Photodynamic targeting of EGFR does not predict the treatment outcome in combination with the EGFR tyrosine kinase inhibitor Tyrphostin AG1478

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Photodynamic therapy (PDT) is a selective treatment modality against cancer. PDT is based on the preferential retention of photosensitizers (PSs), in the tumour and subsequent light exposure which activates the PS and generates reactive oxygen species. Multimodality therapy is increasingly relevant in cancer treatment and PDT has been shown as an effective adjuvant to other anti-cancer modalities. The present study reports on the combination of PDT and an epidermal growth factor receptor (EGFR) specific tyrosine kinase inhibitor (TKI), Tyrphostin AG1478. The combination was studied in two cell lines; A-431 and NuTu-19, expressing EGFR and sensitive to Tyrphostin treatment, but with different sensitivity towards photochemical EGFR damage. A-431 cells were treated with the PS *meso*-tetraphenylporphine with 2 sulfonate groups on adjacent phenyl rings ($TPPS_{2a}$) in order to target mainly the endo/lysosomal compartments (18 h incubation followed by a 4 h chase in drug-free medium) or the plasma membrane (30 min incubation) upon light exposure. The EGFR was inhibited after PDT in A-431 cells only when $TPPS_{2a}$ was located on the plasma membrane, but both treatment regimes resulted in synergistic inhibition of cell growth when combined with Tyrphostin. $TPPS_{2a}$ treatment of NuTu-19 cells, designed for endo/lysosomal localization, followed by light attenuated EGFR phosphorylation but resulted in additive or antagonistic effects on cell growth when Tyrphostin was administered prior to or after PDT respectively. It was therefore concluded that photochemical damage of EGFR does not predict the treatment outcome when PDT is combined with Tyrphostin.

Introduction

Photodynamic therapy (PDT) is a therapeutic modality approved for both neoplastic and vascular diseases.[1] PDT is based on compounds (photosensitizers (PSs)) that absorb energy from visible light and transformation of this energy to molecular oxygen creating reactive oxygen species (ROS) of which singlet oxygen ($^1O_2$) is assumed to dominate.[1] In cancer therapy these photochemically generated ROS can destroy tumours directly, by initiating apoptosis, necrosis or autophagy in the parenchyma cells, or indirectly, by vascular shutdown and immunologic responses.[1–3] PDT is selective to malignant tissue due to the preferential accumulation of the PS in cancer cells and the possibility to direct the light source to the diseased area.[2] Singlet oxygen has a short lifetime in biological systems ($\leq$40 ns),[4] and the photochemical reaction generated during PDT is therefore located in the direct vicinity (10–20 nm) of the PS. The intracellular targets of PDT are, as a result, highly dependent on the physio-chemical properties of the PS and the following intracellular localization of the PS at the time of and during light exposure.[2] The anti-cancer effects of PDT may be further enhanced by combination with other established anti-cancer treatments such as radiation, surgery, and conventional chemotherapy.[5–8] An increased therapeutic effect of such combination therapies may be caused by different modes of cytotoxic actions of the treatment modalities.[9–12]

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Recently, it has been reported that novel anti-cancer drugs such as the cox-2 inhibitor celecoxib,[13] the tyrosine kinase inhibitor imatinib,[14] and the anti-vascular endothelial growth factor (VEGF) antibody avastin[15] can enhance the treatment effect of PDT. It has also been shown that PDT, using the mitochondrial localizing PS benzoporphyrin derivative monoacid ring A (BPD), in combination with cetuximab, an epidermal growth factor receptor (EGFR) antibody, cause synergistic growth inhibition of an ovarian orthotopic cancer model in mice.[9] The EGFR is one of the most investigated targets for selective cancer therapy.[16,17] EGFR is a transmembrane protein overexpressed in several different cancers,[18] and 2 different types of EGFR targeting drugs, monoclonal antibodies and tyrosine kinase inhibitors (TKIs), have obtained marketing authorisation by both EMEA and FDA.

Tyrphostin AG1478 (Tyrphostin) is an EGFR selective TKI, used in cancer research.[19–21] Tyrphostin has structural similarities with the clinical used gefitinib (Iressa™) and erlotinib (Tarceva™). Upon administration Tyrphostin diffuses through the plasma membrane and binds reversibly to the intracellular kinase domain of EGFR where it inhibits receptor activation upon ligand binding,[22] and thereby induces growth arrest and subsequent cell death. The present work on PDT and Tyrphostin is the first report on PDT combined with an EGFR selective TKI. The combination treatment was performed in two cell lines, NuTu-19 and A-431, with different sensitivity towards photochemical EGFR targeting. It is here shown that synergism can be obtained by such combinations, although the effect seems to be highly dependent on the cell type and also on the treatment regimen used.

The treatment outcome after the PDT–Tyrphostin combination seems, however, not to depend on photochemical damage of EGFR. The presented results warrant further investigations of PDT and TKI combination therapy in preclinical studies.

Materials and methods

Materials

$TPPS_{2a}$ (*meso*-tetraphenylporphine with two sulfonate groups on adjacent phenyl rings, Lumitrans) 0.35 mg ml^{-1} in DMSO was kindly provided by PCI Biotech AS (Oslo, Norway) and was stored at −20 °C. Tyrphostin AG1478, abbreviated Tyrphostin in the rest of this report, was obtained from Sigma (St. Louis, MO), dissolved to 31.6 mM in DMSO, aliquotated and stored at −20 °C. α-EGFR antibody, α-phospho(Y1068)-EGFR antibody, α-mouse HRP linked antibody and α-rabbit HRP linked antibody were all received from Cell Signaling Technology (Beverly, MA) and stored at −20 °C. ECL Plus Western Blotting Detection Reagents were obtained from GE Healthcare (Amersham Place, UK). LumiSource from PCI Biotech AS consisting of 4 light tubes ($\lambda = 375$–550 nm, maximum at 435 nm and irradiance = 13.5 mW cm^{-2}) was used as a light source.

Cell culture and cultivation

The studies were performed using two cell lines; A-431 human skin carcinoma cells (ATCC, CRL 1555) and NuTu-19 rat epithelial cancer cells (gift from Dr A. L. Major, University of Geneva, Switzerland). These cell lines have been shown EGFR positive and have been focused on in former work by us on photodynamic therapy and EGFR targeting.[23,24] The cells were subcultured two or three times per week in DMEM (Bio Whittaker Europe, Velviers, Belgium) for A-431 cells and RPMI medium (Sigma, St. Louis, MO) for NuTu-19 cells. Both media were supplied with 10% fetal calf serum (FCS) (GIBCO BRL, Paisley, UK), 100 U ml^{-1} penicillin, 100 U ml^{-1} streptomycin (Sigma, St. Louis, MO) and 2 mM glutamine (Bio Whittaker, Velviers, Belgium). The cells were grown and incubated in 75 cm^2 flasks (Nunc, Roskilde, Denmark) at 37 °C in a humidified atmosphere containing 5% CO_2.

$TPPS_{2a}$ mediated PDT targeted to the endosomes and lysosomes

Unless otherwise described the PS ($TPPS_{2a}$) was targeted to the cells endosomes and lysosomes as illustrated in Fig. 1A, C and D. A-431 and NuTu-19 cells were seeded out in 6 wells plates 80 000 and 50 000 cells per well respectively for the Coulter counter experiments and 200 000 cells per well for the western blot (WB) sample preparation and the MTT assay. After seeding, the cells were incubated 6 h or more, as described for the different combination treatment regimes with Tyrphostin, to allow the cells to attach to the substratum. The cells were then incubated 18 h with 0.1 μg ml^{-1} or 0.2 μg ml^{-1} $TPPS_{2a}$ for A-431 and NuTu-19 cells respectively. After $TPPS_{2a}$ incubation cells were washed twice in drug-free medium and chased in new drug-free medium for 4 h to remove plasma membrane bound PS. The cells were then exposed to 150 and 120 s of light from LumiSource for the A-431 and NuTu-19 cells respectively.

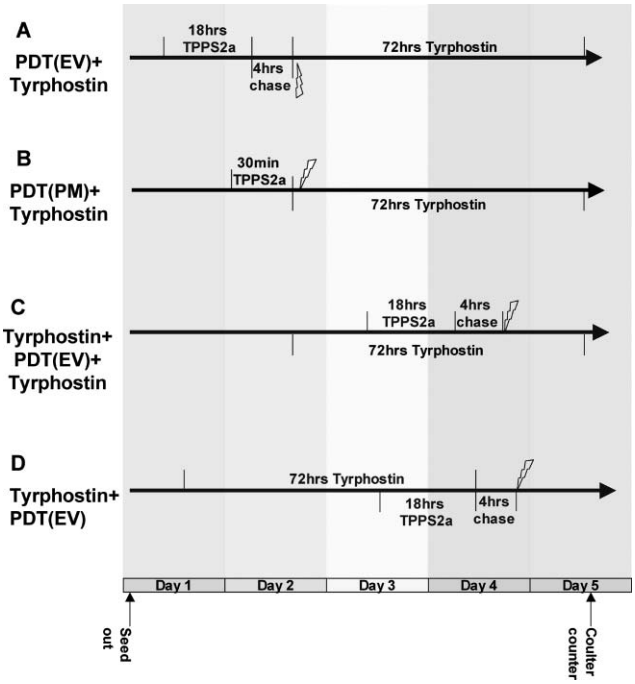

Fig. 1 Treatment schedules for combined PDT and Tyrphostin treatment. Treatment schedule A and B were used for the A-431 cells and treatment schedule A, C and D were used for the NuTu-19 cell line. EV-PS targeted to the endocytic vesicles, PM-PS targeted to the plasma membrane.

$TPPS_{2a}$ mediated PDT targeted to the plasma membrane

In the A-431 cells PDT was also performed with the PS targeted to the plasma membrane as illustrated in Fig. 1B. Cells were then incubated 30 min with 1.0 μg ml^{-1} $TPPS_{2a}$ ~24 h after they were seeded out. The medium was changed to drug-free medium, and the cells were directly exposed to light from LumiSource for 120 s.

Combination treatment with PDT and Tyrphostin

In the experiments where growth inhibition of the A-431 cells was studied by Coulter counter, PDT was performed with the PS targeted either to the endocytic vesicles or to the plasma membrane. Tyrphostin, at indicated concentrations, was added to the cells directly after light exposure and incubated 72 h before the cells were counted (Fig. 1A and B). For the NuTu-19 cells the PS was only targeted to the endo/lysosomal vesicles but the PDT–Tyrphostin treatment was performed with three different treatment-regimens illustrated in Fig. 1A, C and D. The Tyrphostin incubation time in NuTu-19 cells was 72 h for all the experimental setups, however, the timing of administration in relation to the PDT treatment was varied. In one regimen, 20 μM Tyrphostin was administrated to the NuTu-19 cells directly after light exposure and the cells were incubated for 72 h until they were counted (Fig. 1A). In another regimen NuTu-19 cells were incubated with 20 μM Tyrphostin 24 h after the cells were seeded out, and the cells were incubated for 24 h until $TPPS_{2a}$ was added and co-incubated with 20 μM Tyrphostin for 18 h. The medium was replaced with medium containing only 20 μM Tyrphostin for 4 h until light exposure and the Tyrphostin incubation was sustained for an additional 24 h when the cells were counted by Coulter counter (Fig. 1C). In the third experimental setup

20 μM Tyrphostin was administrated to the NuTu-19 cells and incubated for 48 h when $TPPS_{2a}$ was added and co-incubated with 20 μM Tyrphostin for 18 h. The Tyrphostin was removed together with the PS during the washing procedure, light exposure was performed after 4 h, and the cells were counted after an additional 24 h (Fig. 1D). In the sample preparation for SDS-PAGE and western blotting Tyrphostin was only administered directly after light exposure in both cell lines as illustrated in Fig. 1A and B. Toxicity of the PDT given during the western blot sample preparations was evaluated and controlled by the MTT assay as described bellow. The sample preparation for western blotting was performed three times for every experimental setup.

Western blotting analysis

At indicated times after light exposure or incubation with Tyrphostin the cell medium was removed and cells were incubated for 2 min with 100 ng ml^{-1} EGF. The cell dishes were then placed on ice and the sample preparation was performed as previously described.[24] Briefly, the same amount of lysed cells from each sample, as measured by the absorption of nucleic acids at 260 nm, was subjected to gel electrophoresis on 12% SDS–polyacrylamide gels and blotted to Hybond-P PVDF membranes (Amersham Biosciences, Amersham Place, UK) before immunodetection with specific antibodies was carried out. STORM and Image Quant from GE Healthcare (Amersham Place, UK) was used to detect protein bands on the membrane. The experiments were performed at least 3 times.

Evaluation of cytotoxicity

Two different methods were used for the cytotoxicity measurements; cell counting with a Coulter counter, and viability test by the MTT method. Cell counting of both A-431 and NuTu-19 cells was performed with an in house made Coulter counter after trypsination and resuspension in PBS. Two parallels of each treatment regime were made, and the experiments were reproduced at least twice. The MTT assay was used as a control of cytotoxicity in the sample preparation for western blotting. The MTT assay was performed 24 h after PDT when the monolayers were incubated with 0.25 mg ml^{-1} MTT reagent (Sigma) for 3 h. The formazan crystals where then dissolved in DMSO and absorbance measured at 570 nm. Two parallels of each treatment regime were made.

Statistical methods for evaluation of the PDT–Tyrphostin combination therapy

A statistical model was used to assess possible synergistic and antagonistic effects of the PDT–Tyrphostin combination treatments. This model was based on the assumption that the two different treatment modalities had mutually distinct and independent mechanisms of action. In such a system the expected effect of two treatments is the product of the survival fraction (SF) of each treatment separately: $SF_{add} = SF_{Tyrphostin} \times SF_{PDT}$, or $\log SF_{add} = \log SF_{Tyrphostin} + \log SF_{PDT}$, (additive effects). We therefore calculated SF_{add} and compared it to the observed combined effect of the treatment, SF_{comb} using the synergy/antagonism value DL, defined as the difference in logarithms between the observed SF_{comb} and the calculated SF_{add}:

$$DL = -(\log SF_{comb} - \log SF_{add}) = \log SF_{add}/\log SF_{comb} = \log SF_{Tyrphostin} + \log SF_{PDT} - \log SF_{comb}$$

Synergistic effects between PDT and Tyrphostin resulted in positive DL values, antagonistic effects resulted in negative DL values and additive effects resulted in DL values close to zero. Significant deviations of DL from zero were established through *t*-tests using a two-tailed significance level of $p = 0.05$.

Cellular localisation of $TPPS_{2a}$ in A-431 cells by fluorescence microscopy

When the PS was targeted to endosomes and lysosomes A-431 cells were seeded out 150 000 cells per well in 10 cm^2 wells. Cells were incubated 6 h prior to an 18 h incubation with 0.1 μg ml^{-1} $TPPS_{2a}$. Cells were washed twice and chased 4 h in drug-free medium before microscopy was performed. When the PS was targeted to the plasma membrane the A-431 cells were incubated with 1.0 μg ml^{-1} $TPPS_{2a}$ for 30 min and microscopy was performed immediately. Fluorescence microscopy of $TPPS_{2a}$ was preformed as previously described.[24]

Results

In the present work PDT was targeted to the endo/lysosomal compartments in most of the experiments and this was achieved by an 18 h incubation of $TPPS_{2a}$ followed by a 4 h chase in drug-free medium before light exposure. PDT was, however, also targeted only to the plasma membrane by a 30 min incubation directly followed by light exposure to induce photochemical damage of EGFR in A-431 cells. Tyrphostin incubation was in A-431 cells only administered directly after light exposure (Fig. 1A and B) whereas it was given to the NuTu-19 cells directly after, during and prior to exposure to light (Fig. 1A, C and D).

Tyrphostin, but not endo/lysosomal targeted PDT inhibits EGF mediated EGFR phosphorylation in A-431 cells

$TPPS_{2a}$ administered to cells in culture absorbs first to the plasma membrane and is thereafter taken up in the cells by adsorptive endocytosis. $TPPS_{2a}$ can be primarily targeted to the endocytic vesicles by chasing cells in drug-free medium after the PS incubation (regimen A in Fig. 1), as shown by the fluorescence micrographs of $TPPS_{2a}$ in A-431 cells (Fig. 2A), similar to what is found in other cell lines.[25,24] It was recently reported that $TPPS_{2a}$–PDT targeted to endocytic vesicles damaged EGFR in NuTu-19 cells and inhibited the receptors ability to auto-phosphorylate upon EGF stimulation.[24] It was studied whether this was also the case in A-431 cells, but no detectable reduction in phospho-EGFR was observed after PDT with doses that reduced the relative viability by up to 90% (Fig. 2B). $TPPS_{2a}$–PDT utilizing a LD_{50} dose did not inhibit EGFR phosphorylation up to 48 h after light exposure (Fig. 2C and D). Treatment with 4 μM Tyrphostin resulted, however, in a strong and immediate attenuation of EGFR phosphorylation lasting for 24 h, but returned to the control level 48 h after Tyrphostin administration (Fig. 2C and D). A similar decrease in EGFR phosphorylation, as observed after Tyrphostin treatment alone, was observed in cells treated with Tyrphostin in combination with PDT the first 3 h after light exposure (Fig. 2C). The PDT–Tyrphostin combination treatment resulted, however,

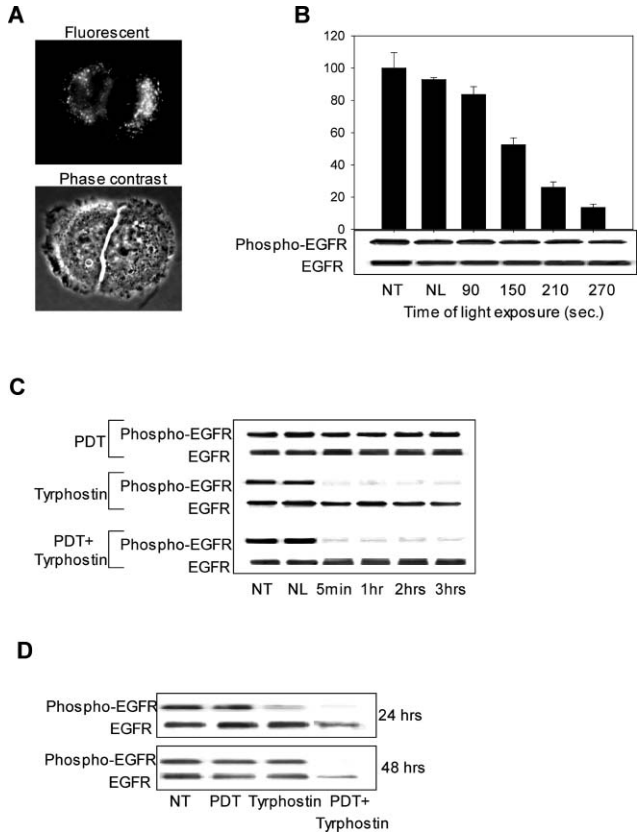

Fig. 2 Effects of endo/lysosomal targeted PDT and Tyrphostin on EGFR in A-431 cells. (A) Fluorescence micrographs showing the intracellular localisation of $TPPS_{2a}$ after 18 h incubation with 0.1 μg ml^{-1} $TPPS_{2a}$ followed by a 4 h wash in PS free medium. (B) The western blots show dose dependent photochemical effects on total and phosphorylated EGFR measured 1 h after PDT. The blots represent 1 of 3 independent experiments. The columns show cell survival, measured by the MTT method 24 h after PDT, plotted as a function of the time of light exposure. The error bars represent the standard error of 3 parallels. (C) and (D) Western blots showing time dependent effects on total and phosphorylated EGFR after PDT, 4 μM Tyrphostin and the combination treatment. The blots represent 1 of 3 independent experiments. The cells were treated with 100 ng ml^{-1} EGF 5 min prior to extraction for WB analysis.

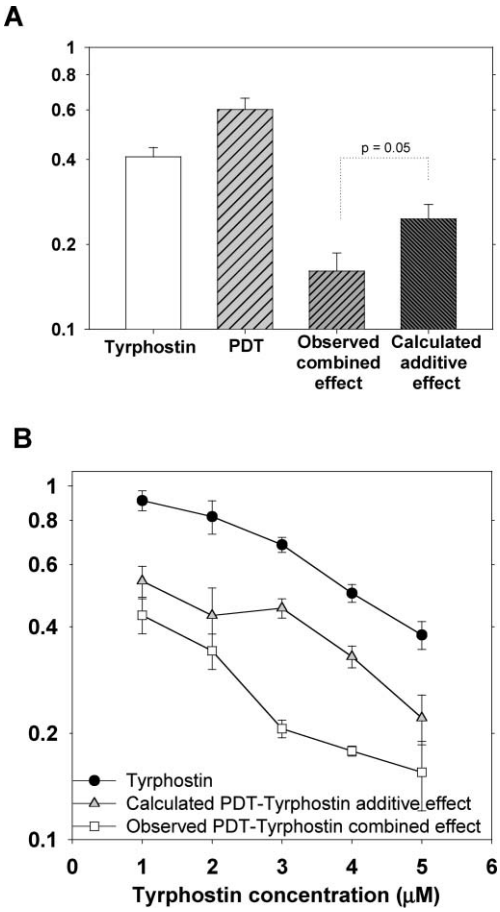

Fig. 3 Combination treatment of endo/lysosomal targeting PDT and Tyrphostin in A-431 cells. 4 μM Tyrphostin was added to the cells directly after PDT and the number of cells was measured by a Coulter counter 96 h after the cells were seeded out as described in Materials and methods. The calculated additive effects of the two treatment modalities are also shown on the figure. (A) shows the combined surviving fractions when 4 and 5 μM Tyrphostin was used. (B) shows the Tyrphostin concentration dependent calculated additive and observed effects of the combined treatments. The data are the mean of three independent experiments. Error bars represent standard errors.

in a prolonged attenuation of EGFR phosphorylation for at least 48 h after light exposure (Fig. 2D). Since no inactivation of EGFR was observed 48 h after the Tyrphostin or PDT mono-treatments, the inactivation of EGFR observed after the combination therapy indicates a synergistic effect on EGFR inhibition 48 h after PDT.

Tyrphostin treatment in combination with endo/lysosomal targeted PDT in A-431 cells

It was studied whether endo/lysosomal targeted $TPPS_{2a}$–PDT could benefit therapeutically from Tyrphostin administration in the A-431 cell line. Cytotoxicity was enhanced compared to the effect of PDT alone when Tyrphostin was administered directly after the photochemical reaction (regimen A in Fig. 1), as revealed by measuring the relative cell number 3 days after light exposure (Fig. 3A). The relative number of cells remaining after the combination therapy was significantly reduced compared to the calculated additive effect of the treatments (Fig. 3A). The synergy/antagonism parameter DL of the experiments was positive and statistically significant from zero as presented in Table 1. The enhanced cytotoxicity of the PDT–Tyrphostin combination in the A-431 cell line seemed stronger when 3 or 4 μM Tyrphostin was administrated compared to 1 and 2 μM (Fig. 3B). In the present statistical model, a treatment combination is accepted as synergistic when the combined effect of Tyrphostin and PDT is greater than the calculated additive effect, *i.e.* when:

$$DL = \log SF_{Tyrphostin} + \log SF_{PDT} - \log SF_{(Tyrphostin + PDT)} > 0$$

As indicated in Table 1 the DL values was significantly higher than zero for Tyrphostin concentrations above 1 μM. However, the difference of the survival fractions in the log plot does not increase uniformly with Tyrphostin concentration. We therefore evaluated the mean and standard error of DL in the concentration range of Tyrphostin treatment and found $DL = 0.13 \pm 0.06$ and $p = 0.03$, indicating that this combination treatment induces a synergistic inhibitory effect on growth of the A-431 cells.

Table 1 Statistical evaluations of the different PDT–Tyrphostin combination treatments in A-431 and NuTu-19 cells. The synergy/antagonism parameter DL is calculated as described in Materials and methods. The significance level p is found by a two tailed t-tests. EV-PS targeted to endocytic vesicles, PM-PS targeted to the plasma membrane

Cell line	PS	Procedure	DL	p	Effect
A-431	EV	PDT → 1 μM AG1478	0.10 ± 0.07	>0.05	n.s.‡
A-431	EV	PDT → 2–3 μM AG1478	0.17 ± 0.08	0.05	Synergy
A-431	EV	PDT → 4–5 μM AG1478	0.18 ± 0.09	0.05	Synergy
A-431	EV	PDT → all concentrations AG1478	0.13 ± 0.06	0.03	Synergy
A-431	PM	PDT → 4 μM AG1478	1.09 ± 0.35	0.004	Synergy
NuTu-19	EV	PDT → 20 μM AG1478	−0.20 ± 0.05	0.003	Antagonism
NuTu-19	EV	20 μM AG1478 → PDT→ 20 μM AG1478	−0.01 ± 0.12	>0.05	n.s.‡
NuTu-19	EV	20 μM AG1478 → PDT	−0.05 ± 0.09	>0.05	n.s.‡

Tyrphostin treatment in combination with plasma membrane targeted PDT in A-431 cells

The PDT targeted to endocytic vesicles, as described above, did apparently not target EGFR in A-431 cells. Attempts were therefore made to reveal the influence of PDT-based targeting of EGFR on the treatment outcome when combined with Tyrphostin treatment. The standard photodynamic treatment used in this study was designed to target the endocytic vesicles. However, $TPPS_{2a}$ can be detected at the plasma membrane of the A-431 cells by avoiding the 4 h washing period prior to light exposure (data not shown) and this is in accordance with previous reports.[24] PDT with this protocol resulted in a dose-dependent decrease in EGFR-phosphorylation in the A-431 cell line at doses reducing the viability by ~70% or more (data not shown) and combination therapy with Tyrphostin still enhanced PDT mediated toxicity synergistically as shown by significant positive DL values (data not shown). The PDT regimen was then changed to a short incubation with $TPPS_{2a}$ directly followed by exposure to light (regimen B in Fig. 1). This protocol targeted the PS to a much higher extent to the plasma membrane as shown by the $TPPS_{2a}$ fluorescence micrograph in Fig. 4A. The plasma membrane targeting PDT regimen was found to reduce the ability of EGFR to phosphorylate upon EGF stimulation in a dose dependent manner, and the reduced EGFR phosphorylation was observed at doses reducing the viability by ~25% or more (Fig. 4B). In addition, combination treatment with Tyrphostin induced an even stronger inhibition of cell growth with the plasma membrane targeting PDT regimen than observed with the endo/lysomal targeting PDT regimen, as indicated by the large positive DL value and the lower p value when the synergism was evaluated (Fig. 4C and Table 1).

Both Tyrphostin and endo/lysosomal targeted PDT inhibits EGFR phosphorylation in NuTu-19 cells

Endo/lysosomal targeting PDT (18 h $TPPS_{2a}$ incubation followed by 4 h wash prior to light exposure, as described in Fig. 1A) at LD_{50} has previously been shown to inhibit the ability of NuTu-19 cells to phosphorylate EGFR upon EGF stimulation[24] and here it was found that PDT with this regimen resulted in a dose-dependent decrease in this EGFR-phosphorylation (Fig. 5A). The attenuation of the phospho-EGFR level was detectable at light doses that reduced the cell viability by about 50% and was further decreased to an almost non detectable level at doses reducing cell viability by 90% or more (Fig. 5A).

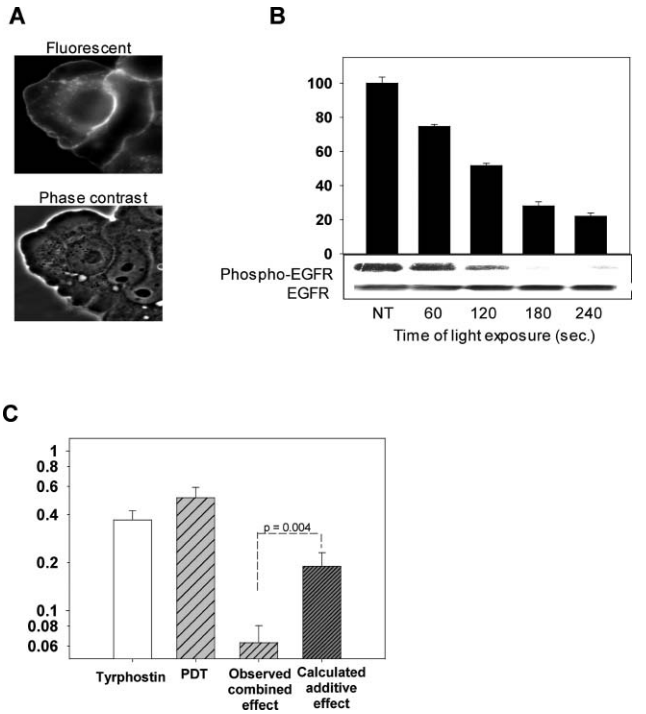

Fig. 4 Effects of plasma membrane targeting PDT and Tyrphostin in A-431 cells. (A) Fluorescence micrographs showing the intracellular localisation of $TPPS_{2a}$ after a 30 min incubation with 1 μg ml^{-1} $TPPS_{2a}$. (B) The western blots show dose-dependent photochemical effects on total and phosphorylated EGFR measured 1 h after PDT following a 30 min incubation with 1 μg ml^{-1} $TPPS_{2a}$. The blots represent 1 of 3 independent experiments. The cells were treated with 100 ng ml^{-1} EGF 5 min prior to extraction for WB analysis. The columns show cell survival, measured by the MTT method 24 h after PDT, plotted as a function of the light exposure time. The error bars represent the standard error of 3 parallels. (C) Surviving fractions of the A-431 cell after treatment with plasma membrane targeted PDT, 4 μM Tyrphostin and the combination therapy where AG1478 was administrated directly after PDT. Number of cells was measured by a Coulter counter 96 h after the cells were seeded out as described in Materials and methods. The calculated additive effects of the two treatment modalities are also shown. The data are the mean of four independent experiments. Error bars represent standard errors.

The direct effects on the EGFR phosphorylation after endo/lysosomal targeted PDT (regimen A in Fig. 1), Tyrphostin and the combined treatments were also studied in the NuTu-19 cell line. The PDT and Tyrphostin single therapies reduced the

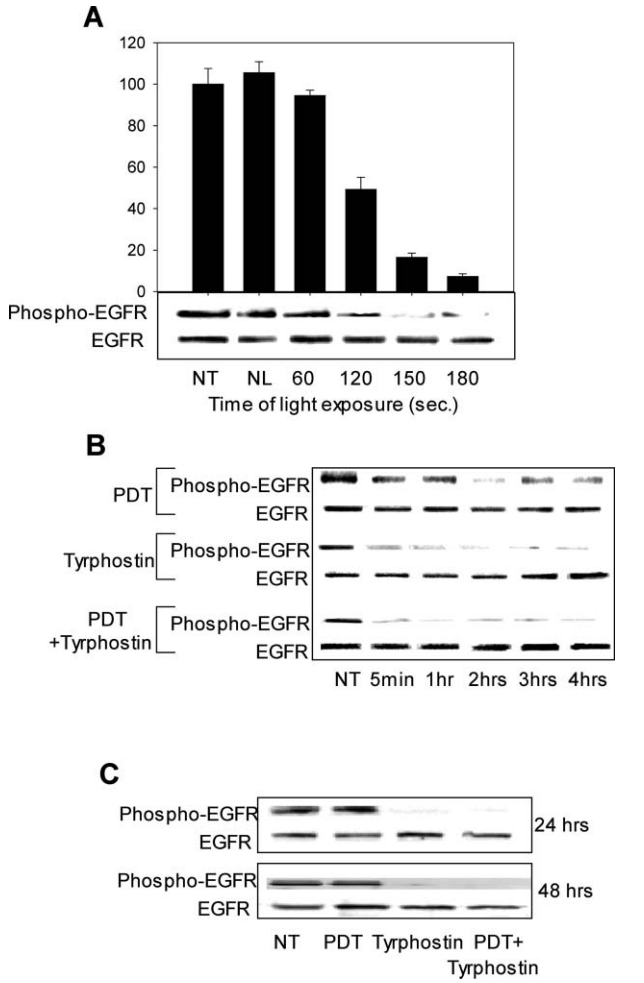

Fig. 5 Effects of endo/lysosomal targeted PDT and Tyrphostin on EGFR in NuTu-19 cells. (A) Western blots showing dose dependent photochemical effects on total and phosphorylated EGFR measured 1 h after PDT following and 18 h incubation with 0.2 μg ml^{-1} $TPPS_{2a}$ and a 4 h chase in PS free medium. The blots represent 1 of 3 independent experiments. The columns show cell survival, measured by the MTT method 24 h after PDT, plotted as a function of the time of light exposure. The error bars represent the standard error of 3 parallels. (B) and (C) Western blots showing time dependent effects on total and phosphorylated EGFR after PDT, 20 μg ml^{-1} Tyrphostin and the combination treatment. Tyrphostin was administrated directly after light exposure in all the combination treatments. The blots represent 1 of 3 independent experiments. The cells were treated with 100 ng ml^{-1} EGF 5 min prior to extraction for WB analyses.

cell viability by ~50% each as measured by the MTT assay 72 h after light exposure or Tyrphostin incubation (data not shown). As reported before,[24] PDT induced an immediate attenuation of EGFR activity. The receptor began, however, to rephosphorylate after 3 h. Administration of Tyrphostin to the NuTu-19 cells caused an immediate and almost complete inhibition of EGFR phosphorylation (Fig. 5B). When the NuTu-19 cells were treated with both PDT and Tyrphostin the attenuation of phosphorylated EGFR seemed to occur to the same extent as in the samples that were treated with Tyrphostin alone (Fig. 5B). As reported previously, the phosphorylation of EGFR returned to the control level 24 h after PDT (Fig. 5C).[24] The EGFR phosphorylation was, however, completely inhibited for at least 48 h in cells treated with Tyrphostin alone or in combination with PDT (Fig. 5C).

Combination therapy of endo/lysosomal targeted PDT and Tyrphostin acts antagonistically or additive in NuTu-19 cells dependent on the treatment regimen

The cytotoxic effect of endo/lysosomal targeted PDT and Tyrphostin was studied in the NuTu-19 cell line by counting surviving cells 3 days after PDT. Surprisingly, no enhanced effect was observed in this cell line when the Tyrphostin treatment was initiated immediately after PDT (Fig. 6A and Fig. 1A). The synergy/antagonism parameter DL was negative and significantly different from zero indicating an antagonistic effect of the combined treatments (Fig. 6A and Table 1). The total cell number measured after the combination therapy when Tyrphostin was administrated after PDT was in addition not significantly different from that of PDT alone (Fig. 6A). It was investigated whether the antagonistic effect in this cell line was dependent on the treatment schedule of the combination therapy, and the PDT–Tyrphostin combination was therefore performed with two other experimental protocols as visualized in Fig. 1C and D. When the Tyrphostin treatment was included in the procedure both before and after the PDT treatment (Fig. 1C), the calculated surviving fraction of these two treatments based on additivity did not differ significantly from the observed combined effect as evaluated by the DL value (Fig. 6B and Table 1, treatment regimen 1C)). Similar results were obtained in the experiments where the Tyrphostin incubation was performed only prior to PDT (Fig. 6C and Table 1, treatment regimen 1D).

Discussion

The present work shows that multimodality therapy between PDT and Tyrphostin AG1478 can cause both synergistic and antagonistic effects. Multimodality therapy is becoming increasing relevant in the treatment of cancer. Drugs with both similar and different mechanisms of action can be combined, but combination therapy is generally considered most effective when the drugs administered have distinct action mechanisms.[9,26,27] Tyrphostin AG1478 is a 4-anilinoquinazolin and has the same backbone structure as the clinically approved TKIs erlotinib, gefitinib and lapatinib.[22,28] The TKIs in this family of drugs are small, lipophilic and function as competitive antagonists for the ATP binding pocket of EGFR.[22] Tyrphostin AG1478 inhibits ligand-induced EGFR activation and blocks EGFR signalling. TKIs may also induce the formation of inactive EGFR/HER2 heterodimers which in turn inhibit HER2 signalling.[29,30] The present results on Tyrphostin induced inhibition of EGFR activation as well as growth inhibition at the used dosages in both NuTu-19 cells and A-431 cells are in agreement with other reports.[31–33]

While Tyrphostin AG1478 is regarded as an EGFR selective drug, PDT is less selective concerning molecular targets. The photochemical reaction generated during PDT oxidize different biomolecules like amino acids (histidine, tryptophan, methionine, cysteine and tyrosine), unsaturated fatty acids and cholesterol.[34] In the present report PDT was preformed with two different strategies targeting the PS to the endo/lysosomal vesicles and the plasma membrane respectively. The experiments are, however,

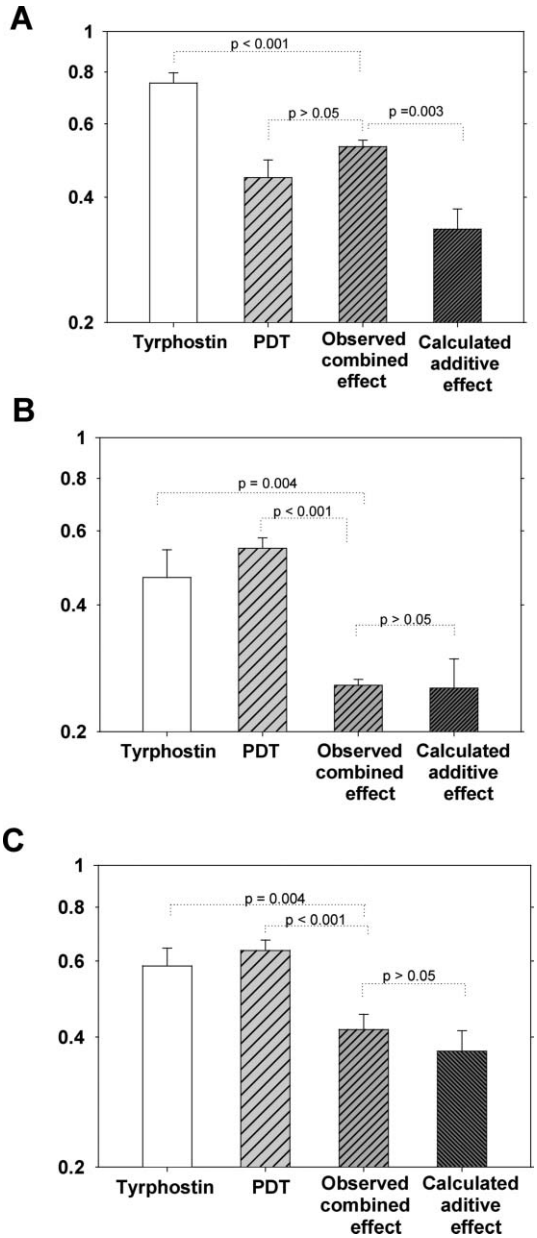

Fig. 6 Combination treatments of endo/lysosomal targeting PDT and Tyrphostin in NuTu-19 cells. Surviving fractions after treatment with PDT with 0.2 μg ml^{-1} $TPPS_{2a}$, 20 μg ml^{-1} Tyrphostin and the combination as measured by the number of viable cells 72 h after treatment. The calculated additive effects of the two treatment modalities are also shown. The columns are the mean of tree independent experiments. Error bars represent standard errors. (A) shows the results from the combination therapy where Tyrphostin was administrated directly after PDT. (B) shows the survival fraction after a combination therapy strategy where PDT was performed during the Tyrphostin incubation. (C) represents the experimental setup where PDT was performed after the Tyrphostin incubation (see Fig. 1).

most focused on endo/lysosomal targeting PDT to continue previous published work from our group on PDT and EGFR targeting drugs.[23,24,35,36]

The cellular signalling following PDT is dependent on the PS, the applied dose and also on the cell line used. The fluorescence microscopy studies indicate that $TPPS_{2a}$ is mainly located in the endo/lysosomal compartments in both A-431 and NuTu-19 cells after 18 h of incubation followed by a 4 h chase period in drug-free medium,[24] Fig. 2A. However, the EGFR phosphorylation is affected by the endo/lysosomal targeting PDT regimen in a dose dependent manner in the NuTu-19 cells, but not in the A-431 cells (Fig. 2 and 5). These analyses were performed shortly (5 min) after light exposure and the inhibition of EGFR phosphorylation is therefore most likely due to a direct targeting of EGFR in the NuTu-19 cells caused by a small fraction of $TPPS_{2a}$ located on the plasma membrane but not detected by fluorescence microscopy.[24]

Previous reports have described PDT-targeting of EGFR and that this may contribute to the cytotoxicity resulting from this therapy.[24,37–39] It was therefore of interest to evaluate how tumour cells responded to a combination of PDT and a tyrosine kinase inhibitor towards EGFR. The $TPPS_{2a}$–PDT and Tyrphostin combination therapy was found to act in an additive to antagonistic manner, depending on the treatment regimen, in the NuTu-19 cells susceptible to photochemical damage of EGFR (Fig. 6). In contrast, the combination resulted in synergistic inhibition of cell growth in A-431 cells when the PDT was optimized for EGFR targeting by increasing the plasma membrane bound fraction of the PS at light exposure time (Fig. 4). Surprisingly, although less pronounced, the PDT–Tyrphostin combination treatment in A-431 cells with the endo/lysosomally located $TPPS_{2a}$ also resulted in a synergistic inhibition of cell growth, even though no photochemical EGFR damage was observed. Thus, in A-431 cells the photodynamic treatment resulted in synergistic effects on cell growth when combined with Tyrphostin irrespective of photodynamic inhibition of EGF-induced EGFR phosphorylation. These results indicate that the outcome of the PDT–Tyrphostin combination therapy cannot be predicted by the PDT-induced effect on EGFR. The synergistic effect of endo/lysosomal targeted PDT and Tyrphostin in A-431 cells may be correlated to the prolonged inhibition of EGFR phosphorylation as observed 48 h after treatment (Fig. 2D). The mechanisms behind this synergistic EGFR inactivation must, however, be further evaluated in future studies. The treatment effects of the applied combination therapies in the present study may also be caused by other cell-specific responses to these treatment regimens. We have recently shown that activation of the mitogen activated protein kinases (MAPKs) have impact on PDT mediated cytotoxicity dependent on the cell line used.[40] Studies have been initiated to investigate the MAPK extracellular regulated kinase (ERK) and also other EGFR downstream proteins after treatments as described here, to evaluate whether these proteins are involved in determining the synergistic or antagonistic outcome of these treatment regimens.

Tyrphostin incubation of NuTu-19 cells inhibited phosphorylation of EGFR in a more sustained manner compared to the A-431 cells (Fig. 2 and 5). The phosphorylation of EGFR returned to the level of untreated A-431 cells 48 h after the Tyrphostin incubation, while inhibition of EGFR phosphorylation was more sustained and lasted for at least 48 h in the NuTu-19 cell line. Surprisingly, in the A-431 cells the PDT–Tyrphostin combination treatment caused a complete inhibition of EGFR phosphorylation for at least 48 h after light exposure, and this inhibition acted synergistically compared to EGFR inhibition by the mono-therapies. No such effect was observed in NuTu-19 cells, where Tyrphostin treatment alone inhibited phosphorylation of EGFR to the same extent as the PDT–Tyrphostin combination treatment at all

time points investigated. The unexpected prolonged inhibition of EGF-induced EGFR phosphorylation after the PDT–Tyrphostin treatment in A-431 cells may thus explain the synergistic effects on growth inhibition after the combination therapy, not observed in NuTu-19 cells. Furthermore, the antagonistic result of the PDT–Tyrphostin combination treatment in the NuTu-19 cells may be related to the long-lasting inhibition of EGFR by Tyrphostin, overriding some of the intracellular death signals induced by PDT.[41–43]

Several TKIs, such as gefitinib, erlotinib and lapatinib, are approved for cancer therapy and PDT may be considered as an adjuvant to established TKI treatment regimens. This requires however that PDT does not counteract the therapeutic response to the TKI treatment. The synergism observed as a result of the PDT–Tyrphostin combination treatment in A-431 is therefore encouraging, but in contrast to the antagonism seen in the NuTu-19 cells. The antagonistic effect observed in the NuTu-19 cell line when PDT was performed only prior to Tyrphostin treatment may be explained by the PDT induced photochemical damage to EGFR. In the other two treatment procedures in this cell line, Tyrphostin is administered to the cells before the photochemical treatment. Both of these procedures therefore provide an action time of Tyrphostin before its target is photochemically destroyed, and this may be the cause of the additive effect of these combination treatments. It may be possible that Tyrphostin bound to EGFR somehow protects the receptor from photochemical damage, and this could explain the additive effect in the procedures where Tyrphostin was administered first compared to the regimen where PDT was performed prior to Tyrphostin incubation. Further studies are, however, needed to conclude on this hypothesis. Patients are usually treated only once with PDT and PDT may therefore be performed prior to, during or after other treatment regimens. In contrast to when PDT was performed before the Tyrphostin administration, PDT applied during or after the Tyrphostin incubation resulted in additive effects on cell growth in the NuTu-19 cell line. The treatment sequence may thus have impact on the treatment outcome and may influence on how PDT can be combined with a TKI treatment regimen. The outcome of combination therapy between PDT and doxorubicin[12] as well as methotrexate[11] has been shown dependent on the treatment schedule, in agreement with the present results.

Two different classes of EGFR targeting drugs, anti-EGFR monoclonal antibodies and EGFR specific TKIs, are used today in the clinic. These two drug types have partly distinct mechanisms of action and the combination of the agents has resulted in both synergistic and antagonistic effects.[29,44] It has been postulated that the outcome of the combination therapy of EGFR mAbs and EGFR TKIs is dependent on the EGFR and HER2 status of the cells[29] and we cannot exclude that the difference in the effect of PDT/TKI combination therapy observed between the NuTu-19 and the A-431 cell line is caused by different levels of EGFR and HER2. High expression of erbB members has been reported to be associated with enhanced sensitivity to EGFR TKIs.[45] Thus, the role of in particular HER2 in combination therapy between PDT and EGFR targeting drugs will therefore be explored in future studies.

We show here synergistic effects on both EGFR dephosphorylation and growth inhibition in A-431 cells when $TPPS_{2a}$–PDT is combined with Tyrphostin AG1478 independent on photochemical EGFR damage. This is in contrast to the antagonistic effect we observe when the same combination regime is used in the NuTu-19 cell line. The synergistic effect obtained in A-431 cells is in agreement with Del Carman *et al.* who reported that BPD–PDT acts synergistically in combination with the EGFR antibody Cetuximab.[9] There are several differences between the work of del Carmen and ours as the PS, the EGFR targeting drug, and the cell model are different. In addition del Carmen's study is performed *in vivo* and, in contrast to the present *in vitro* report, the experiments probably benefit from immunologic reactions caused by both Cetuximab and PDT itself.[46] The presented results show that the outcome of combination therapy between PDT and Tyrphostin AG1478 is cell line dependent and can cause both synergistic and antagonistic effects. The outcome of the PDT–Tyrphostin treatment is, however, not correlated to photochemical damage of EGFR. In general the combination of PDT and EGFR targeted therapy therefore require further evaluation.

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