.8 mL) were added. The reaction mixture was stirred at room temperature for about 2 h. Water was then added to the reaction mixture and extracted with methylene chloride three times and then the organic layer was washed with brine. The methylene chloride was then removed under reduced pressure. The crude mixture recovered was purified by flash chromatography (silica gel) using a hexanes:ethylacetate 2:3 solution as eluent. The protected glycochlorin (60 mg, 82%) was then obtained after precipitation in CH_2Cl_2/hexanes, as a green powder. Glycochlorin (40 mg, 16.5 μmol) was dissolved in methanol/CH_2Cl_2 (3:1, 4 mL) mixture solution and then treated with sodium methoxide (0.5 M solution in methanol, 1.5 mL). The reaction mixture was then stirred at room temperature for about 2 h and then an aqueous citric acid solution was added to neutralize the reaction mixture. The mixture is filtered through Waters Sep-Pak C_{18} 35 cc reverse phase prep column and washed with water. The deprotected $CGlc_4$ was then eluted with methanol and purified by flash chromatography (silica gel) using a mixture of methanol/ethyl acetate (2:3) mixture solution as eluent. Chlorin (27 mg, 93%) was obtained as green powder. ^1H, ^{13}C, and ^{19}F NMR, high-resolution mass spectrometry, and UV–visible spectra were all consistent with previous reports.[20]

3. Results

3.1. Necrosis activation by $CGlc_4$

We previously reported that the tetra-thioglycosylated porphyrin $PGlc_4$ (Scheme 1) accumulates in the ER, and upon activation, it induces apoptosis through a mechanism that initiates from ER damage.[4,21] Photo-activation of $CGlc_4$ can promote cell death by apoptosis, although to a lesser extent than $PGlc_4$. The lower potential of $CGlc_4$ to activate apoptosis is due to its 6-fold increase in fluorescent quantum yield, and concomitant decrease in singlet oxygen quantum yield compared to $PGlc_4$.[20] To better understand the events that occur during $CGlc_4$ uptake, we first performed live fluorescence microscopy