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Photochemical internalization (PCI) of HER2-targeted toxins Synergy is dependent on the treatment sequence

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ABSTRACT

Background: Photochemical internalization (PCI) is a modality for cytosolic release of drugs trapped in endocytic vesicles. The method is based upon photosensitizers localized in the membranes of endocytic vesicles which create membrane rupture upon light exposure by generating reactive oxygen species (ROS), predominantly singlet oxygen ($^1O_2$).
Methods: The human epidermal growth factor receptor 2 (HER2)-targeted immunotoxin (IT), trastuzumab–saporin, was evaluated in combination with PCI using $TPCS_{2a}$ (Amphinex®), a new photosensitizer approved for clinical use.
Results: PCI synergistically enhanced the cytotoxicity of trastuzumab–saporin on trastuzumab-resistant $HER2^+$ Zr-75-1 cells. The PCI effect was only observed when the IT was administered prior to the photochemical treatment ("light after" strategy), while administration of a non-targeted drug may equally well be performed after light exposure. Mechanistic studies showed reduced ligand-induced HER2 phosphorylation and receptor-mediated endocytosis after $TPCS_{2a}$-PDT. Photochemical disruption of the cytoplasmic domain of HER2 was found to be induced by $^1O_2$ generated both by photosensitizer located in the endocytic vesicles and in the outer leaflet of the plasma membrane.
Conclusions: Administration of the HER2-targeted toxin prior to light exposure is a prerequisite for successful PCI-mediated delivery of HER2-targeted toxins.
General significance: PCI of HER2-targeted toxins is demonstrated as a highly effective treatment modality which may overcome trastuzumab resistance. The mechanistic studies of the lack of PCI effect of the "light first" procedure is of outermost importance when designing a clinical PCI treatment protocol for delivery of HER2-targeted therapies.

1. Introduction

Every year, 1.3 million women are diagnosed with breast cancer and over 450,000 will die from the disease [1]. This makes breast cancer the most common type of cancer among women and the second leading cause of cancer deaths in women worldwide (after lung cancer). Overexpression of the human epidermal growth factor receptor 2 (HER2) can be found in ~25% of all breast cancers and is defined as a prognostic factor associated with tumor progression and poor survival [2]. Elevated HER2 expression in tumor tissue combined with low levels in normal tissue makes HER2 an ideal target for therapeutic approaches. Indeed, the HER2 monoclonal antibody (mAb), trastuzumab (Herceptin®), is currently one of the key drugs in the treatment of HER2-positive breast cancer [3]. Significant improvements in disease-free and overall survival have been achieved with trastuzumab-based therapies both in metastatic and adjuvant settings [4]. Acquired resistance to trastuzumab treatment is, however, a great challenge in a majority of the patients [5]. This, in combination with poor selectivity of the conventional therapies utilized (e.g. radiotherapy, chemotherapy), calls for more effective treatment strategies for HER2-positive breast cancer.

Photochemical internalization (PCI) is a new drug delivery modality for cytosolic release of macromolecular therapeutics entrapped in endocytic vesicles. The method is based upon photosensitizers localized in the membranes of endocytic vesicles and subsequent membrane rupture upon illumination so that the drugs can escape into the cytosol [6]. Preliminary data from the first clinical study with PCI of bleomycin report on successful treatment of subcutaneous breast carcinoma with no damage to the overlying tissue [7,8]. PCI provides increased drug toxicity and, in addition, improves the tumor selectivity by preferential retention of the photosensitizer in tumor tissue due to the leakiness of tumor vessels, poor lymphatic drainage and the high LDL receptor level in tumor tissue, and by delivering light only to the cancerous area [8]. However, we expect that coupling of the cytotoxic agent to a targeting ligand (e.g. mAb) that recognizes and binds to a surface antigen specific for cancer cells should offer superior selectivity of the PCI treatment. The PCI technology has been shown to enhance the cytotoxic

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effect of several targeted toxins (both immunotoxins (ITs) and affinity toxins) in a synergistic manner, both *in vitro* [9–11] and *in vivo* [12].

Successful PCI application is dependent on the localization of the macromolecular therapeutics in endocytic vesicles at some stage in the procedure. Targeted toxins based on type I ribosome-inactivating proteins (RIPs) are ideal for delivery by PCI and should induce little side effects, since type I RIPs are highly toxic once entering the cytosol, but unable to penetrate the membranes of endocytic vesicles [13]. In the present study, a HER2-targeted IT was established by linking trastuzumab to a type I RIP, saporin. Trastuzumab is in this connection primarily utilized to deliver saporin to HER2-expressing cells. HER2 binding of trastuzumab induces receptor-mediated endocytosis, which transports the toxin, saporin, into the endocytic pathway of the cell where it may be subjected to PCI-induced relocation into the cytosol [14]. The HER2 endocytosis rate is stimulated by the presence of epidermal growth factor receptor (EGFR) and is reported to be low in cells expressing low levels of EGFR [15]. In order to evaluate the feasibility of utilizing PCI of a HER2-targeted IT, it would be of interest to select $HER2^+/EGFR^-$ cell lines that may not respond efficiently to the therapeutic options. Thus, in this study, the HER2-positive Zr-75-1 cells were selected due to their irresponsiveness to trastuzumab [16] and also due to their low EGFR expression. A slow IT uptake would be expected due to the low EGFR expression and this should represent challenging properties when applying PCI of a HER2-targeted IT in the clinical setting.

Two protocols have been developed for PCI. One is based on illumination of the cells after the macromolecular and photosensitizer treatment (referred to as the "light after" strategy), the other on the photochemical disruption of endocytic vesicles before delivery of the macromolecules (the "light first" strategy) [17]. A proposed mechanism for the "light first" effect is that drug-containing vesicles fuse with photochemically disrupted endocytic vesicles so that the drug can escape into the cytosol [17]. Therapeutics taken up by fluid-phase endocytosis has been successfully delivered by PCI with both treatment strategies [18]. PCI with the "light first" strategy has been demonstrated *in vitro* for delivery of viral and non-viral genes in HeLa [19], HCT-116 [20] and CME-1 cells [21] and on DU145 spheroids [22], and both *in vitro* and *in vivo* for delivery of bleomycin to V79 cells [23] and CT26.CL25 tumors [24], respectively. PCI of the type I RIP gelonin has actually been shown both *in vitro* in THX cells and *in vivo* on WiDr xenografts to be more effective with the "light first" procedure [17,25]. PCI of targeted toxins has, on the contrary, been shown to be highly dependent on the treatment protocol and only minor effects have been reported when PCI of targeted toxins has been performed with the "light first" treatment [9,10,12]. PCI of targeted toxins is dependent on intact and functional receptors that are endocytosed upon drug binding. Photochemical damage to the target receptor may, however, influence on the outcome of PCI with the "light first" strategy. So far, no studies have addressed this hypothesis. We here investigate $TPCS_{2a}$-based photochemical targeting of HER2 with subsequent impact on cytosolic delivery of trastuzumab–saporin, i.e. PCI with the "light first" strategy. We have studied the impact of the localization of $TPCS_{2a}$ and the generation of singlet oxygen ($^1O_2$) on inactivation of HER2. The results in this study indicate that lack of effect after PCI of trastuzumab–saporin with the "light first" strategy can be explained by photochemical HER2 targeting, which prohibits IT uptake by receptor-mediated endocytosis.

2. Materials and methods

2.1. Cell lines and culture media

The cell lines Zr-75-1, MDA-MB231 and WiDr were all obtained from the American Type Culture Collection (LGC Standards AB, Boras, Sweden). All cell lines were maintained and subcultured in RPMI 1640 medium (Sigma, St. Louis MO, USA) as described previously [9].

2.2. Light source and photosensitizer

LumiSource® (PCI Biotech, Oslo, Norway) emitting blue light was used for illumination of the cells as described previously [26]. Disulfonated tetraphenylchlorin with the sulfonate groups on adjacent phenyl rings, $TPCS_{2a}$ (Amphinex®), was used as a photosensitizer [24].

2.3. Intracellular localization of $TPCS_{2a}$

For $TPCS_{2a}$ uptake studies, Zr-75-1 cells ($6.2\times10^4/cm^2$) were seeded in 10-cm^2 Falcon 3001 dishes (BD Biosciences, CA, USA), incubated for 18 h with 0.2 μg/ml $TPCS_{2a}$, washed twice and subjected to a 4 h chase in drug-free medium. $TPCS_{2a}$ fluorescence was recorded as for $TPPS_{2a}$ described by Weyergang et al. [27] using a 40× non-immersion objective lens. In a separate study, the cells were incubated for 30 min with $TPCS_{2a}$ (4 °C), washed and chased for 4 h (37 °C) and illuminated for 8 min (LD_{50}, measured by MTT). $TPCS_{2a}$ relocalization was studied 1 h later. Microscopy was performed using an AxioImager Z1Microscope (Carl Zeiss AS, Oslo, Norway) with the same objective lens and filter set as previously described.

2.4. Preparation of trastuzumab–saporin immunotoxin

The humanized HER2 mAb, trastuzumab, was biotinylated utilizing a biotinylation reagent from Pierce (Pierce Biotechnology Inc., Rockford, IL). Two milliliter of 2 mg/ml trastuzumab (Roche AG, Basel, Switzerland) was mixed with 32 μl of 22.5 mM sulfo-NHS–biotin (Pierce) at room temperature for 4 h. A G-25 Sephadex Medium desalting column (Amersham Biosciences, Amersham Place, UK) was used to separate unconjugated sulfo-NHS–biotin from biotin–trastuzumab. The trastuzumab concentration of the product was quantified spectrophotometrically at 280 nm and the biotin: trastuzumab ratio was determined utilizing a biotinylation quantitation kit (Pierce, #28005). A chemical conjugate of streptavidin and saporin (ATS, San Diego, CA, USA) with an average of 2 saporin molecules per streptavidin molecule was combined with biotin–trastuzumab at a biotin:streptavidin ratio of 4:1 (likely to give several different products with different ratios between trastuzumab and saporin).

2.5. PDT- and PCI-treatment of cells

Zr-75-1 cells ($3.6\times10^4/cm^2$) and MDA-MB231 cells ($4.5\times10^4/cm^2$) were seeded in 96- or 6-well plates (Nunc, Roskilde, Denmark). Unless otherwise described, the cells were incubated for 18 h with 0.2 μg/ml $TPCS_{2a}$, washed twice and chased 4 h in drug-free medium prior to illumination (standard PDT protocol). PCI using the "Light after" protocol was performed according to the standard PDT protocol with co-incubation of $TPCS_{2a}$ with trastuzumab–saporin, streptavidin–saporin or saporin (Sigma) for 18 h. Competition control experiments were performed according to the standard PDT protocol including incubation with 2 nM of trastuzumab for 1 h prior to 3 h incubation with 0.1 nM trastuzumab–saporin during the chase period. The drug-containing medium was exchanged by fresh medium before illumination. For PCI using the "Light first" PCI protocol, standard PDT protocol was followed with the toxin/toxin conjugates administered to the cells immediately after illumination for incubation until the end of the experiment. In a separate study, $TPCS_{2a}$ was targeted to the plasma membrane or to endocytic vesicles according to the treatments described in Fig. 6.

2.6. Fluorescence microscopy of Alexa488–trastuzumab and Alexa488–dextran

Trastuzumab was fluorescence-labeled using Alexa Fluor® 488 Protein Labeling Kit from Molecular Probes (Eugene, OR, USA, A10235). Zr-75-1 ($3.6\times10^4/cm^2$) or WiDr cells ($1.5\times10^4/cm^2$)