Combination of photodynamic therapy with aspirin in human-derived lung adenocarcinoma cells affects proteasome activity and induces apoptosis

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Abstract

Objectives: Photodynamic treatment (PDT) of human lung carcinoma cells A549 ($p53^{+/+}$) and H1299 ($p53^{-/-}$) induces fast but transient stalling of proteasome activity. We have explored the possibility of prolonging this effect by combining PDT with drugs capable of sustaining the stall, and promote apoptosis of surviving cells. We show that aspirin can be used to accomplish this.

Materials and methods: Cells were irradiated at doses ranging from 0.54 to 1.10 J cm⁻², and subsequently were incubated with aspirin at either high (10 and 5 mM) or low concentration (2.5 and 1.5 mM). Photofrin concentration and incubation time were constant (2.5 µg/ml and 16 h). Under these conditions, we analysed cell viability, colony-forming efficiency, cycle profile, expression patterns of specific proteins and ubiquitination state, after individual or combined administration.

Results: Treatment with either PDT or aspirin, rapidly induced proteasome malfunction and accumulation of cells in G₂M, but did not induce apoptosis. However, when aspirin was added to cells (even at low concentrations) after PDT, the proteasome block was sustained. Moreover, significant cytotoxic effects, including apoptosis, were observed along with cytostatic effects (G₂M accumulation/decreased colony formation).

Conclusions: Combination of PDT and low-toxicity drugs (such as aspirin) resulted in protracted inhibition of proteasome activity and induced apoptosis even in apoptosis-resistant cancer cells.

Introduction

Cancer is characterized by virtually uncontrolled tumour growth and spread of abnormal cells. Treatments for cancer are tailored to tumour type, location and stage and, by and large, entail combined medicinal approaches.

Photodynamic therapy (PDT) has high potential to be treatment of choice in certain specific cancers. The procedure involves administration of a photosensitizing agent (which gets trapped in the tumour) whereby, upon activation by light of specific wavelength, causes cell destruction by production of singlet oxygen free radicals. Photodynamic therapy is frequently used to treat conditions on or just under the skin (mainly non-melanoma skin cancers), or in the lining of internal organs, especially some pre-cancerous lesions (for example, Barrett's oesophagus) and some forms of advanced cancers difficult to treat by other means. Low invasiveness is an advantage of PDT, which affords the option of repeated treatment. It is also possible to potentiate efficacy of PDT through its combination with drugs, including those that target molecules involved in cell regulation. The proteasome has emerged as an attractive target for cancer therapy (1); it is essential for numerous cellular processes, it is a highly conserved system and is the chief machinery for non-lysosomal protein digestion in living cells (2). It governs degradation of regulatory proteins such as cyclins A–E, cell cycle inhibitors p27^{KIP1} and CDKN1A (p21), onco-suppressor p53, transcription factor IκB, and cell adhesion molecules (ICAM, VCAM-1) (3). Notably, expression of these proteins is often deregulated in cancer. The proteasome is also involved in degradation of many proteins that become unfolded in response to oxidative damage (4); it recognizes damaged proteins that have been targeted for degradation by their ubiquitin tags (5). Because of the high replication rate of malignant cells (which implies rapid protein synthesis and turnover) and genetic changes that disable diverse protective checkpoint mechanisms, transformed cells are much more sensitive to proteasome

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inhibition than normal cells. This has prompted investigators to develop synthetic and biological proteasome inhibitors for therapeutic purposes. For instance, Bortezomib (Velcade®; Millennium Pharmaceuticals Inc., Cambridge, MA, USA), used to treat multiple myeloma, selectively targets the proteasome. It appears that this drug acts by inducing apoptotic cell death (6,7). However, some cells develop adaptive responses to counteract the assault by proteasome inhibitors (8).

Recently, Bortezomib has been shown to potentiate anti-tumour effects of Photofrin/PDT in mice (9), an observation that contributes also to better understanding of mechanisms involved in PDT cytotoxicity.

In the present study, we report our novel observations resulting from experiments performed with human-derived lung adenocarcinoma cell lines H1299 and A549. The choice of these two cell lines stems from three reasons: first, our research group is very familiar with them, as they have been used in other PDT and cancer studies (10) and references therein; second, both H1299 and A549 are cells derived from human lung adenocarcinoma, a type of cancer, which may be treated with PDT; and third, A549 cells express *p53*, whereas H1299 have no *p53*. We show that PDT had a detrimental effect on proteasome activity, which was identified immediately after light administration. In addition, we report for the first time, the relevant observation that PDT-dependent proteasome arrest can be prolonged by aspirin. Ability of aspirin to halt proteasome activity has been hinted at in a previous study (11), but not directly proven. We demonstrate that, at least *in vitro* PDT followed by aspirin, prolongs proteasome inhibition leading to apoptosis of those cells that eluded the primary lethal effect of PDT.

We suggest that combination of PDT with a low-toxicity proteasome-inhibiting drug, could be a more effective therapeutic approach for treatment of selected cancers.

Materials and methods

Cell cultures

H1299 human non-small cell lung cancer cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI 1640, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/l glucose, 1500 mg/l sodium bi-carbonate, 100 µg/ml streptomycin, 100 units/ml penicillin and 10% foetal calf serum (FCS). H1299 cells are *p53*^{-/-}. A549 human non-small cell lung cancer cell line was also obtained from American Type Culture Collection. These cells were grown in Ham's F12K, 2 mM L-glutamine, 1.5 mg/l sodium bicarbonate, 100 units/ml penicillin and

10% FCS. All media and cell culture reagents were purchased from Life Technologies (San Giuliano Milanese, Italy). A549 cells are *p53*^{+/+}.

Photosensitizer, aspirin, cycloheximide, proteasome inhibitors

Photofrin (haematoporphyrin derivative Porfimer sodium) used in this study was supplied as freeze-dried powder (lot no. 162A6-06; Axcan Pharma, Mont-Saint-Hilaire, Quebec, Canada). Its absorption spectrum consists of various peaks over visible light range. Photofrin stock solution was prepared by dissolving the powder in water containing 5% glucose to obtain final concentration of 2.5 mg/ml. This solution was stored in aliquots at -20 °C in the dark. Before measurements were taken, appropriate aliquots of this solution were diluted to desired concentrations. In all PDT experiments, cells were incubated at 37 °C in the dark with 2.5 µg/ml of Photofrin for 16 h before irradiation.

Aspirin was supplied as powder by Sigma-Aldrich (St Louis, MO, USA). Aspirin stock solution (110 mM) was obtained by dissolving this in 0.1 M TRIS, pH 8.8. Before use, aliquots of the solution were diluted to desired concentrations.

Cycloheximide was supplied as dry powder by Sigma-Aldrich. Stock solution (100 mg/ml) was obtained by directly dissolving it in water.

Proteasome inhibitor MG-132 (Cbz-Leu-Leu-Leucinal) was obtained from Calbiochem (La Jolla, CA, USA). Stock solution of 150 µg/ml of this reagent was prepared in normal medium containing 7.5 µl/ml dimethylsulphoxide (DMSO). Dithiothreitol (DTT) was obtained from Sigma Aldrich. Stock solution (1 M) was obtained by directly dissolving it in water. Proteasome inhibitor Bortezomib (Velcade®) was obtained from Millennium Pharmaceuticals Inc. (Cambridge, MA, USA). This compound was dissolved in dimethyl sulphoxide to obtain 1 µM stock solution.

Individual and combined treatment of cells

Individual treatments. (a) *Aspirin:* Cells were incubated for established times with concentrations of aspirin from 0 to 10 mM and were analysed (cell cycle, cell viability and colony forming efficiency assay, protein extraction followed by immunoblotting).

(b) *PDT:* Cells were routinely irradiated with broad-band radiation delivered from PTL-Penta apparatus (Teclas, Sorengo, Switzerland) consisting of a halogen lamp (Osram 250 W, 24 V, Munich, Germany) equipped with a band-pass filter. Light was delivered through an 8-mm bundle of optical fibres placed at a distance from

cell plates that ensured uniform illumination of the entire cell monolayer. Fluence rate at level of the cell monolayer was fixed at 6 mW cm^{-2} and light was irradiated at doses of up to 1.8 J cm^{-2} . We invariably used a Photofrin concentration of $2.5 \text{ }\mu\text{g/ml}$. Light doses of 0.54 ± 0.02 or $1.10 \pm 0.02 \text{ J cm}^{-2}$ were used in both individual and combination PDT experiments. As described previously (10), under such sub-lethal conditions, $\sim 50\%$ and $\leq 25\%$ cells survived PDT, respectively. In absence of the photosensitizer, illumination (up to 1.8 J cm^{-2}) did not induce any change in cell viability in addition to that caused by aspirin alone (10 mM).

Protein extracts (for western blot profiling) were obtained at established times after irradiation, whereas cells were fixed in aqueous ethanol (70%), for cell cycle evaluation, 6 and 24 h after PDT. Reactive oxygen species generated by PDT were evaluated by cytofluorimetry immediately after irradiation (see below).

Combined treatments. (c) *Administration of aspirin followed by PDT:* H1299 and A549 cells (2×10^4) were incubated 24 h with aspirin alone (controls). When Photofrin (usual concentration) was used in association with aspirin, it was added to cells 8 h after the beginning of incubation with the drug.

Aspirin concentrations were 1.5, 2.5 (low concentration range) and 5 and 10 mM (high concentration range). Controls and samples were washed and irradiated (0.54 J cm^{-2}). Plates were analysed for colony formation (see below) 8 days later.

(d) *PDT treatment followed by aspirin administration:* Approximately 2×10^4 cells/6 cm plate (triplicate) (either H1299 or A549) were incubated with Photofrin and irradiated according to the protocol reported earlier. After PDT (0.54 or 1.10 J cm^{-2}). Cells were incubated with aspirin for 24 (when aspirin concentration was 5 or 10 mM) or 72 h (when aspirin concentration was 2.5 or 1.5 mM). Finally, cells were released in fresh medium and examined for colony formation (see below) ≥ 8 days later.

Cell viability and colony-forming efficiency assays

We measured cell viability using CellTiter 96 Aqueous One Solution Non-Radioactive Cell Proliferation Assay (Promega, Milan, Italy), hereafter indicated as MTS. This is a colorimetric method for determining number of viable cells. According to the supplier's instructions, 5×10^4 cells/well were seeded into 96-well plates. Cells were incubated with aspirin (from 0 to 10.0 mM) for 24 and 48 h to observe effects of aspirin concentration, or incubated with Photofrin for 16 h and then irradiated to test effects of PDT. Each condition was analysed in triplicate.

Absorbance values at 492 nm were corrected by subtracting average absorbance from control wells containing no cells (12,13).

Colony-forming efficiency was assayed in triplicate by seeding $\sim 1 \times 10^4$ cells in six-well plates, and incubating with (a) aspirin (5 and 10 mM for 24 h) or subjected to (b) PDT 0.54 J cm^{-2} or incubating with (c) aspirin (5 and 10 mM for 24 h) followed by PDT (0.54 J cm^{-2}) or (d) subjecting to PDT 0.54 J cm^{-2} followed by aspirin (5 and 10 mM for 24 h). We also measured colony formation in cells that were irradiated with higher light doses (1.10 J cm^{-2}) and then treated with much lower aspirin concentrations (namely 1.5 and 2.5 mM, for 72 h) before being released in fresh medium. After ≥ 8 days, colonies (>50 cells) were stained with 1% methylene blue in 50% ethanol and counted.

Flow cytometry

Dishes (10 cm) containing $\sim 4 \times 10^5$ cells were incubated for 24 h at 37°C in 7 ml of complete medium (controls), or in medium supplemented with (a) aspirin (1.5–5 mM), (b) Photofrin alone to evaluate effects of irradiation, or (c) with 5 or 10 mM aspirin for 6 and 24 h after irradiation (only data at 10 mM are reported in this study).

Cells were detached from dishes by trypsinization, suspended in complete medium, centrifuged, washed twice in 1 ml phosphate-buffered saline (PBS), pH 7.4, and resuspended for storage (20°C) in 70% ethanol. Before analysis, fixed cells were washed twice, centrifuged and resuspended in 1 ml of PBS containing $1 \text{ }\mu\text{g}$ RNase and $100 \text{ }\mu\text{g}$ propidium iodide (10). Samples were stored in the dark for 20 min at room temperature before final readings were taken. Cell orange fluorescence of propidium iodide was detected in a linear scale using a CyAn ADP Flow Cytometer (DAKOCytomation, Ely, UK) and analysed using Summit Software. About 30 000 events (that is fluorescence readings, corresponding to no less than 20 000 cells) were recorded for each sample.

Quantitative aspects of reactive oxygen species production

Reactive oxygen species (ROS) were detected using 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA ; Calbiochem, Milan, Italy). H_2DCFDA diffuses into cells where it is converted into a non-fluorescent derivative (H_2DCF) by endogenous esterases. H_2DCF is oxidized to green fluorescent DCF in the presence of intracellular ROS (14). Photofrin-enriched cells were washed and kept at 37°C in serum-free medium alone or with 1 mM DTT.

After 30 min, they were further incubated with H₂DCFDA in serum-free medium. After an additional 30 min, cells were washed in Hank's solution, irradiated as indicated above, detached by trypsinization and resuspended in PBS. Cells were analysed by flow cytometry as described earlier. Although the observation reported refers to H1299 cells, we obtained similar results with A549 cells (not shown).

Western blot analysis

Total cell protein preparations were obtained by lysing cells in 50 mM Tris (pH 7.5), 100 mM NaCl, 1% NP40, 0.1% Triton, 2 mM EDTA, 10 µg/ml aprotinin and 100 µg/ml phenylmethylsulphonyl fluoride. Protein concentration was routinely measured by the Bio-Rad protein assay (15). Polyacrylamide gels (7.5, 10 and 15%) were prepared, essentially as described by Laemmli (16). Molecular weight standards were from New England Biolabs (Beverly, MA, USA). Proteins separated on the polyacrylamide gels were blotted on to nitrocellulose filters (Hybond-C pure; Amersham Italia, Milan, Italy). Filters were washed and stained with specific primary antibodies and then with secondary antiserum conjugated with horseradish peroxidase (Bio-Rad; diluted 1:2000). Filters were developed using an electro-chemiluminescent western blotting detection reagent (Amersham Italia). Profiles were acquired and grossly quantified (OD integration) by scanning using a Discover Pharmacia scanner equipped with Sun Spark Classic Workstation. Anti-BCL-2 (100SC509), anti-p27^{KIP1} (C-19SC528), anti-IκBα (C-15), anti-ubiquitin (N-19), anti-caspase3 (E-8), anti-PARP (H-250) and actin (C-2) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-tubulin (MCA77G) was from Serotec (Kidlington, UK).

Cycloheximide experiments

(a) *Effect of cycloheximide on IκBα expression in H1299 cells*: Cells were plated in 100 mm cell culture dishes; 24 h later, cycloheximide (40 µg/ml) was added to medium and incubated for 1, 2 and 3 h, then cell proteins were extracted and analysed by western blotting.

(b) *Effect of PDT on IκBα expression in H1299 cells incubated with cycloheximide*: Cells were left to stand in the dark for 16 h with the usual concentration of Photofrin. Cycloheximide was added at concentration of 40 µg/ml for 3 h. Finally, cells were washed, released in fresh medium and irradiated. After 1, 2 or 3 h of irradiation, proteins were extracted from plates and processed for immunoblotting using antibodies against IκBα and anti-tubulin for load control.

Determination of proteasome activity

We evaluated proteasome activity using Proteasome-Glo chymotrypsin-like cell-based assay (G8660) from Promega. This is a homogeneous assay that directly measures chymotrypsin-like protease activity associated with the proteasome complex, in cultured cells. It contains a specific proteasome substrate, suc-LLVY-aminoluciferin, that generates a luminescent signal as consequence of its cleavage, generated by esogenous proteasome activity. The lumino-metric assay allows measurements of cytosolic proteasome activity directly in multiwell plates. This is revealed by emission of a stable luminescent signal proportional to it.

The assay was performed in strict accordance to manufacturer's instructions. Ninety-six multiwells containing around 7000 cells, in 100 µl of medium per well, were used in all experiments. Luminescence signals of triplicate samples were detected using a Glomax microplate luminometer (Promega). The well-known proteasome inhibitor Bortezomib was used as positive control.

Individual treatments. (a) *PDT*: A suitable number of H1299 and A549 cells (7×10^3) were incubated in 96-well plates with the photosensitizer under usual conditions, and irradiated with light doses of 0.54 or 1.10 J cm⁻². Proteasome activity was evaluated at 2 and 6 h after irradiation.

Photofrin dark effect: At 2.5 µg/ml of photosensitizer, Photofrin effect on proteasome activity of both cell lines was undetectable.

(b) *Aspirin*: Suitable numbers of H1299 and A549 cells (7×10^3) were incubated in 96-well plates for 24 h with aspirin, at two concentration levels, namely 1.5 and 2.5 (low level) or 5.0 and 10 mM (high level), and then analysed.

(c) *Bortezomib*: (Stock solution 1 µM in DMSO) was diluted in complete medium to obtain final concentrations of 2.5 and 10 nM. Cells were incubated under these conditions for 3 h before the assay.

Combined treatments. Effects of combined treatment were evaluated using lowest aspirin concentrations associated with higher light dose. To this purpose, H1299 cells were incubated with Photofrin (16 h) and irradiated with light dose of 1.10 J cm⁻². As appropriate, cells were exposed to 1.5 and 2.5 mM aspirin for 72 h, washed and analysed.

Statistical analysis

All data are expressed as mean ± SD. Significance was assessed by Student's *t*-test for unpaired data for comparisons between two mean values.

Results and discussion

Effect of PDT on chymotrypsin-like activity of H1299 and A549 cancer cells

The ubiquitin-proteolytic pathway is a major system for selective protein degradation in eukaryotic cells and is activated in response to a number of environmental stresses, including oxidation, thereby playing an essential role in cell function and survival (2). It has been reported by others (9) that Photofrin/PDT treatment of murine colon adenocarcinoma C26 cells causes delayed induction of chymotrypsin-like activity of proteasomes in whole cell lysates. They suggest that combination of PDT with various proteasome inhibitors leads to accumulation of carbonylated proteins in the endoplasmic reticulum (ER), aggravated ER stress and potentiated cytotoxicity to tumour cells. However, mechanisms and kinetics of PDT cytotoxicity remain elusive. These authors reported increased proteasome activity several hours after photosensitization. This was explained as a consequence of cellular adaptive mechanisms aimed to remove oxidatively damaged proteins. However, these authors did not comment on initial decrease in proteasome activity observed, over the same time course experiment, shortly after Photofrin photoactivation. Thus, we decided to investigate more closely effects of PDT measuring residual chymotrypsin-like activity of cells after treatment, and to look at changes in expression of proteins physiologically processed by the proteasome.

Residual chymotrypsin-like activities in our cells were measured 2 and 6 h, after irradiation at two fluences (0.54 and 1.10 J cm^{-2}) of Photofrin-treated ($2.5 \text{ } \mu\text{g/ml}$ – 16 h) H1299 and A549 cells (Fig. 1a). After 2 h and 0.54 J cm^{-2} , decrease in activity was calculated at 60% for H1299 and at 40% for A549 cells. Irradiation at 1.10 J cm^{-2} yielded a much stronger decrease in proteolytic activity, which approached 65–70% in both the cell lines. A remarkable recovery of proteasome function (85–95%) was observed 6 h after irradiation. This finding is similar to that reported by others (9) on murine EMT6 and C-26 cells, using substantially higher Photofrin concentration ($10 \text{ } \mu\text{g/ml}$). Indeed, in our study, at this concentration, Photofrin was found to be very toxic to both H1299 and A549 cells, even in the dark.

Having observed a transient decrease in chymotrypsin-like activity of our cells, we decided to explore the mechanism of stress response induced by sub-lethal Photofrin/PDT in H1299 and A549 cells, analysing expression profiles of BCL-2, I κ B α and p27^{KIP1} proteins, which physiologically undergo proteasomal degradation (2).

Molecular effects of PDT

For 2 h after irradiation, expression levels of BCL-2, I κ B α and p27^{KIP1} proteins first increased and then gradually faded until they reached basal level or lower (Fig. 1b). These qualitative results are quantitatively represented in the lower section of the same panel (OD obtained by densitometry). It is plausible that under our experimental conditions, Photofrin/PDT induced a pause in proteasome activity in both the cell lines. However, inspection of the figure reveals that the effect is far more pronounced in H1299 cells. The same figure indicates that normal expression levels of all proteins were restored ≥ 3 h after irradiation. Shortly after the crippling effect of PDT was exhausted, proteasome activity resumed and appeared even more active than controls, probably as a result of adaptive responses to remove accumulated oxidatively damaged proteins, as suggested by Szokalska *et al.* (9).

Increase in BCL-2 expression that had started immediately after irradiation is in contrast to a report that BCL-2 declines post-irradiation (17); however, this decline has been observed 24 h after photosensitization. The discrepancy could be due to the nature, concentration and intracellular distribution of the photosensitizer, and experimental conditions of PDT (18–20). There are no previous data concerned with the effect of Photofrin/PDT on p27^{KIP1} and I κ B α expression.

To determine whether production of ROS was involved in this transient effect, we incubated cells with ROS-inhibitor DTT (1 mM) before Photofrin/PDT and measured expression levels of BCL-2, p27^{KIP1} and I κ B α at 1, 2, 3 and 6 h after irradiation. In the presence of 1 mM DTT, initial increase in these proteins and their disappearance 3 h after irradiation, no longer occurred (Fig. 1c, left). This finding suggests that ROS are involved in the stall of proteasome activity. We verified that DTT prevents ROS formation by loading cells with 2',7'-dichlorodihydrofluorescein diacetate and then submitting them to photodynamic treatment (Fig. 1c, right).

These observations prompted us to investigate proteasome function in detail in cells after PDT. When cells are extensively damaged or when the external plasma membrane is damaged, they rapidly proceed to necrosis. When damage is less severe, cells may recover and proceed to apoptosis or they may activate autophagic programmes (21). In the case of oxidative stress, intensity and location of the stress determine which of these fates is evoked. In either case, a number of cell proteins is irreversibly denatured as a result of oxidative stress. Intracellular protein denaturation triggers activation of cell defence systems, including ubiquitination of all proteins that are not rescued. As shown in Fig. 2a, PDT affects ubiquitination

Figure 1. (a) Photodynamic therapy determines reversible arrest of proteasome activity. H1299 and A549 cells were incubated with 2.5 $\mu\text{g}/\text{ml}$ Photofrin for 16 h then irradiated with 0.54 J cm^{-2} (low) or 1.10 J cm^{-2} (high) light fluences. Chymotrypsin-like activity was measured at 2 and 6 h after photodynamic treatment. Columns represent mean of three independent experiments. Statistical analysis was performed using unpaired Student's *t*-test: * $P < 0.01$; ** $P < 0.001$ and *** $P < 0.0001$. Values are expressed as percentage of basal (control) chymotrypsin-like activity. (b) Sub-lethal photodynamic treatment does not permanently inhibit proteasome function. Upper panels: Left and middle: H1299 and A549 cells were incubated with 2.5 $\mu\text{g}/\text{ml}$ Photofrin for 16 h then irradiated (0.54 J cm^{-2}). Right: H1299 cells were incubated with Photofrin as above and irradiated at double light fluence (1.10 J cm^{-2}). Proteins were extracted from lysed cells 1, 2 and 3 h after PDT. Expression of BCL-2, $\text{I}\kappa\text{B}\alpha$ and $\text{p}27^{\text{KIP1}}$ proteins was evaluated using western blotting. Nitrocellulose filters were reprobed with anti-actin antibody to compare gel loads. Lower panels: Relative optical density changes were obtained by integrating electrophoretic bands from immunoblots. Data are expressed as relative changes (per cent of control). (c) Effect of dithiothreitol (DTT) on proteasome activity. Left panel: H1299 cells were treated with 1 mM DTT and irradiated as indicated. Cell proteins were extracted and analysed for expression of BCL-2, $\text{p}27^{\text{KIP1}}$ and $\text{I}\kappa\text{B}\alpha$ at 1, 2, 3 and 6 h after PDT. Nitrocellulose filters were reprobed with anti-actin (BCL-2, $\text{p}27^{\text{KIP1}}$) or anti-tubulin ($\text{I}\kappa\text{B}\alpha$), to compare gel loads. Right panel: Reactive oxygen species formation was cytofluorimetrically evaluated in: (1) untreated H1299 cells (controls); (2) irradiated cells (0.54 J cm^{-2}); and (3) irradiated cells (0.54 J cm^{-2}) pre-treated with 1 mM DTT.

profile of proteins extracted from irradiated cells. In particular, ubiquitinated proteins accumulate in the first 2 h after PDT (Fig. 2, lines 3 and 4). In addition, level of ubiquitination of proteins extracted from cells subjected to PDT is similar to that of cells incubated with proteasome inhibitor MG-132 (Cbz-Leu-Leu-Leucinal), a peptide-aldehyde that hampers ubiquitin-mediated proteolysis (Fig. 2a, line 2) (22,23). This finding, along with the observation that PDT does not permanently inhibit proteasome function, suggests that ubiquitination machinery is well preserved, whereas proteolytic action is transiently halted. In fact, proteasome activity starts recovering from ~3 h after PDT, whereas ubiquitination pattern, as shown by gel electrophoresis, reacquires a profile close to that of the control (Fig. 2a, lanes 1 and 5).

Apparently, photodynamic therapy also up-regulates expression of BCL-2, $\text{I}\kappa\text{B}\alpha$ and $\text{p}27^{\text{KIP1}}$ (Fig. 1b) proteins. Steady-state concentration of protein in a cell depends on the protein's relative rates of synthesis and degradation. Thus, under conditions of inhibition of *de novo* protein synthesis, any change in concentration of a given protein should reflect its rate of degradation. When H1299 cells were incubated in presence of cycloheximide (40 $\mu\text{g}/\text{ml}$), concentration of $\text{I}\kappa\text{B}\alpha$ was reduced in a time-dependent fashion (Fig. 2b, left). However, if H1299 cells were exposed to PDT, immediately after incubation with cycloheximide, $\text{I}\kappa\text{B}\alpha$ expression returned to nearly control levels (Fig. 2b, right) strongly suggesting that its degradation had been hampered. It is possible that observed early increase in $\text{I}\kappa\text{B}\alpha$ is due to instantaneous

Figure 2. (a) Photodynamic treatment promotes transient accumulation of ubiquitinated proteins. Ubiquitination electrophoretic profiles of proteins from H1299 cells incubated with Photofrin for 16 h but not irradiated (line 1); incubated in medium containing MG-132 (10 μ M) for 3 h (line 2); photodynamically treated (0.54 J cm⁻²) and extracted 1, 2 and 6 h after irradiation (lines 3, 4 and 5). Filter was reprobed with anti-tubulin antibody to compare protein loads. (b) Photodynamic treatment affects half-life of specific proteins. Left panel: H1299 cells incubated with 40 μ g/ml of cycloheximide (CycloHex) for 0–3 h. After incubation, cells were washed and protein extracts were analysed for I κ B α expression. Right panel: Cells incubated with cycloheximide for 3 h, washed and immediately subjected to PDT (0.54 J cm⁻²). Protein extracts were analysed for I κ B α expression 1, 2 and 3 h after irradiation. Nitrocellulose filter was reprobed with anti-tubulin antibody to compare gel loads. (c) Effect of a second PDT treatment. H1299 cells were irradiated, washed and incubated in a Photofrin-free medium for 2 h. Then cells were subjected to a second photodynamic treatment at the same dose (0.54 J cm⁻²). Expression profile of three key proteins was evaluated by western blotting 1, 2 and 3 h after the second PDT treatment. Filters were reprobed with anti-actin antibody to compare gel loads.

PDT-induced arrest of protein degradation, and to progressive, but not instantaneous, effects (arrest) on protein biosynthesis caused by cycloheximide.

PDT-mediated proteasome inhibition induces transient G₂/M cell cycle arrest

Proteasome inhibitors that cause changes to the cell cycle hold therapeutic promise (24,25). The rationale being that accumulation of proteins whose degradation depends on proteasome activity would result in cell cycle arrest and possibly apoptosis of cancer cells (26). In this study, we report the novel observation that PDT may cause early proteasome malfunction resulting in transient cell cycle arrest. We irradiated H1299 and A549 cells (see Materials and methods section) and performed cytometric analysis 6 and 24 h later. As shown in Table 1, PDT affected the cell cycle profile of both cell lines. Six hours after irradiation, H1299 and A549 cells had accumulated in G₂/M phase. 24 h after irradiation, cell accumulation in G₂/M was reduced and cell distribution resembled that of control cells. MTS assay (>72 h after irradiation) and clonogenic assay (≥ 8 days) showed substantial recovery of cell viability and proliferation activity (not shown). In line with our previous study (27), also protein expression profiles were restored.

As perturbation of proteasome activity, cell viability and proliferation is limited to a short time frame, following PDT, we explored the possibility of prolonging proteasome arrest by physical or/and pharmacological means. In this respect, we have primarily attempted to stabilize the proteasome stall, and make it irreversible, by irradiating cells twice at 0.54 J cm⁻²: at time 0 and 2 h after the first irradiation. On doubly irradiated cells, we evaluated expression profiles of key proteins (BCL-2, I κ B α and p27^{KIP}) by western blotting after 1, 2 and 3 h.

The second irradiation prolonged effects of the first treatment, but proteasome activity was not irreversibly

Table 1. Effect of PDT (0.54 J cm⁻²) on the cell cycle of H1299 and A549 cells

Treatment	Cell cycle phase		
	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)
H1299			
Control	48	32	21
T = 6 h after PDT	40	21	39
T = 24 h after PDT	44	29	27
A549			
Control	56	30	14
T = 6 h after PDT	56	20	24
T = 24 h after PDT	58	25	17

arrested (Fig. 2c). Surviving cells (>25% of the original, 24 h after both irradiations) regained their proliferative capacity, as estimated by colony-forming assay (not shown) similar to cells subjected to single PDT treatment.

We also combined PDT with a drug that could prolong the proteasome stall for enough time for the cell to proceed to apoptotic death. Several possibilities are offered in this regard. For instance, MG132 or Bortezomib have been used by others (9). These drugs pose serious problems as potential therapeutic agents. Indeed, MG132 is notoriously restricted to *in vitro* use because of its toxicity, while chemotherapeutic agent Bortezomib is very expensive and not devoid of important side effects. For example, it cannot be administered to patients with liver disease, infiltrative pulmonary disease or pericardial disease (as suggested by the European Medicines Agency, EMEA note 139443/2008) and induces thrombocytopenia in ~30% of patients.

Dikshit (11) suggested that aspirin could induce apoptosis through inhibition of proteasome function; however, this observation was not direct and has not been confirmed since. If we could prove that aspirin is a suitable molecule, this drug would certainly appear preferable compared to other molecules, especially when used at low concentration.

We are now able to directly prove that aspirin has an effect on the proteasome at concentrations from 10 mM down to 1.5 mM, that is, quite low and considered to be a harmless concentration.

Aspirin at concentrations between 1.5 and 10 mM induced transient proteasome arrest in H1299 and A549 cell lines

To investigate effects of aspirin on H1299 and A549 carcinoma lung cancer cells, we began to analyse effects of high aspirin concentration, 10 mM. This high concentration has been customarily used in *in vitro* studies by several authors [for example, (28,29)].

We evaluated expression levels of BCL-2 and p27^{KIP1} proteins by western blot analysis after 1, 2, 3 and 6 h exposure to 10 mM aspirin. After 1 h incubation with aspirin, expression of both proteins seemed to increase, and to be sustained for up to 3 h, and appeared to fade after 6 h (Fig. 3a, lines 2, 3, 4 and 5). These qualitative results are quantitatively represented in the lower section of the same panel (OD obtained by densitometry). These patterns did not significantly differ from those observed after PDT treatment (see Fig. 1, panel b). Findings depicted in Fig. 3a, clearly imply transient aspirin-mediated inhibition of proteasome activity. This held true for both cell lines.

This suggestive hypothesis of aspirin-mediated proteasome arrest has been fully confirmed by us by exploiting an assay that directly measured chymotrypsin-like activity

in cells. It is interesting that the inhibitory effect of aspirin was observed not only at high and expectedly toxic dose (10 mM) but also at much lower and quite harmless aspirin concentrations. In such regard, we incubated both cell lines with 10 (high), 2.5 and 1.5 mM (unusually low for *in vitro* studies) aspirin concentrations, for 1, 3 and 6 h, and evaluated cell proteasome chymotrypsin-like activity. As shown in Fig. 3b, proteasome activity of H1299 cells was reduced after 1 and especially at 3 h of aspirin treatment. In particular, observed reduction spanned from 40% to 60% at 1.5 mM and from 60% to 70% at 2.5 mM. At 10 mM, inhibition was expectedly much higher (~85%). However, evident recovery of chymotrypsin-like activity 6 h after treatment, even at higher aspirin concentrations (i.e. 10 mM), unequivocally demonstrated that the arrest of proteasome activity was rapid, not irreversible, but transient. Reversibility of this cell response (proteasome arrest) was further demonstrated by observation that cell viability of both cell lines at 24 and 48 h after treatment did not significantly differ from that of respective controls (Fig. 3c,d). As expected, however, viability of cells treated with potentially toxic aspirin concentrations (10 mM) was appreciably abated. Despite such toxicity, however, aspirin even at this high concentration did not appear to induce apoptosis, as indicated by total absence of procaspase activation and lack of PARP cleavage (not shown). Observed transient proteasome block and absence of apoptosis were confirmed by cell cycle profiling. As expected, incubation of both cell lines with 5, 2.5 and 1.5 mM aspirin for 6 h effected a significant accumulation of cells in G₂/M phase, but hallmarks of apoptosis (sub-G₁) were absent (Table 2). Analysis of cell cycle patterns of both the lines after 24 h of aspirin exposure showed profiles quite superimposable on those of controls, in agreement with the hypothesis of transient proteasome block (Table 2). Temporary nature of such a block was also appreciable even at higher aspirin dose (10 mM); thus, release of these cells into aspirin-free medium restored their capacity to proliferate and to form colonies in a short time (not shown).

Dividing cells depend on ubiquitin-mediated protein destruction for cell cycle progression and therefore the proteasome is intimately involved in regulation of progression through the cell cycle from G₂/M into mitosis, through temporal degradation of regulators of this process (30). It appears that in our cells the effect of aspirin at concentrations <10 mM was not steady but only short-lasting, as indicated by recovery of proteasome activity and resumption of normal proliferation once cells were released into drug-free medium.

It must be underlined that recovery of proteasome activity and cells subjected to PDT or treated with aspirin re-entry into the cell cycle, are two phenomena that pro-

Figure 3. (a) Aspirin induces reversible arrest of proteasome activity. Upper panel: H1299 cells treated with 10 mM aspirin for 1, 2, 3 and 6 h. Expression profiles of BCL-2 and p27^{KIP1} obtained by western blot analysis after protein extraction. Filter was reprobbed with anti-tubulin antibody to compare gel loads. Lower panel: Relative optical density changes obtained by integrating electrophoretic bands from immunoblots. Data expressed as relative changes (per cent of control). (b) Aspirin affects chymotrypsin-like activity of H1299 cells in a time- and concentration-dependent manner. Chymotrypsin-like activity of untreated H1299 cells and cells incubated for 1, 3, 6 h with aspirin at three different concentrations (1.5, 2.5 and 10 mM). Experiment described in detail in Materials and methods section. Columns represent mean of three independent experiments. Statistical analysis was performed using unpaired Student's *t*-test: ***P* < 0.001 and ****P* < 0.0001. (c, d) Influence of aspirin on viability of H1299 and A549 cells. Cells (H1299, panel c and A549, panel d) incubated for 24 or 48 h with 1.5, 2.5 and 10 mM aspirin. Cells were then released in aspirin-free medium and analysed by MTS assay 24 h later. Columns represent mean of three independent experiments. Statistical analysis was performed using unpaired Student's *t*-test: ***P* < 0.001 and ****P* < 0.0001.

ceed in accord with each other and can be documented (Figs 1 and 3 and Tables 1 and 2). Most likely, the two effects are not synchronized in a timely fashion in that re-entry into the cell cycle appeared to be a slower process in relation to proteasome activity resumption.

Combined therapy

At present, PDT is a stand-alone therapy for selected tumours (31,32). However, *in vitro* studies have shown that PDT combined with other therapeutic measures might enhance tumour cell death (10). Here, we have compared molecular effects induced by stand-alone treatments (PDT or aspirin) versus combined PDT plus aspirin treatment. At the doses administered, PDT and aspirin were partially but reversibly harmful to cells. Individually neither PDT nor aspirin (at all concentrations tested) were able to induce apoptosis.

To investigate effects of combined treatment on H1299 and A549 carcinoma lung cancer cells, we first

subjected them to sub-lethal PDT treatment (at fluence rates of 0.54 and 1.10 J cm⁻²) and then incubated them with aspirin at high (10 or 5 mM) or low concentrations (2.5 or 1.5 mM) for 24 or 72 h, respectively. It must be underlined that PDT in human therapy is used at fluences much higher than 1.10 J cm⁻² without significant detrimental side effects. In this report, higher aspirin concentrations (≥5 mM) were used to exalt and enlighten drug effects. Such concentration range has been widely used in *in vitro* experimentation as stated earlier, but cannot be used *in vivo*. Lower concentration range (2.5 and 1.5 mM, in particular), in turn has been used to explore the opportunity of potential therapeutic use.

First, we studied expressions of BCL-2 and p27^{KIP1} proteins by western blot analysis in H1299 and A549 cells. Both proteins seemed to rapidly increase their expression after combined treatment suggesting enduring proteasome malfunction (Fig. 4a, lines 1–6). More importantly, superimposable behaviour was observed at higher fluence (1.10 J cm⁻²) and lower drug (aspirin) concentra-

Table 2. Effect of aspirin (0–5 mM) on the cell cycle of H1299 and A549 cells

Aspirin (mM)	Cell cycle phase			
	Incubation time (h)	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)
H1299				
0	0	48	32	21
1.5	6	38	32	30
2.5		34	33	33
5.0		35	32	33
1.5	24	48	29	23
2.5		49	31	21
5.0		47	33	20
A549				
0	0	56	30	14
1.5	6	41	28	31
2.5		39	30	32
5.0		40	30	31
1.5	24	57	21	22
2.5		59	21	20
5.0		54	32	16

tion, 2.5 and even 1.5 mM (Fig. 4a, lines 7 and 8). These qualitative results are also quantitatively represented in the lower section of the same panel (OD obtained by densitometry). To substantiate this hypothesis, we again took advantage of the specific assay that quantitatively evaluates proteasome chymotrypsin-like activity in cells. To this end, we assayed both H1299 and A549 cells that survived individual (PDT or aspirin) or combined (PDT + aspirin) treatments. It appears that treatment of H1299 cells with Photofrin alone (indicated as Ph in the Fig. 4b) did not change proteasome activity compared to that of untreated cells. In contrast, PDT at 1.10 J cm⁻² (but also at lower fluence, 0.54 J cm⁻², not shown) and PDT followed by 2.5 or even 1.5 mM aspirin (combined treatment) all clearly abated this activity (Fig. 4b, upper panel). Bortezomib (2.5) has been included as positive control. It is interesting that Bortezomib induced effects similar to those caused by aspirin and PDT in that it was able to stabilize and favour amassing of BCL-2 (Fig. 4b, lower panel) at concentrations ranging from 2.5 to 10 nM. Effects of aspirin on proteasome activity have already been discussed (see Fig. 3b).

Further characterization by cytofluorimetry revealed that after combined treatments, there was increased PDT-dependent G₂/M accumulation, concomitant depletion of the G₁ phase (specially with 10 mM aspirin, Table 3), statistically significant abatement of colony formation (Fig. 5a,b, upper panels) and more importantly, appearance of measurable sub-G₁ fractions, suggestive of incipient apoptosis. This was observed in both the cell lines.

Under these conditions, expression of uncleaved pro-caspase 3 decreased, in parallel with PARP fragmentation (Fig. 5a,b, lower panels). Although surprising, this finding may not have real impact because of high aspirin concentration. However, we observed that combined therapy may exert similar effects using much lower concentrations of aspirin (as low as 1.5 mM) by administering PDT at higher fluence, 1.10 J cm⁻². This fluence, in stand-alone modality, spares ~30% (10) of cells within 24 h post-irradiation and does not inhibit resumption of proliferation. Combined treatment, in which cell lines were first subjected to PDT (1.10 J cm⁻²) and then incubated with 2.5 or even 1.5 mM aspirin for 72 h, produced effects comparable to those obtained at higher aspirin concentrations and lower light doses (Fig. 5c,d, upper panels). This behaviour is in agreement with findings that expression of both BCL-2 and p27^{KIP1} proteins in H1299 cells, remained sustained (Fig. 4a, lanes 7 and 8). Since the combination of PDT with aspirin, even at the lowest concentration, induces apoptosis in both cell lines, we think that this observation may be attractive for future clinical use.

However, use even of aspirin is not devoid of drawbacks. As efficacy of PDT is mediated through direct vascular effects (33,34), these can be a major contributor to response observed *in vivo*. In this regard, aspirin has the potential to alter the response through its anti-thrombotic properties because it may add unpredictable consequences, whose therapeutic relevance definitely deserves further investigation. Salicylism may be another problem. However, according to the well-known Toxicology handbook of Klaassen (35), serum concentration of aspirin not exceeding ~50 mg/100 ml (~2.8 mM), does not induce salicylism. Indeed, we have now shown that combination of a single relatively low aspirin dose (1.5 mM) with photodynamic treatment that uses at least fluence of 1.10 J cm⁻², induces two effects. Since combination of PDT with Aspirin, even at the lowest concentration, induces apoptosis in both cell lines, we think that this observation may be attractive for future clinical use. Nevertheless, it is clear that use of aspirin in humans remains problematic inasmuch as that it has inhibitory effects on platelet function that would greatly complicate (by risk of bleeding) its use in patients with lung cancer patients undergoing photodynamic treatment. Bearing this in mind, while opening a potential therapeutic window, our observation remains restricted for the time being, to cell *in vitro* systems.

One additional point deserves particular attention is that when we investigated effects of combination applications by inverting the order in which PDT and aspirin were administered, no therapeutic effects were observed, in either H1299 or A549 cell lines. This is clearly demon-

Figure 4. Combined treatments: persistence of proteasome malfunction. (a) Upper panel: Effect of combined treatment on expression of BCL-2 and p27^{KIP1} in H1299 and A549 cells. Extracts obtained from a) non-irradiated H1299 and A549 cells (lines 1 and 2); (b) cells irradiated with a light dose of 0.54 J cm⁻² and incubated with 5 or 10 mM aspirin for 24 h (lines 3 and 4 refer to H1299, lines 5 and 6, to A549); (c) H1299 cells irradiated with light dose of 1.10 J cm⁻² and incubated with 1.5 or 2.5 mM aspirin for 72 h. Lower panel: Relative optical density changes obtained by integrating electrophoretic bands from immunoblots. Data expressed as relative changes (per cent of control). (b) Upper panel. Effect of combined treatment on chymotrypsin-like activity in H1299 cells. From left: (a) untreated cells (control), (b) cells treated with 2.5 nM Bortezomib (used as positive control), (c) cells treated for 16 h with 2.5 µg/ml Photofrin (Ph) but not irradiated (dark effect), (d) cells irradiated using a fluence of 1.10 J cm⁻² and (e) and (f) cells subjected to combined treatment (1.10 J cm⁻² followed by 72 h treatment with 1.5 and 2.5 mM aspirin). Lower panel. Expression profile of BCL-2 evaluated by western blotting after treatment with Bortezomib at 2.5 and 10 nM. Nitrocellulose filter was reprobbed with anti-actin antibody to compare gel load. Statistical analysis performed using unpaired Student's *t*-test: **P* < 0.01; ****P* < 0.0001.

strated by absence of changes in colony-forming assay, in both cell lines (Fig. 5a,b, upper panels, bars 4 and 5). Lack of effect may have several possible explanations. For example, aspirin itself may work as a scavenger for oxygen radicals generated by sensitizer photoactivation (36). Alternatively, as suggested by Podhaisky *et al.* (37), aspirin may exert cytoprotective effects by up-regulating expression of proteins that physiologically counteract oxidative stress. Whichever the correct explanation is, the observation that patients under treatment for choroidal neovascularisation, taking aspirin, required more PDT treatments than patients not taking aspirin (38), is in accord with our *in vitro* observation. In this regard, we

show that combined treatment *in vitro* is therapeutically effective only when a specific sequence of administration is respected.

Photodynamic therapy exerts a directly lethal effect on cells that have taken up a sufficient amount of photosensitizer that when light reaches the cell is appropriate in dose and energy. However, the effect may not be fatal in cells that have not taken up sufficient amounts of photosensitizer or are not reached by enough light. Under our conditions (which are evidently sublethal), PDT inhibited proteasome activity, albeit transiently, and influenced the cell cycle. Aspirin, which confirms its surprisingly wide biological/therapeutic power, exerted a

Table 3. Effect of 10 mM aspirin on the cell cycle of pre-irradiated H1299 and A549 cells

Treatment	Cell cycle phase			
	Sub G ₁ (%)	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)
H1299				
Control	0	48	32	21
Aspirin	1	43	19	37
PDT + Aspirin	4	36	25	35
A549				
Control	0	56	30	14
Aspirin	2	48	22	28
PDT + Aspirin	5	37	25	34

similar effect. This, not only at concentrations that were clearly toxic (salicylism), but even at much lower concentrations than could be considered much less harmful, in view of the fact that only a single administration is apparently necessary. As protracted stall of proteasome activity may trigger apoptosis (38), combination of PDT with aspirin may have therapeutic relevance. It may be envisaged that aspirin induces apoptosis in tumour cells that, being far from the surface of a neoplasm or at the border of the light beam, are not sufficiently photosensitized and elude direct effect of PDT. This is important because transformed cells are believed to be more sensitive to proteasome inhibitor-mediated apoptosis than

Figure 5. Combined treatments: clonogenic survival and induction of apoptosis. Panels a

and b: Effects of high aspirin concentration and low light fluence. Upper graphs: H1299 (left) and A549 (right) incubated for 24 h with 5 or 10 mM aspirin and then irradiated (aspirin + PDT), or first subjected to PDT then incubated with 5 or 10 mM aspirin for 24 h (PDT + aspirin). After treatments, cells were washed and incubated in fresh complete drug-free medium. Colonies were stained with methylene blue ≥ 8 days later. Columns represent means of three independent experiments. Statistical analysis performed using unpaired Student's *t*-test: ***P* < 0.001; ****P* < 0.0001. Colonies are expressed as per cent of untreated cells (dashed line). Lower panels: expression of pro-caspase 3 and PARP in cells irradiated at 0.54 J cm⁻² then incubated for 24 h with aspirin (5 and 10 mM). Nitrocellulose filter was reprobed with anti-tubulin antibody to compare gel load. Panels c and d: Effects of low aspirin concentrations using higher light fluence. Upper graphs: H1299 (left) and A549 (right) cells irradiated with higher light fluence (1.10 J cm⁻²) washed and incubated for 72 h with 1.5 or 2.5 mM aspirin. Colonies stained with methylene blue ≥ 8 days later. Columns represent means of three independent experiments. Statistical analysis performed using unpaired Student's *t*-test: ****P* < 0.0001. Colonies expressed as per cent of untreated cells (dashed line). Lower panels: expression of PARP in cells irradiated at 1.10 J cm⁻² and then incubated for 24 h with aspirin (1.5 and 2.5 mM). Nitrocellulose filter reprobed with anti-tubulin antibody to compare gel load.

non-transformed cells (39) and this may have significant implications in cancer therapy.

The possibility of prolonging inhibition of proteasome activity using combination treatment, which does not cause or at least reduce severe adverse side effects, may be an additional strategy to enhance death of apoptosis-resistant cancer cells, thus reinforcing the concept that PDT, especially in combination, may be a practicable cancer therapy. Aspirin and Bortezomib may or may not be the best drugs to this purpose, but the principle appears to be valid.

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