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Research Paper

The diverse roles of glutathione-associated cell resistance against hypericin photodynamic therapy

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ABSTRACT

The diverse responses of different cancers to treatments such as photodynamic therapy of cancer (PDT) have fueled a growing need for reliable predictive markers for treatment outcome. In the present work we have studied the differential response of two phenotypically and genotypically different breast adenocarcinoma cell lines, MCF7 and MDA-MB-231, to hypericin PDT (HYP-PDT). MDA-MB-231 cells were 70% more sensitive to HYP PDT than MCF7 cells at LD_{50}. MCF7 were found to express a substantially higher level of glutathione peroxidase (GPX4) than MDA-MB-231, while MDA-MB-231 differentially expressed glutathione-S-transferase (GSTP1), mainly used for xenobiotic detoxification. Eighty % reduction of intracellular glutathione (GSH) by buthionine sulfoximine (BSO), largely enhanced the sensitivity of the GSTP1 expressing MDA-MB-231 cells to HYP-PDT, but not in MCF7 cells. Further inhibition of the GSH reduction however by carmustine (BCNU) resulted in an enhanced sensitivity of MCF7 to HYP-PDT. HYP loading studies suggested that HYP can be a substrate of GSTP for GSH conjugation as BSO enhanced the cellular HYP accumulation by 20% in MDA-MB-231 cells, but not in MCF7 cells. Studies in solutions showed that L-cysteine can bind the GSTP substrate CDNB in the absence of GSTP. This means that the GSTP-lacking MCF7 may use L-cysteine for xenobiotic detoxification, especially during GSH synthesis inhibition, which leads to L-cysteine build-up. This was confirmed by the lowered accumulation of HYP in both cell lines in the presence of BSO and the L-cysteine source NAC. NAC reduced the sensitivity of MCF7, but not MDA-MB-231, cells to HYP PDT which is in accordance with the antioxidant effects of L-cysteine and its potential as a GSTP substrate. As a conclusion we have herein shown that the different GSH based cell defense mechanisms can be utilized as predictive markers for the outcome of PDT and as a guide for selecting optimal combination strategies.

1. Introduction

Photodynamic Therapy is a photomedical treatment using a photosensitive substance, photosensitizer (PS), which upon irradiation by light at the appropriate wavelength, interacts with biomolecules or molecular oxygen to produce reactive species. PDT has been approved for several indications, in particular for cancerous diseases, but mainly as a palliative end point. Despite that PDT has been evaluated in clinical trials for more than 30 years and despite approvals for clinical use, PDT has not so far become part of standard clinical practice. In recent years molecular biology-based research, including deep sequencing analyses, has documented the large tumor heterogeneity even between tumors of the same origin and sub-classification [1]. This has led to development of personalized medicine based on predictive markers for treatment response and is expected to result in the use of approved drugs and treatment modalities on only a subfraction of tumors today treated uniformly [2]. This has already shown to be a good strategy for treating EGFR positive colon cancer depending on their ras status [3]. The differences in tumor sensitivity to PDT have been shown to be large although little has been done to reveal the mechanistic basis for this difference [4]. Studies of mechanisms influencing the cell sensitivity to PDT may be utilized in the search for predictive markers for PDT response and better selection of patients to undergo PDT. In this way the clinical benefit of PDT may be more easily documented and lead to better selection of patients and therapeutic regime.

PDT is under consideration for treatment of breast cancers [5]. However, molecular expression profiles have shown that breast cancers may be divided in several subgroups with different sensitivity to various treatments [6] that may also include PDT. In addition,

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standard therapies include ionizing radiation, various chemotherapeutic and hormone based therapies as well as mAbs such as Herceptin. These treatment modalities induce resistance mechanisms that could influence second line treatments such as PDT. Thus, it is of outermost importance to reveal the predictive markers for PDT sensitivity of the cancer cells and in this way select eligible patients for PDT.

In order to seek knowledge regarding possible predictive markers for sensitivity to PDT, two invasive ductal/breast carcinoma cell lines (MCF7 and MDA MB 231) have been selected in this study, for the following reasons: (i) MCF7 cells are estrogen and progesterone receptor positive, while MDA-MB-231cells are triple negative; (ii) MCF7 cells prefer oxidative phosphorylation for ATP production at normoxic conditions (Pasteur type metabolism) and switch to glycolysis under hypoxia, whilst MDA-MB-231 cells rely on glycolysis for ATP production in both normoxic and hypoxic circumstances (Warburg type metabolism) [7] and (iii) MCF7 cells express the epithelial phenotype while MDA-MB-231 cells express mesenchymal characteristics [8]. Also, MDA-MB-231 breast cancer cells have been documented for their multidrug resistance [9], and this has been attributed to e.g P-gp upregulation [9]. The lack of estrogen receptors (ER) has rendered MDA-MB-231 cells insensitive to treatments with antiestrogens, such as tamoxifen [10], which is widely used in breast cancer chemoprevention [11], but also as an adjuvant in treatment of primary disease [12].

In the present project, we endeavoured to mechanistically elucidate the different responses to hypericin (HYP)-PDT between MCF7 and MDA-MB-231 cells, which we observed in the course of experiments, based on their relevant differences. It has to be noted that the recorded difference in photocytotoxicity between the two cell lines was also detected previously, in our recent work on bimodal porphyrin-cyclodextrin system [13]. In that work the phototoxicity was conferred by 5,10,15,20-Tetrakis(*m*-hydroxyphenyl)-21,23*H*-porphyrin. A profound discrepancy between the responses of the two cell lines to $TPCS_{2a}$ PDT was also observed elsewhere [14]. In that study MCF7 cells were practically insensitive to $TPCS_{2a}$ PDT, while MDA-MB-231 were very efficiently decimated by the same treatment.

2. Materials and methods

Chemicals and reagents RPMI 1640 without phenol red, L-Glutamine, penicillin/streptomycin, trypsin, dimethylsulfoxide (DMSO), Antimycin A (ANTI-A), *N*-Acetyl-L-cysteine (NAC), Ethylenediaminetetraacetic acid (EDTA), trizma® hydrochloride (TRIS-HCL), trizma® base (TRIS-BASE), , polyethylene glycol sorbitan monolaurate (TWEEN 20), bovine serum albumin (BSA), Buthionine sulfoximine (BSO), Carmustine (BCNU), Triton X-100, thiazolyl blue tetrazolium bromide (MTT), β-Nicotinamide adenine dinucleotide reduced disodium salt (NADH), metaphosphoric acid (MPA), triethanolamnine (TEAM), anti-γ-tubulin, Glutathione S-Transferase (GST) Assay Kit and sodium pyruvate were purchased from Sigma-Aldrich Norway AS (Oslo, Norway), Anti-GPX-4 (H-90) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.) and anti-GSTP1 (3F2) from Cell Signalling Technology, Inc. (Danvers, MA, U.S.A.). Hypericin (HYP, 99.3%) was obtained from Planta Natural Products GmbH (Vienna, Austria). The total glutathione (GSH) assay kit and 17β-Estradiol (E2) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

2.1. Cell culture

The MCF7 and MDA-MB-231 (triple negative) human breast adenocarcinoma cell lines were purchased from ATCC. Both cell lines were grown in RPMI 1640 media without phenol red, supplemented with 10% FBS, 100 U/mL penicillin/100 μg/mL streptomycin and 2 mM L-Glutamine at 37 °C in a 5% CO_2, humidified atmosphere. Cells were inoculated into 96-well plates (20×10^3 cells/100 μL media/well) or 6 well plates (1×10^6 cells/2 mL media/ well), 24 h prior to treatment.

2.2. Cell treatment

BSO was added to cells overnight and was subsequently maintained in the appropriate treatment groups until cytotoxicity assessment. NAC was added to the appropriate cell groups together with HYP (4 h before irradiation) and maintained up to the cytotoxicity assessment (24 h after irradiation). The DMSO content (where applicable) was at all times kept ≤0.25%. Following cell incubation with HYP for 4 h, all treatment groups were washed twice. The cells were irradiated from the plate underside by means of a *Lumisource* lamp (PCI Biotech AS, Oslo, Norway) through a 530 nm cut-off longpass filter (Roscolab Ltd, London, U.K.), at an irradiance of 4 mW/cm^2.

2.3. Cytotoxicity assessment

Cells were inoculated (20×10^3) into 96-well plates and left to incubate in complete media containing 10% FBS for 24 h. Cells were then treated with media only or HYP (2 μM), in the presence of modulators (where appropriate) and irradiated. The cell viability was assessed by the MTT assay 24 h post-irradiation. The assay was performed by replacing cell media with complete media containing 0.5 mg/mL MTT and incubating at 37 °C in a 5% CO_2 humidified atmosphere for 3 h. MTT media were subsequently aspirated from all cells and the produced formazan crystals solubilized with 100 μL DMSO per well. The plates were shaken for 10 min at ~300 rpm in a Heidolph Titramax 101 orbital shaker (Heidolph Instruments GmbH & Co.KG), and the endpoint absorbance measurements at 570 nm were performed in a BioTek PowerWave XS2 plate reader (BioTek Instruments, Inc.). Blank values measured in wells with DMSO and no cells, were in all cases subtracted.

2.4. Hypericin loading

Fluorescence spectroscopy was employed to monitor HYP loading in MCF7 and MDA-MB-231 cells. Cells were seeded in 96 well plates. The designated groups were treated with 100 μM BSO overnight. Subsequently cells were treated with vehicle, HYP, BSO+HYP, NAC +HYP and BSO+NAC+HYP for 4 h. All cells were consequently washed twice and all cell groups were placed in complete media. The fluorescence was read in a Biotek synergy 2 platereader (BioTek Instruments, Inc.) using a 530 ± 25 nm bandpass excitation filter and a 590 ± 35 nm bandpass emission filter. Empty wells incubated with HYP and washed twice were used as blanks and subtracted from all data groups.

2.5. GSH measurements

The total GSH was measured according to the Tietze recycling assay [15], following the manufacturers' (Cayman Chemical) instructions. Cells seeded in 96 well plates (20×10^3 per well) were left overnight to attached and then the designated cell groups were treated with BSO (again overnight). Next, the cells were treated with HYP for 4 h, in the presence and absence of BSO and NAC and then irradiated. The corresponding dark controls were included. Irradiated samples were assayed immediately and 2 h post-irradiation. In all samples 200 μL of 0.5 w/v metaphosphoric acid (MPA) was added to the cells for protein precipitation together with 1 mM EDTA to prevent GSH oxidation from transition metals at the desired assay point, and the cells were kept at −20 °C until the GSH assay was performed. 3 μL of 4 M triethanolamine (TEAM) was added to each well to neutralize the MPA prior to the assay. Fifty microliters of each well supernatant was assayed for GSH content for all treatment groups.