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Abstract

Pancreatic ductal adenocarcinoma remains one of the worst types of cancers mainly due to its late diagnosis, lack of effective therapies for advance disease and high chemoresistance. Novel therapeutic options that could improve patient quality of life and overall survival are therefore imperative. In this study, we describe the use of an original strategy based on photochemical internalisation (PCI) technology for pancreatic cancer treatment. Subcellular localisation of the photosensitiser meso-tetraphenylporphine-disulfonate ($TPPS_{2a}$) was performed in PANC-1 cells, showing its preferential accumulation in lysosomes. Treatments with increasing concentrations of the ribosome-inactivating protein saporin or $TPPS_{2a}$ alone were compared with PCI-saporin. Metabolic activity and cell viability of PANC-1 cells were determined 96h post-illumination by MTT and trypan blue assays, respectively. Our results show that PCI using the photosensitiser TPPS2a, synergistically enhances the cytotoxic effects of saporin in PANC-1 cells and could offer more effective treatment options for pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains a leading cause of cancer related death despite its low incidence. Late stage diagnosis and lack of effective therapeutic options account for the high mortality rates associated with this disease.[1-3] While surgical resection offers the highest chances for survival, only 20% of cases are deemed operable at time of diagnosis, out of which only around 8% will be medically fit for resection.[3,4] Gemcitabine-based chemotherapy has long been used to treat both resectable and non-operable patients. However, recent studies suggest that combination chemotherapy regimens including agents such as FOLFIRINOX, GEMOXEL, nab-paclitaxel and capecitabine, can improve survival rates. The overall prognosis however, remains dismal.[2,5,6]

A characteristic dense, fibrotic stroma composed of a mixed population of pancreatic stellate cells, cancer associated fibroblasts, nerve cells, endothelial cells, and inflammatory cells as well as extracellular matrix components, is a hallmark of PDAC.[7] This complex tumour microenvironment challenges efficient chemotherapeutic drug delivery by posing a physical barrier and reducing drug bioavailability.[8-10]

Photochemical internalisation (PCI) is a light-triggered drug delivery technique derived from Photodynamic therapy.[11,12] PCI has been shown to enhance the delivery of a variety of therapeutic molecules including drugs such as doxorubicin or bleomycin, which are otherwise subject to lysosomal degradation and therefore fail to exert their full potential therapeutic effects.[13-16] The successful use of Saporin-PCI in a 5- Fluorouracil chemoresistant pancreatic cancer cell line (Panc03.27) has recently reported by Lund et al.[17] However, to our best knowledge, PCI of saporin has never been studied for the treatment of chemoresistant PANC-1 PDAC cells.

In PCI, amphiphilic photosensitisers (PSs) that accumulate in endocytic vesicle membranes are used. Following light-mediated activation, reactive oxygen species (ROS) are generated and induce endolysosomal membrane disruption, leading to the release of the entrapped drugs into the cytosol (see **Fig.1**).[18,19] The combined use of PSs together with therapeutic macromolecules, enables a synergistic enhancement of the cytotoxic effect and therefore, optimal drug doses can be minimised to sublethal concentrations. The fact that PSs require light illumination for their activation, ensure a site-specific induction of the cytotoxic effect which is localised to the illuminated targeted tumour area.
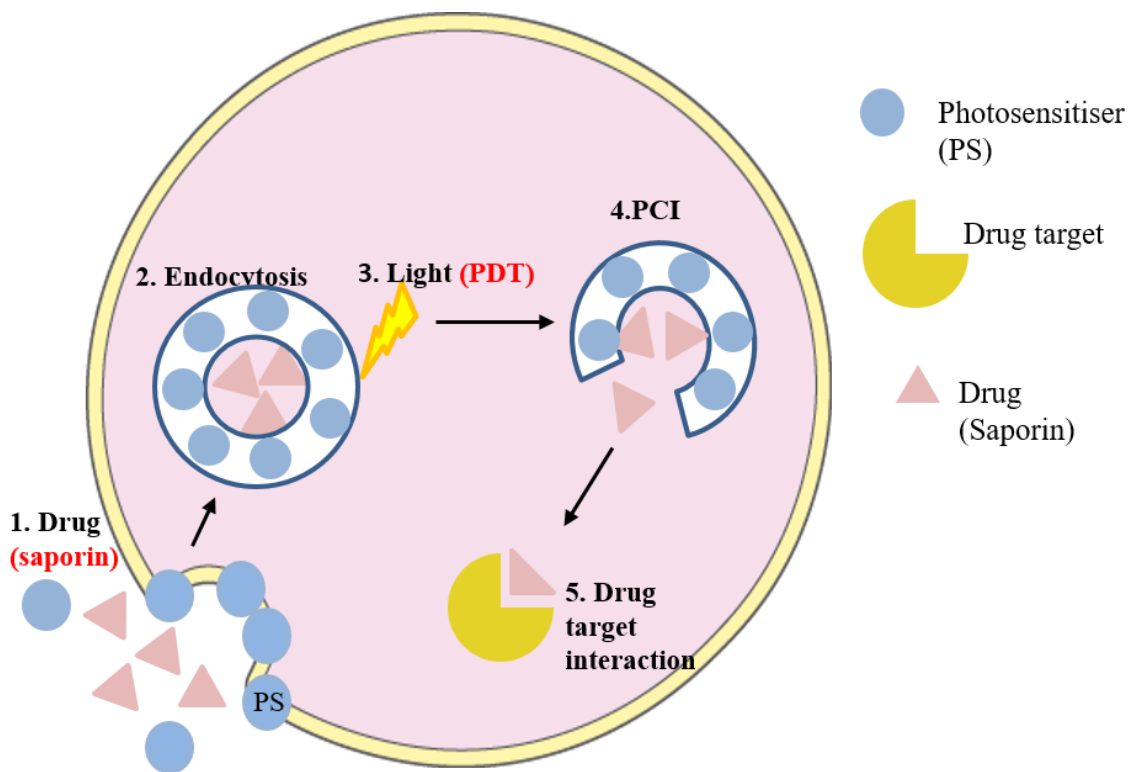

Figure 1: Principles of PCI therapy. The drug is endocytosed by the cells (1) is transported to endocytic vesicles where the PS is localised in the membrane (2). The activation of the PS by light irradiation (3) causes the disruption of the endocytic membrane (4), releasing the drug into the cytosol which can now interact with its target (5).

Type-I ribosome-inactivating proteins (RIPs) are attractive PCI agents as they enter into the cells via endocytosis, and are further transported to the lysosomes for degradation.[20-22] In this study we present our encouraging results of the synergistic augmentation of the cell killing effect of saporin, (a type I RIP isolated from the seeds of the plant Saponaria officinalis, commonly known as Soapwort), together with TPPS2a in an *in vitro* model of PDAC. These studies are a proof of concept to support further experimental work using more clinically relevant chemotherapeutic agents and PDAC models.

Methodology

Cell culture

The gemcitabine resistant PDAC cell line PANC-1 (ATCC® CRL-1469™) was used in this study. Cells were grown in $75cm^2$ tissue culture flasks (TPP, Switzerland) and were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Sigma Aldrich, UK) supplemented with 10% fetal bovine serum (FBS) (Gibco, UK) and 1% Penicillin-Streptomycin (Gibco, UK). Cells were kept in a BINDER incubator (Tuttlingen, Germany) at 37°C in 95% humidity and 5% CO_2. Once a confluence of 70% was reached, cells were either sub-cultured or seeded for the below described protocols.

Spectroscopic studies of TPPS$_{2a}$

The absorption and emission spectra of the amphiphilic photosensitiser TPPS$_{2a}$ in methanol (**Fig. 2a and 2b**) were analysed using a Jasco V-630 UV (Jasco, UK) spectrophotometer and a Spex Fluoromax-4 spectrofluorometer (Horiba Jobin-Ybon, USA) respectively, according to manufacturer guidelines.

Sub-cellular localisation of TPPS$_{2a}$ using fluorescence microscopy

We investigated the subcellular localisation of TPPS$_{2a}$ by fluorescence microscopy. To corroborate its accumulation into lysosomes the lysosomal probe, LysoTracker® Green DND 26 (Life technologies™) was used. A Leica DMI4000b inverted fluorescence microscope was used for image acquisition ((excitation light wavelengths used were 405nm (TPPS$_{2a}$) and 488nm (LysoTracker Green)) at 60X magnification.

TPPS$_{2a}$ redistribution following light exposure

TPPS$_{2a}$ redistribution following light exposure was studied using an Olympus IMT-2 epi-fluorescence inverted microscope with 20X and 60X magnification objectives. Cells were seeded onto FluoroDishTM (World Precision Instruments, Inc.) glass bottom dishes at 20,000 cell/dish density. Cells were incubated with TPPS$_{2a}$ for 18h and the control group only supplemented with culture media. Following incubation with TPPS2a, the plates were washed twice using PBS, fresh media was added and fluorescence was immediately analysed. A 2mW 405 nm blue diode laser module (Thorlabs Inc.) was used for TPPS$_{2a}$ excitation. Images prior to excitation and at timed intervals (3 and 5 minutes) after light illumination were captured using a 512x512 pixel cooled charge-coupled device (CCD) camera (PIXIS 512F, Princeton Instruments Inc.) using a 660 nm bandpass detection filter (Omega Optical Inc.).

MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric cell viability assay was used to assess the % of reduction in cell viability (metabolic activity) achieved at different times after the end of the treatments. An absorbance microplate reader (ELx800 BioTek, UK) was used to detect optical density at 562nm. MTT assays were performed at 48h and 96h time intervals following treatment.

Saporin and TPPS$_{2a}$ monotherapies dose response analysis

In order to investigate the possible cytotoxic effects induced by saporin or TPPS$_{2a}$ monotherapies (**Fig. 2c**), as well as determine the optimal doses for further experiments, PANC-1 cells were treated with increasing concentrations of saporin (0.1-80nM) or TPPS$_{2a}$ (0.05-0.8μg/ml) for 18h. In the case of TPPS$_{2a}$ treatments, two illumination time intervals were used 3 or 5 min corresponding to 1.26 and 2.1 J/cm^2 light doses, respectively. The illumination of the samples was performed using a LumiSource Lamp (PCI Biotech, Norway). The spectrum of the blue light ranges between 375-450nm with a peak wavelength at approximately 420 nm (fluence rate: 7 mW/cm^2). Studies were also performed in the dark (without light illumination) to validate the light-mediate induction of cell toxicity.
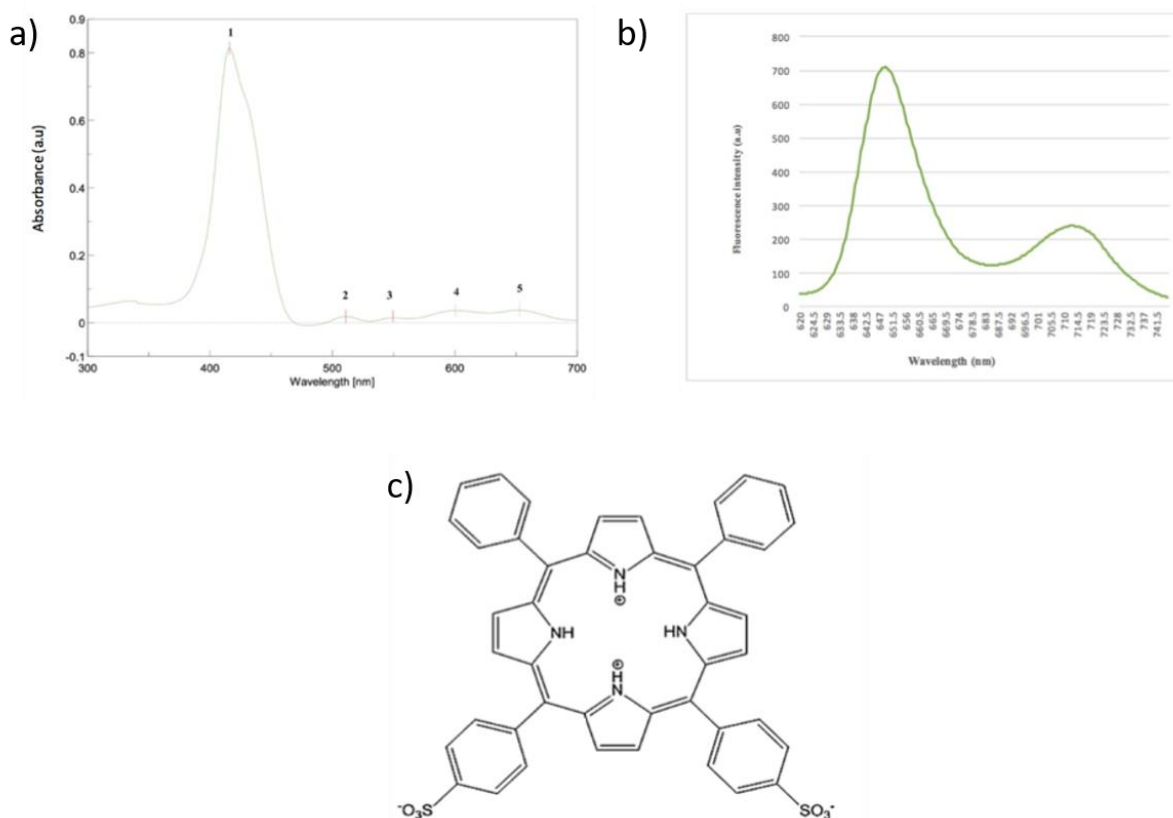

Figure 2. a) Chemical structure of TPPS$_{2a}$.[23] b) TPPS$_{2a}$ absorption spectra in methanol. A major absorption peak was observed at 416nm (corresponding to the Soret band) and 4 smaller (Q band) peaks (2-5) were seen closer to the near red region of the spectra. c) TPPS$_{2a}$ fluorescence emission spectra. TPPS2a excitation achieved using a 415nm wavelength. Two fluorescence emission peaks (650nm and 715nm) were detected.

Photochemical internalisation protocols

The effects of saporin-PCI (saporin + $TPPS_{2a}$ + light combination protocol) in PANC-1 proliferation capacity were assessed 48 and 96 h following treatment using the MTT assays. Optimal drug concentrations were chosen based on the monotherapy dose response experiments. The following experimental groups were established: control (untreated cells), saporin only (saporin monotherapy: 10, 20 or 40 nM), PS only ($TPPS_{2a}$ monotherapy: 0.1 or 0.2µg/ml) and combination (PCI groups). After an 18h treatment, cells were illuminated with blue light for 1.26 and 2.1 J/cm^2 total light doses. One plate was not exposed to light and defined as "dark toxicity" control group. MTT assays were performed 48h and 96h after illumination.

Validation of reduction in cell viability and induction of cell death following PCI using the trypan blue dye exclusion test

Morphological tests and analysis of cell viability using the Trypan Blue (TB) dye exclusion test, were performed following monotherapies and PCI protocols to determine whether the reduction in cell viability observed by MTT assays was due to inhibition of cell proliferation (cell cycle arrest) or due to induction of cell death. TB test was performed 24, 48 and 96h after treatment. An EVOS (Thermo Fisher Scientific) inverted microscope was used to analyse cell morphology and dye uptake at the above mentioned timepoints.

Assessment of the effects of combination treatments

The effects of combined treatments using saporin and the $TPPS_{2a}$ were assessed for synergism after PCI protocols using the Valeriote and Lin method.[24,25] When: [A+B] < [A] X [B] / 100 the interaction is synergistic (where [A+B]- cell viability following PCI, [A] – cell viability following saporin treatment, [B]- cell viability following $TPPS_{2a}$ treatment).

Statistical analysis

All experiments were repeated at least three times for statistical accuracy. Raw data using mean average of optical densities was normalised against control data and analysed with Prism 7.0 statistical analysis software. An independent t-test and a One-way ANOVA test were applied, where two groups and three (or more) independent groups were compared, respectively. A significance level of <0.05 was set for all experiments in order to determine significant difference between groups. P-values are represented in graphs as follows: p-value <0.05 = *; p <0.01 = **; p<0.001 = ***.

Results

Absorption and emission spectra of $TPPS_{2a}$

Absorption spectra analysis demonstrated a major absorbance peak (the Soret band) at 416nm within the blue light spectrum (**Fig. 2a**) and further four minor (Q band) absorption peaks at 511nm, 550nm, 600.5nm, and 652.5nm in the near red region of the spectra (**Fig. 2a** peaks 2-

5, respectively). Fluorescence analysis using spectrofluorometry showed two emission peaks following TPPS$_{2a}$ excitation at 405nm. The first emission peak was observed at 650nm while the second emission peak at 715nm (**Fig. 2b**).

TPPS$_{2a}$ localises in the lysosomes of PANC-1 cells

The subcellular localisation of TPPS$_{2a}$ within PANC-1 cells was analysed using fluorescence microscopy. Excitation of the PS using a 405nm wavelength yielded intense red fluorescence emission with an intracellular granular pattern of distribution, suggesting uptake into endosomes and lysosomes (**Fig. 3a**). Detection of the specific endosomal/lysosomal (acidic organelles) probe LysoTracker Green DND26 (green fluorescence) confirmed TPPS$_{2a}$ internalisation and accumulation in endosomes/lysosomes as apparent in the same figure when the overlap between the red and green channels was made (yellow fluorescence).

TPPS$_{2a}$ is redistributed from the endosomes/lysosomes to the cytoplasm following light exposure

Following confirmation of TPPS$_{2a}$ internalisation and subcellular localisation within lysosomes, the redistribution of this PS after light excitation was studied. Images were acquired using an epi-fluorescence wide field microscope, subsequent to TPPS$_{2a}$ light excitation at a wavelength of 405nm. A similar intracellular granular pattern of TPPS$_{2a}$ distribution in lysosomes was observed prior to light excitation (**Fig. 3b**), confirming our previous findings. Five minutes following light exposure, the distribution pattern of TPPS$_{2a}$ became diffuse (**Fig. 3b**) compared to images taken immediately following light exposure (**Fig. 3b**), demonstrating TPPS$_{2a}$ redistribution within the cell. No fluorescence was detected in PANC-1 control cells that were not incubated with TPPS$_{2a}$ (**Fig 3b**).
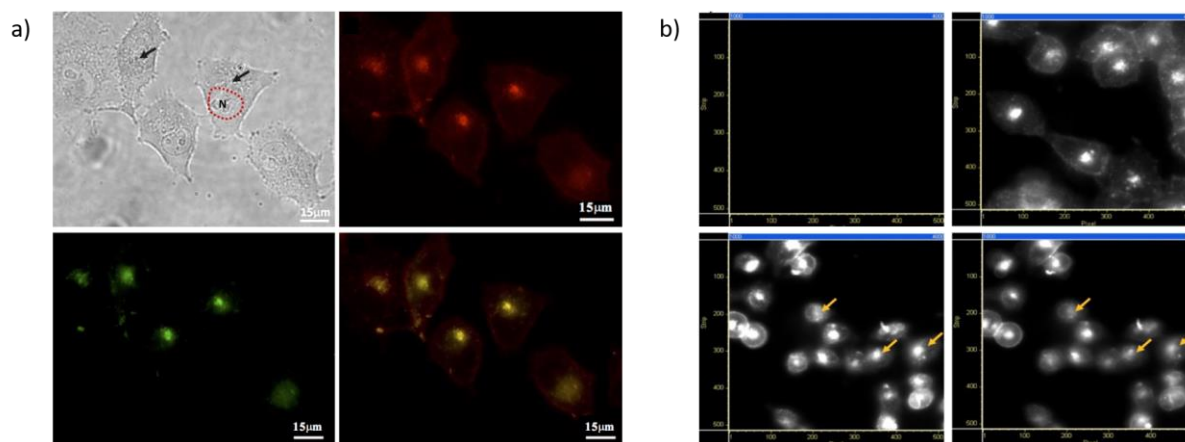

Figure 3. a) Subcellular accumulation of TPPS$_{2a}$ in endolysosomes of PANC-1 cells analysed by fluorescence microscopy (60X magnification). Cells were incubated with 1µg/ml TPPS$_{2a}$ for 18h. 50nM LysoTracker Green was added 1h prior to imaging. 60X magnification light microscope (top left) (N-nucleus, black arrows mark endolysosome location adjacent to the nucleus). TPPS$_{2a}$ distribution (red fluorescence). Lysotracker green staining lysosomes in green. Merged image of TPPS$_{2a}$ (red) and Lysotracker (green) fluorescence giving a yellow fluorescence pattern (bottom right image). Scale bar: 15 µm. b)

Subcellular redistribution of TPPS$_{2a}$ following light exposure. Control cells (not treated) (top left). Images acquired using an Olympus IMT-2 epi-fluorescence inverted microscope demonstrated intracellular localisation of TPPS$_{2a}$ (top right) in a granular pattern in lysosomes, while no signal was detected in the untreated control cells (top left image). TPPS$_{2a}$ redistribution within the cytoplasm (marked by arrows) was observed 5 minutes following blue illumination (bottom right image), compared to images acquired immediately after light exposure (bottom left). Images were taken with a 60X (top row) and 20X magnification objectives (bottom row) using a 250X250 micron scale.

High concentrations of saporin and TPPS$_{2a}$ are needed to inactivate PANC-1 cells when used as monotherapies

The optimal saporin concentration for PCI experiments as well as its cytotoxic effect on PANC-1 cells, were determined after cell incubation with increasing concentrations of saporin (0.1-80nM). Results of MTT assays performed 48 and 96h following each treatment are represented in **Figure 4a** by means of % survival. A statistically significant cytotoxicity was observed only when using saporin concentrations higher than 40nM, suggesting dose dependent cytotoxicity. A statistically significant reduction in cell viability was observed with 60nM (p-value = 0.04) and 80nM (p-value = 0.03) only in the 96h experiments, when compared to control cells (not treated). Saporin concentrations of 10, 20 and 40nM were chosen for further PCI experiments as sub-lethal concentrations of the drug to minimise systemic side effects.

No significant toxicity was observed in the TPPS$_{2a}$-treated but non-illuminated (0min/dark) cells compared to the control group, regardless of the concentrations used, confirming the absence of dark toxicity and safety of the photosensitiser (data not shown). Time- and concentration-dependent increase of cell toxicity was observed at both 48h (data not shown) and 96h (**Fig. 4b**). A significant reduction ($p<0.001$) in cell proliferation was detected with a concentration as low as 0.1 µg/ml TPPS$_{2a}$ following 3 min light exposure (10% reduction compared to untreated control cells). Treatment using a TPPS$_{2a}$ concentration of 0.2 µg/ml, resulted in a reduction of cell proliferation of 32% and 54% following 3- and 5-min illumination in the 48h plates, and 29% and 67 % following 3- and 5-min illumination, respectively, in the 96h plates. Using TPPS2$_a$ concentrations of 0.3 µg/ml or higher, 80-90% reduction in proliferation was detected. Similarly, near complete cell inactivation was observed at these concentrations in the 96h plates.

PCI protocols induced a synergistic growth inhibition and cell death of pancreatic cancer cells

Analysis of the PCI effect in PANC-1 cell proliferation using 10, 20, and 40nM saporin together with 0.1 or 0.2 µg/ml TPPS$_{2a}$ were compared to saporin and TPPS$_{2a}$ monotherapies at 48 and 96h following treatment (**Fig. 4c**). PCI enhanced the cytosolic delivery of saporin and its effect was increased as a function of illumination time and saporin concentration. At 96h post treatment, a more pronounced augmentation of the PDT effect was observed and therefore only these will be described below.

No toxicity was observed in the PCI plates that were not exposed to illumination (data not shown). PCI effect using combinations of saporin 10nM with 0.1 or 0.2 µg/ml TPPS$_{2a}$, resulted in significant cell viability reduction compared to monotherapies (**Fig. 4c**). With three-minute light exposure (1.26J/cm^2), PCI using 0.1 µg/ml TPPS$_{2a}$ + 10nM saporin resulted in a 30%

decrease in cell viability, while an increase in $TPPS_{2a}$ concentration to 0.2 μg/ml, resulted in a 59% reduction 48h after the end of the treatment. Compared to PDT alone ($TPPS_{2a}$ monotherapy), a significant augmentation of the cytotoxic effect was demonstrated in both cases using PCI, with a 2.7 ($p<0.001$) and 1.7 ($p<0.001$) fold increase in effect respectively. Maximal reduction in cell viability was seen when cells were illuminated for five minutes (2.1 J/cm2 light dose), achieving 51% and 77% cell killing effect for 10nM saporin with 0.1μg/ml and 0.2μg/ml, respectively. PCI using 0.1μg/ml $TPPS_{2a}$ showed a 2.1-fold increase ($p<0.001$) in cell killing effect compared to PDT alone ($TPPS_{2a}$ monotherapy), at this concentration of $TPPS_{2a}$. The use of 0.2μg/ml together with saporin 10nM, led to a 1.4-fold augmentation of the PDT effect at this concentration of PS ($p<0.001$). No increase in cell proliferation (suggesting cell recovery after treatment) was observed 96h following the PCI treatment. This fact could be explained by an efficient induction of either cell cycle arrest or cell death following PCI.

In order to determine whether PCI using higher concentrations of saporin results in greater enhancement of the effect achieved using $TPPS_{2a}$ alone, 20nM and 40nM of saporin were tested next. PCI using 20 or 40nM of saporin did not result in a significant reduction in cell viability compared to PCI using 10nM (and therefore this data has not been included in this manuscript). However, PCI with 40nM of saporin resulted in significantly higher enhancement of the PDT effect. A factor of 4.65 was the highest enhancement of PDT effect ($PCI_{\%reduction\ in\ cell\ viability}/PDT_{\%reduction\ in\ cell\ viability}$) observed in this study, where 0.1μg/ml TPPS2a was used together with 40nM saporin, subject to 3-minute light exposure, 96h after treatment. A 46.5% reduction in viability with PCI compared to 10% with PDT was observed.

In all cases, the use of PCI showed a synergistic effect over PDT ($TPPS_{2a}$ monotherapy) and saporin monotherapies. This was determined using the Valeriote and Lin method of assessing synergism in anticancer treatments[24] and is detailed in **Table 1**.

Table 1. The synergistic effect of PCI over $TPPS_{2a}$ and saporin monotherapies as a function of treatment doses and illumination time, was assessed using the Valeriote and Lin method. In brief, if **[A+B] < [A] X [B] / 100** the interaction is synergistic. A= cell viability after $TPPS_{2a}$ treatment; B= cell viability after saporin monotherapy, [A+B] = cell viability after PCI. PCI shows a clear synergistic effect when $TPPS_{2a}$ is combined with saporin.

Assessment of synergism 96h following PCI treatment				
Illumination time	**Treatment dose $TPPS_{2a}$+saporin**	**[A+B]**	**[A]X[B]/100**	**Effect**
3 minutes	0.1μg/ml + 10nM	60	83	Synergistic
	0.2μg/ml + 10nM	27	60	Synergistic
	0.1μg/ml + 40nM	54	86	Synergistic
	0.2μg/ml + 40nM	18	67	Synergistic
5 minutes	0.1μg/ml + 10nM	46	63	Synergistic

	0.2µg/ml + 10nM	7	32	Synergistic
	0.1µg/ml + 40nM	33	65	Synergistic
	0.2µg/ml + 40nM	5	40	Synergistic

The trypan blue (TB) cell-death assay analysis 24h following treatment did not show any significant morphological changes neither significant TB uptake and therefore is not further described. Images were acquired 48h or 96h following incubation with saporin only (10, 20 or 40nM), treatment using 0.1µg/ml or 0.2µg/ml TPPS$_{2a}$ subject to 3- or 5-minute illumination or PCI combination therapy. A higher ratio of TB uptake and reduction in cell density was observed as a function of increased concentrations of saporin, TPPS$_{2a}$ and illumination time following PDT and to a higher extent, after PCI. Cytotoxicity was clearly enhanced when PCI therapy using 40nM and 0.2µg/ml TPPS$_{2a}$ was applied (see **Fig. 4d**). This effect correlates with the reduction in cell viability observed and described previously following MTT assays. Analysis performed 96h following PDT and PCI treatment demonstrated the highest cytotoxic effects. Cells that were subject to 5 minutes of light exposure showed a higher degree of cell detachment and cell death following PCI treatments. All detached cells after PCI therapy were positive for trypan blue staining indicating a loss of plasma membrane integrity after treatment and induction of cell death.
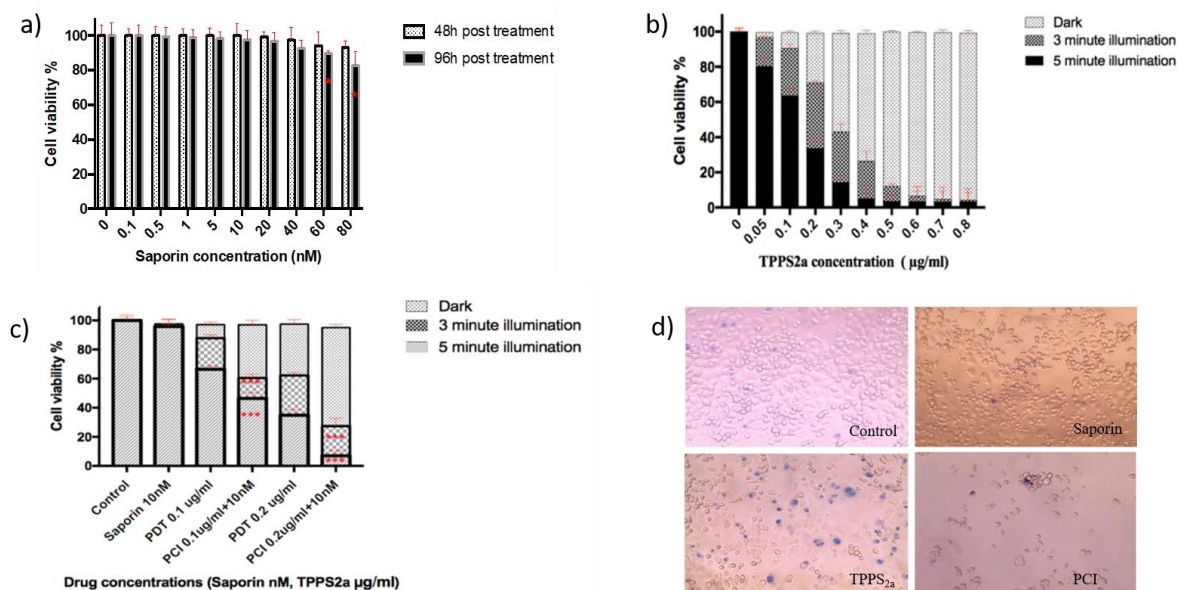

Figure 4. a) Saporin dose response 48h and 96h after the end of the treatment. Dose dependent toxicity was observed over 40nM saporin. b) TPPS$_{2a}$ dose response analysis performed 96h after illumination. Cells were treated with increasing concentrations of photosensitiser for 18h. Two illumination times were tested: 3 and 5 min (corresponding to 1.26 and 2.1

J/cm^2 respectively). c) PCI effect of 10nM saporin combined with 0.1 or 0.2µg/ml TPPS$_{2a}$ analysed 96h after the end of the treatment. Cell viability is expressed as percentage of survival compared to control (not treated cells). Error bars represent standard deviations. Statistically significant differences observed between groups are expressed as follows: p-value <0.05 = *; p <0.01 = **; p <0.001 = ***. d) Trypan blue assay analysed at 96h following treatment subsequent to three-minute light exposure (2.1 J/cm^2). Control cells (top left). Cells treated with saporin 40nM only (top right), showing minimal TB uptake (viable cells). Cells treated with 0.2µg/ml TPPS$_{2a}$ monotherapy (bottom left), showed a higher degree of reduction in cell density as well as of dye uptake. PCI combining 40nM of saporin and 0.2µg/ml TPPS$_{2a}$ resulted in significant reduction in cell adhesion and subsequent cell death induction (bottom right).

Conclusions

We successfully demonstrated the uptake of the photosensitiser TPPS$_{2a}$ by PANC-1 cells and its accumulation into endolysosomes as an essential requirement for its use in PCI. Additionally, we demonstrated redistribution of TPPS$_{2a}$ subsequent to light exposure in line with lysosomal disruption. Saporin monotherapy induced minimal toxicity to PANC-1 cells while TPPS$_{2a}$ alone demonstrated a concentration and light dose dependent toxic effect. Remarkably, the combination of saporin + TPPS$_{2a}$ + light (PCI protocol) in sub-lethal doses, resulted in almost a five-fold increase in the cytotoxic effect compared to both monotherapies. At the same time, PCI enhanced the cytosolic delivery of saporin and its effect was increased as a function of illumination time and saporin concentration. Altogether our results suggest that TPPS$_{2a}$-PCI of cytotoxic drugs such as saporin achieves a synergistic effect greater than single therapies being more effective in inactivating chemo-resistant pancreatic cancer cells. Further analysis involving clinically approved chemotherapeutic agents and more complex biological models are needed but, PCI has shown promise as a possible novel pancreatic cancer therapy.

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