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Optimization of photodynamic therapy response by survivin gene knockdown in human metastatic breast cancer T47D cells

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ABSTRACT

Photodynamic therapy (PDT) leads to the generation of cytotoxic oxygen species that appears to stimulate several different signaling pathways, some of which lead to cell death, whereas others mediate cell survival. In this context, we observed that PDT mediated by methyl-5-aminolevulinic acid as the photosensitizer resulted in over-expression of survivin, a member of the inhibitor of apoptosis (IAP) family that correlates inversely with patient prognosis. The role of survivin in resistance to anti-cancer therapies has become an area of intensive investigation. In this study, we demonstrate a specific role for survivin in modulating PDT-mediated apoptotic response. In our experimental system, we use a DNA vector-based siRNA, which targets exon-1 of the human survivin mRNA (pSil_1) to silence survivin expression. Metastatic T47D cells treated with both pSil_1 and PDT exhibited increased apoptotic indexes and cytotoxicity when compared to single-agent treated cells. The treatment resulted in increased PARP and caspase-3 cleavage, a decrease in the Bcl-2/Bak ratio and no participation of heat shock proteins. In contrast, the overexpression of survivin by a survivin-expressed vector increased cell viability and reduced cell death in breast cancer cells treated with PDT. Therefore, our data suggest that combining PDT with a survivin inhibitor may attribute to a more favorable clinical outcome than the use of single-modality PDT.

1. Introduction

Photodynamic therapy (PDT) of cancer is based on the administration of a photosensitizing compound with tumor-localizing properties. Subsequent irradiation of the tumor or cells with light of an appropriate wavelength, in the presence of oxygen, leads to the generation of cytotoxic oxygen species and consequently to cell death and tissue destruction [1]. The Food and Drug Administration-approved photosensitizing agent, Photofrin as well as several second-generation photosensitizers are used today in a variety of clinical trials. A wide applied PDT treatment involves the generation of endogenous Protoporphyrin IX (PpIX) through the heme biosynthetic pathway after administration of 5-aminolevulinic acid (ALA) [2,3]. ALA is frequently administered topically or systemically for PDT treatment of several tumor varieties [2,3]. ALA-induced PpIX accumulation has been shown to be preferentially greater in certain tumor cells primarily due to the reduced activity of ferrochelatase, the enzyme responsible for the conversion of PpIX into heme [4] and a relative enhancement of deaminase activity [5]. At this time only MAL, a methyl ester of ALA, has been approved for clinical use in Europe, in combination with red light, for treating actinic keratosis, superficial and nodular basal cell carcinoma (BCC), and Bowen disease. MAL accumulates selectively as ALA in neoplastic tissue and seems to penetrate the skin more rapidly and deeply. In addition, ester derivatives more rapidly achieve the maximum in intracellular protoporphyrin concentration which leads to a shorter incubation time compared with ALA [6].

PDT-mediated oxidative stress induces a transient increase in the downstream early-response genes (*c-fos, c-jun, c-myc* and *egr-l*) and stress genes (coding for heat shock proteins [Hsp], glucose-regulated proteins and heme oxygenase) in mammalian cells [7–12]. The early-response genes function as transcription factors and act by regulating the expression of a variety of genes via specific regulatory domains. PDT appears to stimulate several different signaling pathways, some of which lead to cell death, by caspase-dependent [13] and -independent [14] apoptosis, whereas others mediate cell survival such that the ultimate survival of a given cell results from the combined action or interaction (or both) of these different pathways [11,12,15,16]. Therefore, survival cells may cooperate in tumor recurrences following PDT and underline the need to more fully understand the molecular responses initiated by PDT. In this context, there have been reports that showed that PDT induces the expression of heat-shock proteins (HSPs) such as HSP70 [17], HSP47 [18] and HSP60 [10], as well as other stress-inducible proteins [7,19]. HSPs are abundantly

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expressed in most cancer cells, and its over-expression (HSP27, HSP70 and HSP90) correlates with a poor prognosis in acute myeloid leukemias and myelodysplastic syndromes [20,21].

Conversely, it was recently observed that PDT induces over-expression and phosphorylation of survivin in human cancer cells and tumors [22]. Phosphorylation of survivin extends the lifetime of this IAP protein and enhances protein activity [23]. Survivin is a member of the inhibitor of apoptosis protein (IAP) family, and it has received increasing attention as a target for anticancer therapies due to its selective expression in tumors versus normal tissues, its distribution and sub-cellular localization, and the emerging evidence for its dual role in the control of both cell apoptosis and proliferation [24–27]. Moreover, up-regulated expression of survivin has been consistently associated with tumor resistance to chemotherapeutic drugs [28,29], decreased survival of patients, and poor prognosis [30,31].

The aim of the present study was to understand crucial molecular events that allow for increased effectiveness following PDT treatment.

Results obtained ruled out the possibility that IAPs mediated prevention of apoptotic and cytotoxic responses of PDT. We provide evidence of the altered expression of the cytoprotective protein, survivin, after PDT-treatment in T47D breast cancer cells. Thus, its silence triggers hypersensitivity of tumor cells to apoptosis-mediated PDT. Our results also demonstrate that strategies in targeting protective proteins in metastatic cells have the potential to increase the clinical effectiveness of PDT-mediated cancer treatments.

2. Materials and methods

2.1. Drug

The drug used, delta-aminoleavulinic acid methyl ester hydrochloride (MAL) was purchased from SIGMA. The drug was dissolved in sterile water to make a 10 mM stock solution and stored at 4 °C. Stock solution of photosensitizer was diluted in DMEM medium (Gibco) without fetal bovine serum (FBS) to obtain the desired concentrations.

2.2. Cell cultures

The metastatic human breast carcinoma cell line T47D was cultured in DMEM complete growth medium: DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 2 mM glutamine (Sigma), and antibiotic–antimycotic mixture (Gibco). Cells were maintained in a humidified 37 °C incubator with 5% CO_2.

2.3. Photodynamic treatment

T47D cells were seeded in Petri dishes (60-mm diameter) and incubated for 24 h in DMEM complete growth medium. The growth medium was removed and replaced with DMEM without FBS and the desired MAL concentration; the cells were incubated at 37 °C for 4 h. After incubation, the cells were irradiated in the same MAL-containing medium for various periods of time with monochromatic light source (635 nm ± 17 nm) from a MultiLED system (coherent light) as described by Gaullier et al. [32] and Wyld et al. [33]. The light fluence rate on the cell monolayer was 1.6 mW/cm^2 (as measured by Coherent Laser mate power meter). The MAL-containing medium was removed; cells were washed twice with 2 mL of PBS and replaced with complete growth medium. After 24 h of photosensitization, cells were processed for different studies. References made to PDT henceforth will mean MAL photosensitization plus LED light exposure.

2.4. Plasmid vectors and transfection

A DNA vector-based siRNA was utilized to knockdown Survivin. Design of specific siRNA was performed as previously published [34]. The 21-nucleotide sequence, pSil_1, targets sequences to exon 1 (50-GGACCACCGCATCTCTACATT-30) of the human SURVIVIN mRNA. This sequence is directed against all Survivin mRNA splicing variants. We used siRNA plasmid vectors against green fluorescent protein (GFP) as a control (50-gctgaccctgaagttcatct-30); referred to as pSil_C. We then used survivin-expressed vector (pSURV) to over-expression survivin. For transfection, cells were cultured in antibiotics-free medium before the start of transfection. Survivin-specific siRNA or pSil_C was mixed with transfection reagent LipofectamineTM Plus in OptiMEM medium (Invitrogen) according to the manufacturer's instructions. After transfection for 6 h at 37 °C, the cells were cultured in DMEM GlutaMAX medium (Gibco) containing 10% FBS (Gibco). At 72 h after transfection for pSil_1 and pSil_C and 48 h after transfection for pSURV, cells were processed for different studies.

2.5. Immunoblot analysis

Total cell lysates from control and treated cells were extracted with lysis buffer containing 20 mM HEPES pH 7.5; 1.5 mM KCl; 1 mM EDTA; 1 mM EGTA; 0.15% Triton-X100; 1 mM PMSF; 1 mM DTT; and a cocktail of protease inhibitors (Sigma). The samples were centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatants were collected and protein concentration was determined by the MicroBCA assay according to the manufacturer's instruction (Pierce). Proteins were separated on 14% sodium dodecyl sulfate polyacrylamide gels. PARP was separated on 10% sodium dodecyl sulfate polyacrylamide gels. Subsequently, the resolved proteins were transferred onto Immobilon-P membranes (Sigma) to 250 mA for 1 h. The immunoblots were sequentially incubated in 5% milk blocking solution at room temperature for 1 h. The primary antibodies were mouse monoclonal anti-survivin (6E4; Cell Signaling Technology), rabbit polyclonal antibody to phosphorylated survivin (Thr34; Santa Cruz Biotechnology), rabbit monoclonal anti-cleaved caspase-3 (Asp175; Cell Signaling Technology), rabbit polyclonal anti-Bcl-2 (sc-783; Santa Cruz Biotechnology), rabbit polyclonal anti-Bak (sc-7873; Santa Cruz Biotechnology), mouse monoclonal anti-caspasa-8 (Chemicon International), PARP-1 (H-300; Santa Cruz Biotechnology), rabbit polyclonal anti-Hsp-90 (SPA-846; Stressgen Bioreagents Corp.), or rabbit monoclonal anti-GAPDH (14C10; Cell Signaling Technology). After washing three times with 0.05% Tween-PBS solution at room temperature for 15 min, membranes were incubated with anti-rabbit (1:3000; Cell Signaling Technology) and anti-mouse (1:4000; Cell Signaling Technology) secondary antibodies. Finally, detection was carried out using the enhanced chemoluminescence (ECL) kit (Amersham) according to the manufacturer's instructions.

2.6. Immunofluorescence

T47D cells were grown on culture slides (BD Falcon), and when reached 60–80% confluence, the PDT protocol was performed. Twenty-two hours after treatment, cells were washed three times with PBS and fixed with 2% of paraformaldehyde for 20 min at 4 °C. Cells were permeabilized with ethanol: acetic acid 2:1 for 5 min at 20 °C and then washed and incubated in PBS blocking solution (10% FBS, 1% BSA) for 30 min at RT. Next, cells were incubated with rabbit polyclonal anti-survivin (FL-142) for 45 min at 37 °C. After washing, cells were incubated with FITC-conjugated anti-rabbit-IgG (1:200; Sigma) for 45 min at 37 °C. Samples were mounted on microscope slides with ProLong Antifade Kit (Molecular Probes).

The fluorescence signal was visualized in Olympus fluorescence microscope.

2.7. MTT cell viability assay

The effect on cell survival was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 3×10^3 cells in 100 μL of complete growth medium were seeded onto 96-well plates for 24 h before treatments. We then added 20 μL of MTT (1 mg/mL) into each sample and incubated for 4 h, under 5% CO_2 and 37 °C. Cleavage of MTT and formation of MTT formazan was determined by absorbance at 540 nm using a microplate reader. Survival of treated cells was normalized to control cells. Eight samples were analyzed for each experiment, and all experiments were done in duplicate.

2.8. Detection of apoptosis

Cellular apoptosis was evaluated using the Cell Death Detection ELISA Plus kit (Roche) according to the manufacturer's instructions. This assay quantifies mononucleosomes and oligonucleosomes from cell lysates using monoclonal antibodies targeting DNA and histones in a quantitative photometric sandwich enzyme immunoassay. Cells were analyzed 24 h after treatment. Two samples were analyzed for each experiment, and all experiments were done in duplicate. Apoptotic enrichment factors were calculated from absorbance ratios of treated versus control cells. Results were normalized with respect to protein concentrations.

2.9. Cellular and nuclear morphology

Control and treated cells were washed with PBS followed by fixation with cold methanol (−20 °C) for 10 min. The cells were stained either with 10 μg/mL Hoechst 33258 or 0.5 mg/mL toluidine blue (TB) for 1 min. Stained cells were then washed and mounted on slides using DePeX.

Nuclear morphology of the cells was visualized with an Olympus fluorescence microscope using 330–380 nm excitations and measuring fluorescence at 420–450 nm. Three different fields of 200 cells per group, per treatment were scored.

2.10. Statistical analysis and determination of combination index

Data are represented as the mean ± standard deviation (SDs) for the indicated number of separate experiments. Statistical analysis was carried out using Duncan's multiple range tests ($P < 0.05$) by using software STATISTICA (version 6.0). Combination index (CI) was analyzed with the program Compusyn 1.0, by Ting-Chao Chou and Nick Martin based on the median-effect analysis according to the method of Chou [36].

3. Results

3.1. Determination of T47D human metastatic carcinoma cells viability and phenotype after MAL-PDT

It was observed that PDT stimulates several different signaling pathways, some of which lead to cell death whereas others mediate cell survival [11,12,15,16]. Thus, survival cells may cooperate in tumor recurrences following PDT and the phenotype of these cells might determined the long term effects of the treatment.

Firstly, we established the photocytotoxicity of MAL-PDT on T47D human metastatic mammary carcinoma cells, measuring the ability of viable cells to reduce MTT and convert it to non-water-soluble violet formazan crystals. Cell survival was not affected by light or MAL alone. However, when cells were exposed to the combination of 1 mM MAL incubation followed by irradiation with light doses ranging from 0.2 to 0.5 J/cm^2, a decrease in cell survival from 60% to 40% was observed (Fig. 1). The following experiments were performed with 1 mM MAL and a light dose of 0.2 J/cm^2, which induced around 40% cell death (an LD_{40} dose).

Then, we decided to analyze the phenotype of PDT-treated cells that mediated their sensibility to treatment. In response to MAL-PDT in T47D cells, we determined both expression and phosphorylated survivin because its over-expression has been demonstrated by several anti-cancer treatments [35]. Western blot analysis revealed that exposure of T47D cells to a LD_{40} dose of MAL-PDT induced an increase in survivin and phosphorylated survivin expression after 24 h of treatment (Fig. 2a). To confirm these results, fluorescence microscopy analysis was performed. PDT-treated cells showed strong cytoplasmatic expression of survivin (Fig. 2b). On the other hand, non-treated cells (NC), irradiated cells (IC) and cells treated with MAL alone (MAL) showed poor immunoreactivity of survivin (Fig. 2b). These results are in agreement with those obtained by immunoblot analysis. Although, our findings demonstrated that metastatic T47D cells seem to be sensitized in a PDT dose-dependent manner.

On the other hand, there are some reports that showed that PDT induces the expression of heat-shock proteins such as HSP70 [17], HSP47 [18] and HSP60 [10] as well as other stress-inducible proteins [7,19] that collaborated in tumor therapy resistance. When we examined the level of HSP60, HSP70 and HSP90 in MAL-PDT T47D cells, we did not observe alterations in the expression of heat-shock proteins of above control cell levels (Fig. 2c).

3.2. Efficient down-regulation survivin protein by pSil_1 in metastatic T47D cells

Due to the fact that we observed PDT only enhanced survivin activation and moreover, has emerged its targeting by virtue of over-expression in most tumor cell types, we decided to down-regulate survivin with a previously designed DNA vector-based siRNA, named pSil_1 [34]. With the aim of increasing the efficiency of pSil_1 to silence expression of the antiapoptotic protein survivin in tumor cells, T47D human breast cancer cells were transfected with different concentrations of pSil_1, and a control plasmid (psil_C) by liposomal reagent. The plasmids concentrations used were 1.5, 2.5 and 5 μg/mL, and survivin expression level was evaluated 72 h post-transfection by Western blot analysis. As shown in Fig. 3a, pSil_1 down-regulated survivin protein expression in a

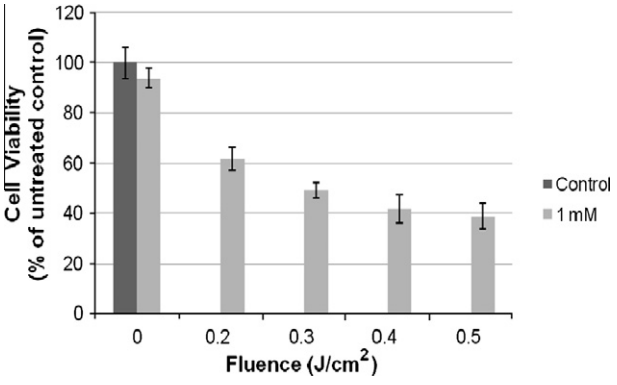

Fig. 1. Effect of MAL-PDT on T47D cells. Photocytotoxicity effects of MAL-PDT. T47D cells were incubated with 1 mM of MAL for 4 h and irradiated with different light doses (0.2–0.5 J/cm^2). After phototreatment, cells were left 24 h in the incubator with complete growth medium and survival was evaluated by the MTT assay. Values are expressed as means ± SDs of three separate experiments.

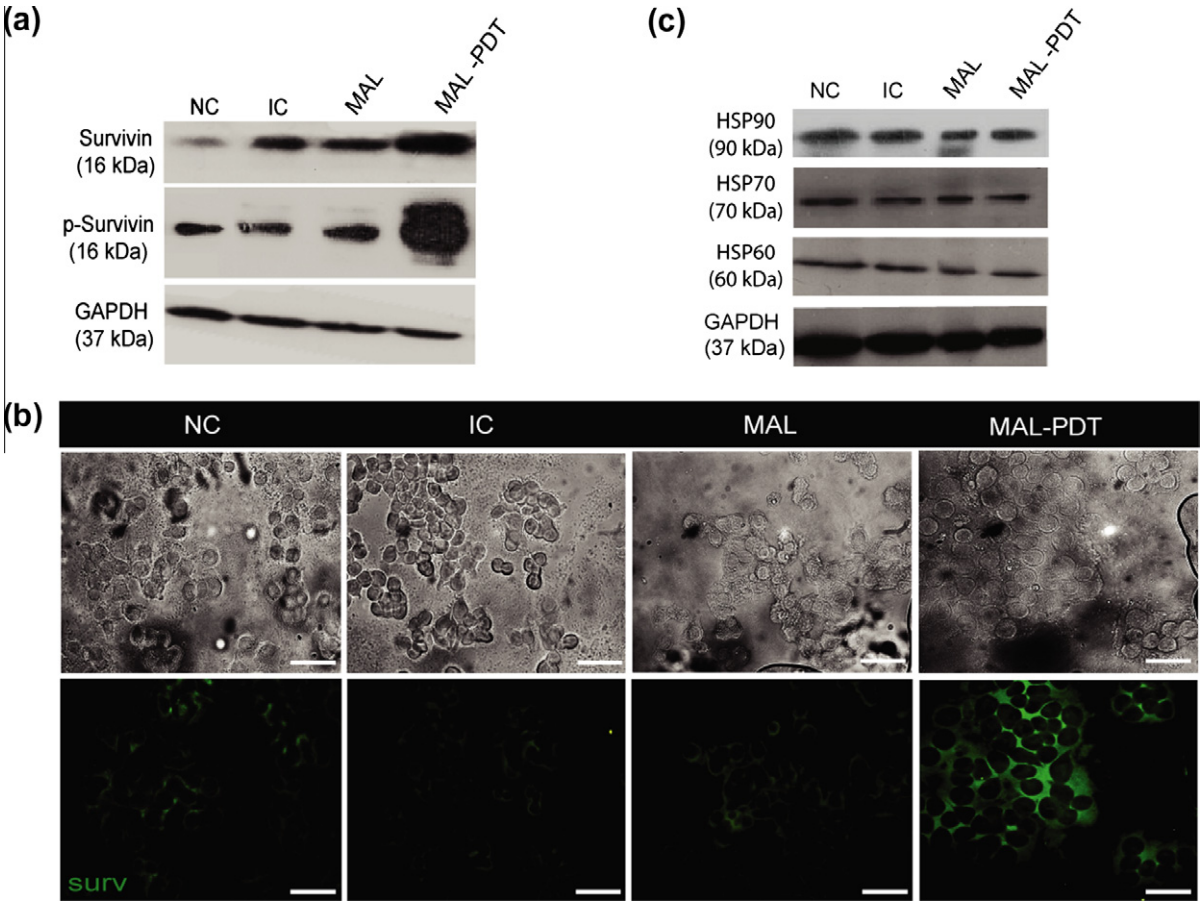

Fig. 2. Survivin and HSPs expression after PDT treatment. Western blot analysis were carried out for survivin, phosphorylated survivin (a) and HSPs (b) expression after treatment. T47D cells were incubated with 1 mM of MAL for 4 h and then exposed to 0.2 J/cm^2. After 24 h of light treatment, whole extract from non treated cells (NC), irradiated cells (IC), cells treated with MAL alone (MAL) and PDT-treated cells (PDT) were prepared and analyzed by Western blot to determinate survivin, phosphorylated survivin (Phospho-Survivin), and GAPDH (internal control) levels. (c) Fluorescence microscopy of survivin expression in non treated cells (NC), irradiated cells (IC), cells treated with MAL alone (MAL) and PDT-treated cells (PDT). Scale bars 20 μm. Data are representative of three independent experiments performed in triplicate.

dose-dependent manner. The highest down-regulation (90% of the initial protein level) was achieved with 5 μg/mL of pSil_1. Transfection of pSil_C, a control vector expressing siRNA against the green fluorescent protein, had no effect on survivin expression (Fig. 3a).

To analyze the biological effect associated with down-regulation of survivin expression, proliferation of T47D cells treated with pSil_1 was assessed using the colorimetric *MTT assay*. As shown in Fig. 3, 72 h post-transfection, pSil_1 had reduced T47D cell growth dose dependently, with an LD_{40} of 5 μg/mL.

The implementation of siRNA to silence gene expression of survivin generates a more specific target gene and response than inhibitor techniques that are, possibly indirectly based on survivin protein.

3.3. Consequence of survivin silence on cell survival and apoptosis in MAL-PDT-treated cells

Since PDT stimulates survivin expression within cells with metastatic behavior, we studied the possibility that PDT treatment could result in a selective treatment for a more aggressive and invasive population of cell. Indeed, these cells might constitute a future potential risk for tumor recurrences and multidrug resistant. In this sense, we performed a combined treatment; down-regulating survivin expression by pSil_1 and MAL-PDT to avoid resultant cells with a more aggressive phenotype and ensure highly sensitized cells by treatment. For this purpose, T47D cells were pre-transfected with pSil_1 (5 μg/mL) for 72 h and then incubated with 1 mM of MAL for 4 h and exposed to 0.2 J/cm^2. Fig. 4a shows that co-treatment of pSil_1 and MAL-PDT significantly reduced cell viability of T47D cells by 88%, compared to control; the cyto-toxicity achieved represents twice that of MAL-PDT treatment alone. To determine the combined effect of pSil_1 and MAL-PDT in T47D cells we applied a method which is based on the median-effect principle of the mass action law [36]. This method was supported on a dose–response curve of combining treatment and compares it with the dose–response curves of individual treatment. The Combination Index (CI) calculated using Chou and Talalay analysis describes the interaction between two therapies and quantifies the synergism (CI < 1), antagonism (CI > 1) or additive effects (CI = 1) [34].

To obtain the dose–response curve of combining treatment, T47D cells were transfected with different concentrations of pSil_1 (1.5, 2.5 and 5 μg/mL). After 72 h of transfection, cells were treated with 1 mM MAL and light 0.2 J/cm^2. The PDT dose used was that at which we had observed over-expression and phosphorylated survivin (see Fig. 2a). Post-24 h hours of photodynamic treatment, cell viability was determined using the MTT assay. As depicted by CI-effect plot, the CI of the combination (5 μg/mL pSil_1 + 1 mM MAL + 0.2 J/cm^2) was 0.3 (Fig. 4b), showing a strong synergism when survivin was most down-regulated. Furthermore, this tumor cells utilized in these experiments became round and slightly en-

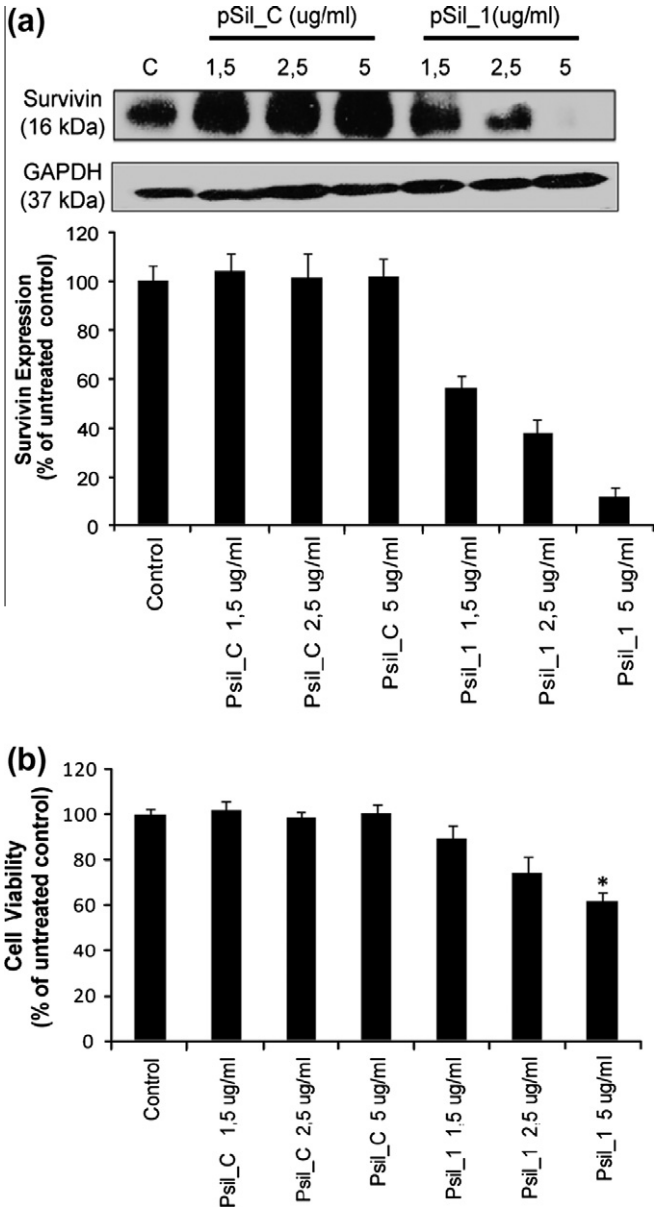

Fig. 3. Effect of a plasmid vector encoding siRNA against survivin on T47D cells viability. Cells were transfected with different concentrations of pSil_C and pSil_1 (1.5, 2.5, 5 μg/mL). (a) Immunoblot analysis of survivin or GAPDH (internal control) protein expression performed 72 h after transfection. Densitometric analysis represented the average signal intensity of survivin protein and was normalized to GAPDH. Data are representative of three independent experiments. (b) MTT metabolism of T47D cells treated with different concentrations of pSil_1 and pSil_C. Values are expressed as means ± SDs of eight separate experiments. A statistically significant difference in the cytotoxicity level between cells treated with pSil_1 5 μg/mL and control cells is denoted by "*" ($p < 0.05$).

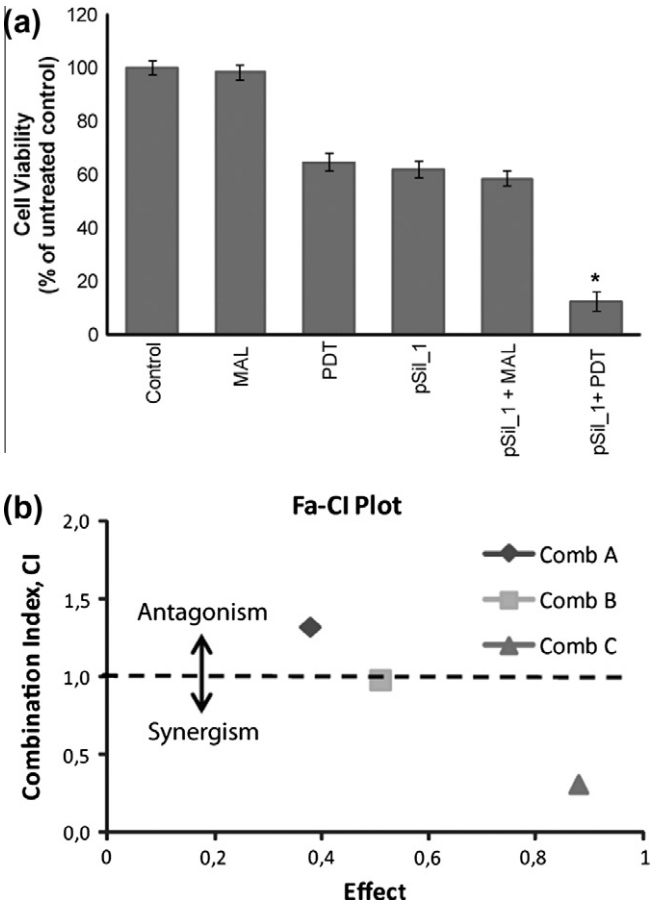

Fig. 4. Evaluation of cytotoxicity on PDT-treated human T47D breast cancer cells survivin down-regulated. Cells were pre-transfected with pSil_1 (5 μg/mL) for 72 h and then incubated with 1 mM of MAL for 4 h and exposed to 0.2 J/cm^2. (a) T47D cells viability was measured using a MTT assay 24 h after MAL-PDT. Values are expressed as means ± SDs of eight separate experiments. A statistically significant difference in the level of viability between cells treated with pSil_1/PDT combination therapy and pSil_1 or PDT monotherapy is denoted by "*" ($p < 0.05$). (b) CI–effect plot. Different combining treatment of pSil_1 and MAL-PDT to determine combination effect in T47D cells using CompuSyn software. Combination A: 1.5 μg/mL, Combination B: 2.5 μg/mL, Combination C: 5 μg/mL. Fa: fraction affect. Each dot represents eight separate experiments.

larged in size. Moreover, the multinucleated cells we observed allowed us to confirm a specific role of survivin in modulating PDT, since the therapeutic response correlated with survivin expression level in metastatic T47D cells.

Similarly, nuclear fragmentation, condensation and detachment from the culture surface were also increased in combination-treated cells compared with single-agent treated cells (Fig. 5a). Furthermore, treatment with PDT alone produced few cells containing condensed and fragmented chromatin into apoptotic bodies (Fig. 5a, lower panel).

Quantitative levels of apoptosis were analyzed for control and treated T47D cells 24 h after MAL-PDT using an ELISA assay. Both PDT and treatment with pSil_1 alone caused measurable levels of apoptosis. However, combining PDT with pSil_1 further increased apoptosis to 3-fold higher when compared with single-agent treated cells (Fig. 5b).

3.4. Transfection with a survivin-expressed vector reduces PDT-induced cytotoxicity in the human breast carcinoma cells

The role of survivin on the PDT-induced cytotoxicity and apoptosis was investigated by transfection with a survivin-expressed vector (pSURV). T47D human breast cancer cells were transfected with different concentrations of pSURV (2.5, 5 and 10 μg/mL) and survivin expression level was evaluated 48 h post-transfection by Western blot. pSURV vector increased survivin proteins via a concentration-dependent manner and semi-quantification shows that pSURV significantly increased survivin protein levels (Fig. 6a). Moreover, we observed that T47D cells transfected with 10 μg/mL of pSURV and then treated with PDT were more resistant to apoptotic death than PDT-treated cells (Fig. 6b and c).

3.5. MAL-PDT in survivin knockdown cells resulted in apoptosis-related protein activation

Finally, we examined cellular responses following the combination of pSil_1 and PDT using human metastatic T47D breast cancer cells. Firstly, we analyzed the expression of apoptosis-related proteins, as well as Hsp-90 by Western blot. Survivin is an Hsp-90 client protein and the binding of the two proteins allows survivin to fold and madurate properly [37]. At the time of collection, we did not observe a correlation between Hsp-90 levels and the increase of survivin expression. Combining pre-transfection of pSil_1 with PDT induced PARP cleavage, from the native 116-kDa enzyme to an 89-kDa fragment, as well as enhancing cleavage of caspase-3 (17-kDa) (Fig. 7a). T47D cells treated with PDT alone showed a very modest cleavage of PARP and caspase-3. Densitometric analysis revealed that the increase in cleavage of PARP or caspase-3 was 3-fold higher in cells treated with pSil_1/PDT compared to PDT alone (Fig. 7b and c).

One of the mechanisms involved in the induction of apoptotic death in cells is a decrease in the level of B-cell lymphoma 2 protein (Bcl-2) and an increase in the level of Bcl-2 homologous antagonist/killer (Bax) [38]. To investigate the participation of the forementioned pro- and anti-apoptotic proteins in all protocols, we carried out Western blot analysis. Our results revealed an enhancement of Bax and inhibition of Bcl-2 on T47D cells which were transfected with pSil_1 and survivin was silenced (Fig. 7a). Fig. 7d shows the densitometric analysis of Bcl-2 (an apoptosis inhibitor) and Bax (an apoptosis promoter) ratio, which was diminished when cells were treated with a combined treatment of pSil_1 and PDT. Therefore, the decrease in the Bcl-2/Bax ratio might play a role in the apoptosis observed previously (Fig. 5b).

To examine whether combination treatments affect the extrinsic apoptotic pathway, we also evaluated the expression of the proapoptotic protein caspase-8. Remarkably, pSil_1 and PDT resulted in a weakly detectable cleavage of the caspase-8 (Fig. 7a). Thus, our findings indicate that the combination of down-regulating survivin and PDT treatment, involves mainly intrinsic apoptotic markers, such as cleavage of PARP, caspase-3 and a decrease of Bcl-2/Bak ratio, leading to tumor cell death by apoptosis.

4. Discussion

PDT uses non-toxic dyes and harmless visible light in combination with oxygen to produce highly reactive oxygen species that kill cells. In addition, PDT destroys tumor tissue by a process that can produce cellular apoptosis, necrosis and an acute inflammation [17]. On the other hand, it was reported that PDT also induces the expression of growth factors, matrix metalloproteinases, cytokines, and prostaglandins in the tumor-treated microenvironment [17,39,40]. These later treatment-related reactions can be associated with a survival phenotype that could cooperate in tumor

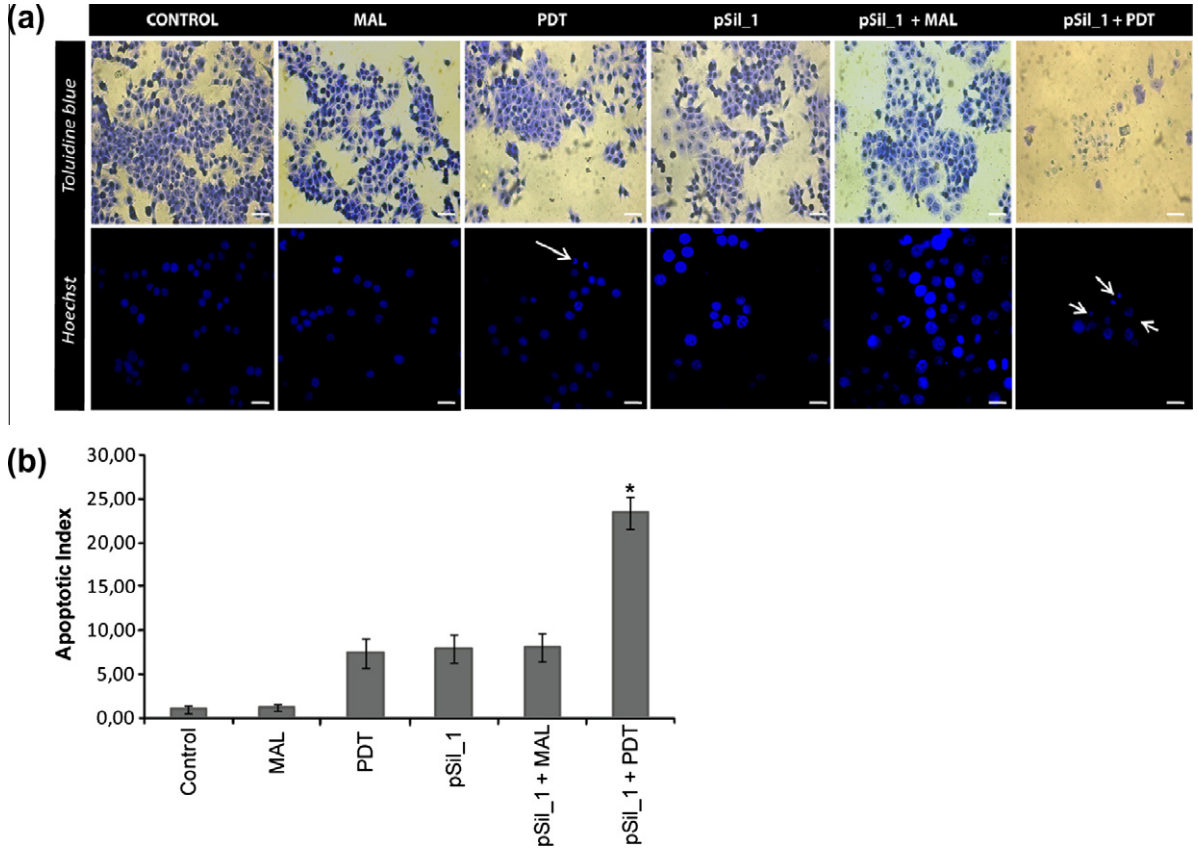

Fig. 5. pSil_1/MAL-PDT combination therapy induced cellular and nuclear morphology of apoptotic T47D cells. (a) Cells growing on cover slips were treated or not (Control) with MAL and then irradiated (PDT), MAL alone (MAL), pSil_1 alone (pSil_1), pSil_1 and MAL without irradiation (pSil_1/MAL), and pSil_1 and then MAL-PDT (pSil_1/PDT); the cells were fixed, stained with Hoechst 33258 or TB (toluidine blue), and photographed. Images of TB-stained nuclei became observable at 200× and for Hoechst 33258-stained nuclei became observable at 400×. White arrows indicate apoptotic cells. Data are representative of three independent experiments performed in triplicate. Scale bars 10 μm. (b) Apoptotic indexes were measured 24 h after MAL-PDT using the Cell Death Apoptosis Detection ELISA Plus kit. Values are expressed as means ± SDs of two separate experiments. A statistically significant difference in the level of apoptosis between cells treated with pSil_1/PDT combination therapy and pSil_1 or PDT monotherapy is denoted by "*" ($p < 0.05$).

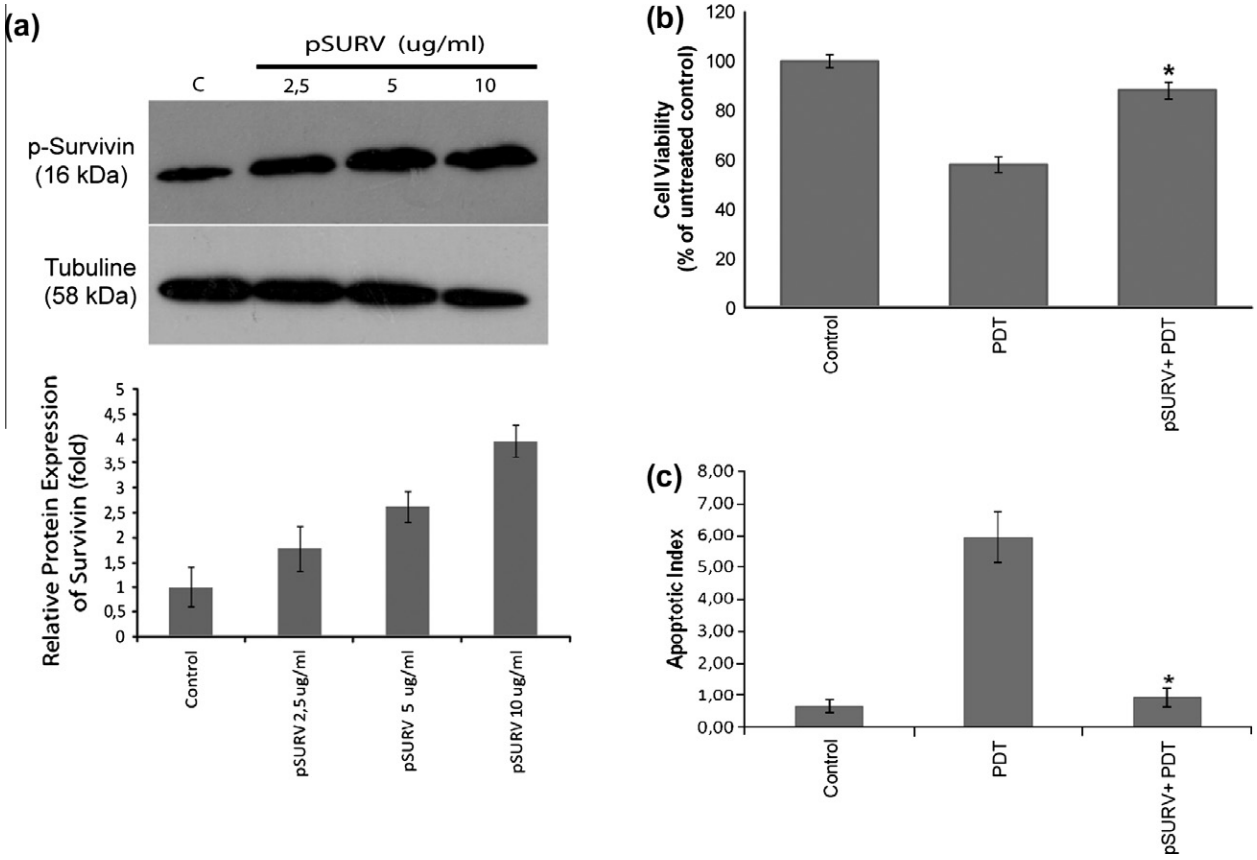

Fig. 6. Effect of over-expression of survivin by a survivin-expressed vector on PDT-induced cytotoxicity and apoptosis. (a) T47D cells were transfected with 2.5, 5 and 10 μg/mL of survivin expressed vector (pSURV) for 48 h. Total protein extracts were subjected to Western blot analysis and we used GAPDH as a internal control. Densitometric analysis represented the average signal intensity of survivin protein and was normalized to GAPDH. Data are representative of three independent experiments. (b) T47D cells were pre-transfected with 10 μg/mL pSURV for 48 h and then incubated with 1 mM of MAL for 4 h and exposed to 0.2 J/cm^2. Cell viability was measured by MTT assay. Results were obtained from 3 separate experiments. The bar represents the mean ± SDs $^*p < 0.05$ indicate significant difference between PDT and pSURV + PDT. (c) Apoptotic indexes were measured 24 h after MAL-PDT using the Cell Death Apoptosis Detection ELISA Plus kit. Values are expressed as means ± SDs of two separate experiments. A statistically significant difference in the level of apoptosis between cells treated with pSil_1/PDT combination therapy and pSil_1 or PDT monotherapy is denoted by "*" ($p < 0.05$).

recurrences following PDT. In the present study, we analyzed the phenotype of PDT-treated cells.

Due to the fact that there have been reports that showed PDT-induced expression of heat-shock proteins [17], we demonstrated the induction of the expression of HSP70, HSP60 and HSP90 by MAL-PDT treated T47D cells. The results shown clearly indicate that treatment in T47D cells does not involve expression of HSPs. Conversely, Ferrario and co-workers [22] reported that PDT treatments such as Photofrin- and NPe6-PDT, increase the expression/activation of survivin in non-metastatic malignant cells and experimental tumors. Survivin has received increasing attention as a candidate target for anti-cancer therapies due to its selective expression in tumors versus normal tissues and the emerging evidence for its dual role in the control of both cell apoptosis and proliferation [24–27]. Moreover, up-regulated expression of survivin has been consistently associated with tumor resistance to chemotherapeutic drugs [28,29], decreased survival of patients, and poor prognosis [30,31]. In the present study, we found that MAL-PDT increased both expression of survivin and the phosphorylated form of survivin in metastatic breast cancer cells. Our results are in accordance with another study that observed increased survivin expression after PDT [41]. This suggests the possibility of interfering with cellular response to photochemical therapy. Moreover there are many signaling molecules up-regulated by PDT, including phosphatidylinositol 3-kinase/Akt, mitogen-activated protein kinase, hypoxia inducible factor-1a, activator protein-1, and nuclear factor-nB, are inducers of survivin expression [39]. Similarly, inflammatory cytokines, vascular endothelial growth factor, vascular injury, and hypoxia are associated with increased expression and/or stability of survivin and these responses are also increased following PDT.

Conversely, PDT appears to stimulate several different signaling pathways, some of which lead to cell death, whereas others mediate cell survival such that the ultimate survival of a given cell results from the combined action or interaction (or both) of these different pathways. Therefore, in the present report we proposed that before treatment, the population of T47D has a level of survivin expression that is similar in all cells (see Fig. 1B, NC). We argued that the increased expression observed on CL and MAL is attributed to some stressing condition given to the cells. But is clear that only after treatment, certain percentage these cells could triggers signaling pathways which lead to cell survival involving survivin overexpression.

Enhanced survivin after PDT in T47D cells leads us to hypothesize that this anti-apoptotic protein would be potentially relevant to PDT outcome in sensitized cells. To confirm this issue, down-regulated survivin was applied. In an attempt to demonstrate the crucial role of survivin on moderate PDT response in metastatic breast cancer cells, we targeted specifically mRNA survivin instead of regulatory pathways. We successfully inhibited 90% of this protein expression in T47D cells by siRNA gene-silencing technology (pSil_1). Indeed, growth-inhibitory effects in T47D cells in the absence of any further cytotoxic stimulus was observed, and this

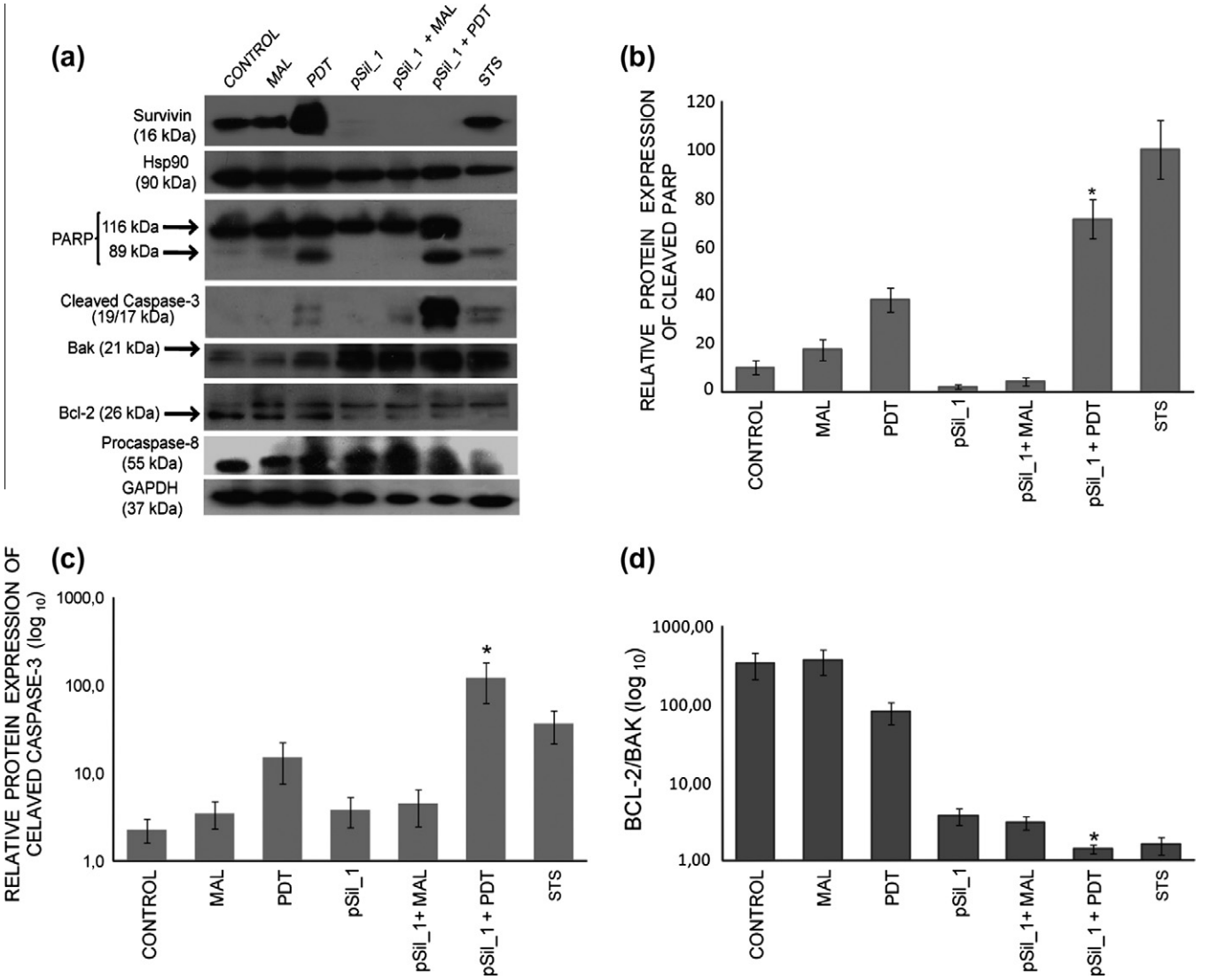

Fig. 7. Analysis of proteins involved in MAL-PDT apoptotic cells. (a) Cell lysates from control (Control), photosensitizer alone (MAL), pSil_1 alone (5 μg/mL; pSil_1), and PDT-treated cells in the absence (PDT) or presence (pSil_1 + PDT) of pSil_1 were collected 24 h after light exposure. Expression of survivin, Hsp-90, PARP and caspase-3 cleaved, Bcl-2, Bak, caspase-8, and GAPDH (internal control) were determined by Western blot analysis. Staurosporine (STS; 1 μM) treated T47D cells were used as positive control of apoptosis. Data are representative of three independent experiments. Densitometric analysis of (b) Percentage of cleaved PARP, $^{*}p < 0.01$ versus PDT. (c) Cleavage of capsase-3, $^{*}p < 0.01$ versus PDT. (d) Bcl-2/Bak ratio, $^{*}p < 0.05$ versus pSil_1. The average signal intensity of the different proteins was normalized to GAPDH.

agrees with results previously reported [34]. Moreover, we performed a dose–response curve by combining treatment of T47D cells transfected with pSil_1 with MAL-PDT. We found the condition to sensitize T47D cells to PDT synergistically, suggesting a survivin specific role in modulating PDT. In contrast to our study, Ferrario and co-workers [42] reported that Hsp-90 plays an active role in modulating tumor responsiveness following PDT by stabilizing client proteins such as survivin, and targeting Hsp-90, thereby enhancing the therapeutic effectiveness of PDT.

The synergistic combination (pSil_1 and PDT) increased apoptosis and cytotoxic effect when compared with single treatments. This procedure also led to enhanced PARP- and caspase-3 cleavage, a strong decrease in Bcl-2/Bax ratio and activation of caspase-8.

On the other hand, we have demonstrated that over-expression of survivin by a survivin-expressed vector can increase cell viability and reduce cell death in breast cancer cells treated with PDT. Therefore, we suggest that survivin plays an important role in modulating cancer cell survival by PDT treatment during cancer therapy.

It has been proposed that survivin may inhibit apoptosis through suppression of caspase activity [43], but we have previously observed that silencing survivin T47D cells by pSil_1 trigger apoptosis in a caspase-independent manner, involving nuclear translocation of mitochondrial AIF [34]. Interestingly, when combined treatment was applied, apoptosis was triggered in a caspase-dependent manner. Therefore, our results demonstrated that in PDT protocols survivin directly or indirectly could interfere with caspase-3 activity.

Curiously, as a consequence of pSil_1 transfection, with or without PDT, a diminished Bcl-2/Bax ratio was observed. It has been suggested that an alternatively spliced survivin variant, called survivin-ΔEx-3, which localizes in mitochondria, interacts with Bcl-2 [44]. Since anti-apoptotic Bcl-2 proteins function as inhibitors of mitochondrial permeability transition, this recognition would position survivin, or at least one of its spliced variants, in the regulation of mitochondrial membrane integrity. Alternatives of this pathway have been suggested, involving hyperphosphorylation of Bcl-2, and a reduced activation of proapoptotic Bax by survivin, potentially upstream of caspase activation [45], thus further dampening mitochondrial permeability. Furthermore, survivin-ΔEx-3 was recently shown to maintain mitochondrial membrane poten-

tial and to control the production of reactive oxygen species in response to cell-death stimuli [46]. Since pSil_1 targets exon 1 of the human survivin mRNA, survivin-ΔEx-3 was blocked in T47D cells transfected with pSil_1, that would explain how survivin modulates the response of cancer cells to PDT.

Our findings provide crucial insight into the application of a FDA-approved photosensitizer prodrug (MAL) on PDT-treated metastatic breast cancer cells. We also demonstrate that MAL-PDT induced over-expression of survivin results in a more aggressive phenotype for certain tumor cells. On the other hand, we were able to restore the MAL-PDT sensitivity when the survivin cytoprotective effect was abolished.

In summary, the intricate relationship between programmed cell death and cell survival may directly or indirectly be dependent on survivin. Since this protein is overexpressed in MAL-PDT treated cells, this could may result in aggressive tumor behavior yielding a poor survival rate and resistance to cancer therapies. We also showed that pSil_1 efficiently down-regulates the expression of survivin effectively suppressing tumor growth in human breast cancer cells at the metastatic site. Moreover, down-regulation of survivin sensitizes breast cancer cells to PDT-induced cytotoxicity.

Since a number of different strategies are now employed to treat metastatic breast cancer, it is promising to demonstrate that a combined modality and sequential therapy can prove beneficial to treatment [47]. Therefore, our data suggests that emerging strategies in targeting protective proteins may increase the clinical effectiveness of cancer treatments. Although, it remains to be clarified the exact role of survivin isoforms that could be implicated in mediates tumor cell viability and PDT effectiveness.

Conflict of interest

None declared.

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