Photodynamic therapy with 5-aminolaevulinic acid and DNA damage: unravelling roles of p53 and ABCG2

I. Postiglione* | F. Barra* | S. M. Aloj | G. Palumbo

Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Naples, Italy

Correspondence

G. Palumbo, Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Naples, Italy.
Email: palumbo@unina.it

Abstract

Objectives: In spite of high sensitivity of A549 cells (p53^{+/+}) to lethal effects of photodynamic therapy with 5-aminolaevulinic acid (5-ALA/PDT), DNA damage was observed only in H1299 cells (p53^{-/-}), suggesting that p53 may exert a protective effect. Studies on human colon adenocarcinoma cell lines HCT-116, and their cognate knockouts for p53, were not entirely consistent with the assumption above. Exploring alternative explanations for such conflicting behaviour, we observed that expression of the ATP-binding cassette G2 (ABCG2), a regulator of cell component efflux, had important effects on PDT-generated DNA injury in PC3 cells (prostate) which are p53^{-/-} and positive for ABCG2. Addition of an ABCG2 inhibitor in ABCG2 positive A549 (p53^{+/+}) and PC3 (p53^{-/-}) cells eliminated resistance to DNA damage.

Materials and methods: All cell lines investigated were incubated with 5-ALA and irradiated. Effects of PDT were evaluated assessing residual cell viability, cell-cycle profiles, PplX localization, comet assay and Western blotting. Identical measurements were made in the presence of ABCG2 inhibitor, in cells expressing the transporter.

Results: Our data show that cell aptitude to defend its DNA from PDT-induced injury was mainly ruled by ABCG2 expression. These findings, while providing helpful information in predicting effectiveness of 5-ALA/PDT, may indicate a way to shift PDT from a palliative to a more effective approach in anti-cancer therapy.

1 | INTRODUCTION

Photodynamic therapy exploits the properties of compounds, introduced into cells as such or metabolically produced by the cells from precursors, to become cytotoxic when exposed to light of proper wavelength. There is a general consensus that the cytotoxic effect observed after a photodynamic treatment finds its origin in the generation of ROS, such as singlet oxygen and other free radicals, upon light activation of the photosensitizer.¹ Activation may give rise to two types of reactions referred to as types I and II. In type I photoreaction, the excited photosensitizer transfers one electron to a substrate causing the formation of radical species (radical or ion-radical), which, in the presence of oxygen, yield reactive oxygenated products. Alternatively, the direct transfer of the extra electron to oxygen generates a superoxide radical anion. In the type II reaction, the excited sensitizer may

form excited state singlet oxygen (¹O₂), by transferring its excess energy to ground-state molecular oxygen. Singlet oxygen, then, reacts with the substrate to generate oxidized products. Interestingly, the photosensitizer is not destroyed through this process.^{2,3}

Considering the short life and the limited diffusion of oxygen radicals from the site of their formation, the effects of PDT occur primarily at the site of intracellular localization of the photosensitizer; thus, they depend on its intracellular distribution.² Although photosensitizers accumulate almost everywhere within the cell, mitochondria and endoplasmic reticulum appear to be their preferential targets.^{4,5} The affinity of a photosensitizer for a specific cellular compartment depends on their physicochemical nature and specific cell/tissue⁴; therefore, also the nucleus can be target of reactive oxygen species^{6,7}; nevertheless, studies of nuclear involvement in PDT have been limited^{7,8} with only a few observations reporting PDT-associated DNA injury.^{9–13} It has been reported that photo activation of a porphyrin-derivative caused direct DNA damage,¹⁴ as well as production of 8-oxo-Guanine, a typical product of

*These two authors contributed equally.

DNA oxidative damage.¹⁵ To date, the extent of nuclear damage, the circumstances in which it may occur, and the possible ways to predict and control its effects for therapeutic purposes remain to be established.

A better understanding of the extent DNA damage caused by PDT is important in qualifying its use as anti-cancer therapeutic approach. In this regard, a targeted delivery of photosensitizers to the nucleus should be seen as a powerful way to potentiate the effectiveness of PDT as tumour-cell killing strategy.⁷

In this study, we used δ -aminolaevulinic acid (5-ALA), a naturally occurring intermediate in haem biosynthesis that is largely converted within cells into protoporphyrin IX (PpIX), a powerful photosensitizer.^{16,17} There are several advantages in using 5-ALA for PDT: first, porphyrin metabolism is strongly accelerated in tumours^{18–22}; second, PpIX is cleared from the body within ~48 hours subsequent to systemic 5-ALA administration; third, 5-ALA is far less toxic than photosensitizers that are active per se without requiring metabolic transformation.

A set of five cell lines of human origin have been selected as experimental model, including two lung adenocarcinoma cell lines, namely H1299 and A549, two versions of the same colon adenocarcinoma cell line HCT-116, that differ for p53 expression (p53^{+/+} and p53^{-/-})²³ and a prostate adenocarcinoma cell line, PC3. These cell lines display different level of expression of two key proteins, which appear relevant in steering cellular response to PDT: p53 and the transporter ABCG2. Indeed, it is well established that p53, as the main stress sensor,²⁴ plays a central role in cellular defence against DNA damage, whereas ABCG2, a regulator of cellular efflux of specific substrates, plays a key role in clearing the cell from obnoxious substances.

ABCG2 is a member of G-subfamily of human ATP-binding cassette transporters, originally named breast cancer resistant protein (BCRP).²⁵ Overexpression of ABCG2 in cell lines confers resistance to a variety of chemotherapeutic drugs, suggesting a role for ABCG2 expression in cancer cells as a mechanism of resistance to chemotherapy.^{25–28}

ABCG2 has been proposed to be a strong determinant of the effectiveness of PDT^{29,30} because PpIX is a recognized substrate for the transporter, whose overexpression induces decreased intracellular PpIX fluorescence associated with reduced response to PDT.^{31,32}

This work dwells on the ability of 5-ALA/PDT to target DNA on a panel of five cell lines characterized by different combinations of p53 and ABCG2 expression, as summarized in Table 1. We tried to identify the molecular effectors involved in DNA damage to better circumstantiate the conditions in which a photodynamic treatment may exert its cytotoxic effect also at nuclear level.

TABLE 1 The cell lines used in this study and the relative status of p53 and ABCG2 expression.

Cell lines	p53	ABCG2
H1299	Negative	Negative
A549	Positive	Positive
HCT116 (+/+)	Positive	Negative
HCT116 (-/-)	Negative	Negative
PC3	Negative	Positive

No special attention has been paid to mechanisms of cell death triggered by 5-ALA/PDT, since this issue, has been discussed in some details in a previous study from this laboratory.³³

2 | MATERIALS AND METHODS

2.1 | Cell cultures

Non-small-cell-lung-cancer cell lines H1299 (p53^{-/-}) and A549 (p53^{+/+}) were obtained from ATCC (Rockville, MD, USA). H1299s were cultured in RPMI 1640 medium, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 2.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% FCS and 1% penicillin/streptomycin; A549s were cultured in F12K medium, 0.75 g/L sodium bicarbonate, 2 mmol/L L-glutamine, 10% FCS and 1% penicillin/streptomycin.

The colorectal cancer cells HCT-116 were kindly provided by B. Vogelstein²³ and cultured according to ATCC recommendation in complete McCoy's 5A medium, consisting of 10% foetal bovine serum, 1% penicillin/streptomycin and 2 mmol/L L-glutamine. The two lines are p53^{+/+} and p53^{-/-} (the latter obtained by knocking out the p53 gene). The prostatic adenocarcinoma cell line PC3 was obtained from ATCC and cultured in RPMI, 10% FCS, 2 mmol/L L-glutamine and 1% penicillin/streptomycin. Saline solutions, media and serum were all obtained from Gibco (Invitrogen, Waltham MA, USA).

2.2 | Reagents

5-aminolaevulinic acid (Sigma Aldrich, St Louis, MO, USA) stock solution (300 mmol/L) was dissolved in water and stored at -20°C. The ABCG2 inhibitor Ko143^{34,35} (Sigma Aldrich) stock solution (5 mmol/L) was prepared by solving the powder in DMSO and stored at -20°C.

2.3 | PDT treatment

All photodynamic treatments performed throughout this work were carried out on cells as previously described by us and others.^{33,36} All cells were incubated in the dark for 3 h in serum-free medium in the presence of an excess of 5-ALA (0.5 or 1.0 mmol/L). After incubation, cells were washed three times with Hank's solution (Sigma Aldrich) and then exposed to light. In particular, PDT was administered to cells 72 (H1299 and A549) or 48 hours (HCT-116 (+/+ and -/-) and PC3) after seeding. Cells were irradiated using a LED array (S-630; Alpha Strumenti, Melzo, Italy) designed specifically for photodynamic therapy. The red-light source was placed at a distance to ensure uniform illumination of the entire cell monolayer. Light fluences used ranged from 0 to 20 Jcm⁻². Before further analyses, irradiated cells were released into drug-free complete medium and kept in the incubator as required.

2.4 | Protoporphyrin IX measurements

Determination of protoporphyrin IX production was performed according to Wild et al.³⁷ For this purpose, 1.0×10⁵ H1299 or A549 cells, 3.0×10⁵ HCT-116 (+/+ and -/-) cells, 3.5×10⁵ PC3 cells were seeded

in 60 mm tissue culture dishes. All steps of these protocols were performed in the dark to avoid photo-bleaching of protoporphyrin IX. Intracellular protoporphyrin IX was measured in cells detached by mild trypsinization, washed in PBS, and collected by centrifugation (1200 rpm, 5 min). Cells were resuspended in 1:1 methanol (HPLC grade): perchloric acid (0.9 M) and homogenized using a homogenizer (Ultra-Turrax T25-IKA Germany). The suspension was then centrifuged at maximum speed and supernatants quickly transferred into quartz cuvette for the measurement of fluorescence spectra, between 550 nm and 700 nm, in a Perkin-Elmer spectrofluorometer with the excitation wavelength at 406 nm and the excitation and emission slits at 10 nm.

2.5 | Cell viability and trypan blue assay

All cells were seeded in 35 mm tissue culture dishes (A549 and H1229, $\sim 5.0 \times 10^4$ and HCT-116 and PC3, $\sim 1.2 \times 10^5$) in triplicate and exposed to PDT as indicated above. The different initial cell density was chosen because of the different growth rate of the individual cell lines. In all cases, cell viability was evaluated by trypan blue assay (Sigma Aldrich, diluted 1:10 in a trypsinized cell suspensions) 24 hours after PDT. Residual viability was expressed as percentage (mean $\pm$ SD) of trypan blue-negative cells versus untreated controls. Incubation of cells with either 1.0 or 0.5 mmol/L 5-ALA did not cause significant changes in cell viability and light fluence dose-response to PDT. This suggested that both concentrations were in excess with respect to the cells ability to produce sufficient PpIX (data not shown). However, since 1 mmol/L 5-ALA is slightly toxic to PC3 cells, even in the dark, the experiments with PC3 cells, reported herein, were performed with 0.5 mmol/L 5-ALA only.

Assessment of cell viability was carried out using dose-response curves at increasing light fluences for each cell line. Eventually, light fluences for subsequent experiments were chosen such to determine $\sim 25\%$ or $\sim 50\%$ cell death in each cell line. These fluences were 6 and 12 Jcm $^{-2}$ for H1229, 4 and 8 Jcm $^{-2}$ for A549, 7.5 and 15 Jcm $^{-2}$ for HCT-116 $^{+/+}$, 10 and 20 Jcm $^{-2}$ for HCT-116 $^{-/-}$, and 5 and 10 Jcm $^{-2}$ for PC3 cells, respectively.

2.6 | Cell-cycle analysis

Cells (1.0×10^5 H1229 and A549, 3.0×10^5 HCT-116 and 3.5×10^5 PC3) were seeded in 60 mm tissue culture dishes in triplicate and exposed to PDT as detailed above. As stated before, the two light fluences used for each cell line were chosen to generate either $\sim 25\%$ or $\sim 50\%$ dead cells. Six hours after PDT, cells were detached by trypsinization, washed in PBS and fixed in 70% ethanol. Before analysis, cells were washed in PBS, resuspended in a PBS solution containing RNase (Roche, Milan, Italy) and propidium iodide (Sigma Aldrich) and stored in the dark for 20 min at RT. Fluorescence was detected using the 488 nm laser line with a CyAn ADP Flow Cytometer (DAKO Cytomation, Milan, Italy). Not less than 20 000 events (≥ 15 000 cells) were recorded for each sample. Cell-cycle profiles were analysed using ModFit/LT 3.2 version (Verity Software, Topsham, ME, USA). Data were obtained from three independent experiments (triplicate samples) and expressed as mean $\pm$ SD.

2.7 | Alkaline comet assay

Cells were dealt exactly as for cell-cycle analysis (see above). Six hours after PDT, cells were detached by trypsinization, washed once with PBS, centrifuged and resuspended in PBS (1.0×10^5 cells/mL). Alkaline comet assay was performed according to the instructions provided by the kit manufacturer (Trevigen, Helgerman, CT, USA). Cells were combined with molten agarose (at 37°C) and pipetted onto Comet Slide™. Slides were incubated at 4°C for 20 min in the dark for the agarose to gel and adhere to the slide. Slides were then immersed in pre-chilled lysis solution for 45 min and subsequently in alkaline unwinding solution for 45 min in the dark. Slides were transferred in a horizontal electrophoresis apparatus (30 min, 1 V/cm, 300 mA), covered with alkaline electrophoresis solution, washed twice with H $_2$ O, incubated in ethanol for 5 min, air-dried, stained with SYBR™ Green and analysed by fluorescence microscopy. For each sample, more than 100 cells were collected. Only undamaged DNA retains round shape, while damaged DNA migrates away from the nucleus, depending on the molecular weight of its fragments, acquiring the characteristic comet shape. Cell images were analysed using Comet Score™ (TriTek, Annandale, VA, USA) to quantify DNA damage through measurements of the comet tail moment, Olive moment and % DNA tail.³⁸ The fluorescence (arbitrary units) of tail moment, olive moment, and % DNA tail have been reported as function of applied light fluences (mean $\pm$ SD).

2.8 | Histone γ -H2AX phosphorylation assay

Cells, dealt with as for cell-cycle analysis (see above), were washed (three times) with a colourless Hank's solution (Sigma Aldrich), irradiated and released in fresh complete culture medium for 6 hours, detached with trypsin, centrifuged, washed with PBS (twice) and resuspended in 70% ethanol. To detect γ -H2AX, cells were incubated with anti- γ -H2AX (ser139) monoclonal antibody (Upstate) in 4% FBS TBS-T 0.1% for 2 hours at RT. Cells were washed with TBS-T 0.1% and incubated with a fluorescein-tagged anti-mouse secondary antibody (Sigma Aldrich) in 4% FBS TBS-T 0.1% for 1 hours at RT in the dark. After washing with TBS-T 0.1%, cells were resuspended in TBS and then analysed in a CyAn ADP Flow Cytometer (DAKO Cytomation) equipped with an excitation laser line at 488 nm. The data collected were analysed using the Dako Summit Software version 4.3. The observed shift of the fluorescence signal (referred to the curve mean) was referred to the position in the untreated control and expressed as percent (mean $\pm$ SD). About 20 000 events (i.e. fluorescence readings, corresponding to not less than 15 000 cells) were recorded for each sample (triplicates).

2.9 | ABCG2 inhibition

ABCG2 protein inhibition was evaluated treating cells with either 1.5 or 2.0 μ mol/L Ko143.³⁴ The inhibitor was added to the culture medium together with 5-ALA standard incubation time (3 hours) in serum-free medium. Subsequently, cells were washed, exposed to light and resuspended in complete culture medium containing the appropriate concentration of Ko143 until analysis.

2.10 | PpIX intracellular localization

H1299 (1.0×10^5) and PC3 (3.5×10^5) cells were grown onto glass coverslips, allowed to adhere and then exposed to PDT (in the absence or in the presence of $2 \mu\text{mol/L}$ Ko143); fluences used were 6 and 12 Jcm^{-2} for H1299 and PC3 cells, respectively. After irradiation cells were washed with PBS and fixed for PpIX analysis immediately or released in fresh-culture medium for analysis at 6 hours.

Fixation was performed in PBS/4% formaldehyde for 10 minutes in the dark. After further washes with PBS, samples were mounted and viewed with an Axioplan fluorescence microscope (Zeiss).

2.11 | Western blot analysis

Appropriate quantities of protein extracts were obtained from the following cell numbers: 1.0×10^5 H1299 or A549; 3.0×10^5 HCT-116 or PC3; exposed to the following fluences: 6 and 12 Jcm^{-2} for H1299; 4 and 8 Jcm^{-2} for A549; 10 and 20 Jcm^{-2} for HCT-116^{-/-}; 7.5 and 15 Jcm^{-2} for HCT-116^{+/+}; and 5 and 10 Jcm^{-2} for PC3 cells.

Cells were harvested 0.5, 1, 3 and 6 hours after PDT (additional details are in the figure legends). Cellular pellets were suspended in lysis buffer; the proteins extracted were measured as previously reported.^{39,40} Polyacrylamide gels were prepared as described by Laemmli.⁴¹ Proteins separated on polyacrylamide gels were blotted on to nitrocellulose filters (Perkin Elmer). Molecular weight standards were from Fermentas Life Sciences (M-Medical, Milan, Italy). Filters were washed in TBS-T 0.1%, soaked in 5% non-fat dry milk in TBS-T 0.1%, incubated with specific primary antibodies and then with secondary antibodies (Santa Cruz, Dallas, TX, USA) conjugated to horseradish peroxidase (BioRad, Segrate (MI), Italy). The primary antibody against ABCG2 was from Santa Cruz catalogue no sc-58224; anti-tubulin was provided by ABM (Richmond, BC, Canada). Finally, filters were developed using an electro-chemiluminescent Western blotting detection reagent (Roche).

2.12 | Statistical analysis

Significance was assessed using unpaired Student's *t* test for comparison between two means (* $P < .05$; ** $P < .01$; *** $P < .001$).

3 | RESULTS

3.1 | 5-ALA/PDT affects cell viability

We wanted to find out whether p53 had a role in protecting cells from the photodynamic effect brought about by light activation of PpIX. Accordingly, cell lines expressing p53 (A549 and HCT-116) were compared to cells constitutively lacking that protein (H1299 and PC3) or p53 knockouts (HCT-116^{-/-}). All cell lines studied, independent of p53 expression, take up exogenous 5-ALA. The precursor is metabolically converted to the actual photosensitizer PpIX, as indicated by the characteristic fluorescence spectra of cell extracts, obtained as detailed in Methods, which reveal the two maxima (at 604 nm and 670 nm) typical of the photosensitizer (Fig. 1, insets).

To evaluate cell viability, cells were incubated with 5-ALA in serum-free medium for 3 hours and then irradiated at increasing light fluences. Trypan blue assay was performed 24 hours after the cells were released into drug-free complete medium. Viability was reduced in all cell lines tested and was light-dose dependent (Fig. 1). With the exception of A549 cells (p53^{+/+}), which are more sensitive to PDT, response is remarkably similar in the other cell lines, regardless of p53 expression. As indicated in Methods, subsequent experiments were performed using only two light fluences for each cell line. These light fluences were chosen such that they would induce ~25% or ~50% cell death, and they were as follows: 6 and 12 Jcm^{-2} for H1299, 4 and 8 Jcm^{-2} for A549, 7.5 and 15 Jcm^{-2} for HCT-116^{+/+}, 10 and 20 Jcm^{-2} for HCT-116^{-/-}, 5 and 10 Jcm^{-2} for PC3 cells.

3.2 | 5-ALA/PDT causes cell-cycle alterations

The effects of 5-ALA/PDT on the cell cycle were evaluated in all cell lines treated as detailed above. Cell-cycle profiles were analysed by cytofluorimetry 6 hours after PDT.

In H1299 cells, 5-ALA/PDT determines a significant shift of cells from G₁ to the S phase. Indeed, it appears that cells escape G₁, enter the S phase and stop cycling. The fraction of cells in the S-phase increases with increasing light fluences. Such increases are statistically significant (Fig. 2, first row).

Equally significant cell population shifts, albeit more complex since also a G₂-phase accumulation was also observed at 10 Jcm^{-2} (Fig. 2, 3rd and 4th rows), occurred in HCT-116 cells, although significantly smaller in HCT-116 p53^{+/+} at 7.5 and 15 Jcm^{-2} (Fig. 2, 4th row).

In contrast, no G₁/S-phase shifts after 5-ALA/PDT were detected in A549 and in PC3 cells (Fig. 2, 2nd, 4th and 5th rows).

G₁ progression to S phase is a major checkpoint for proliferating cells. Low levels of p21 are required for cyclin/Cdk complexes to be active, while high levels of p21 block Cdk activity and cell-cycle progression.⁴²⁻⁴⁴ Indeed, expression levels of p21 after PDT in A549, HCT-116 p53^{+/+} and PC3 cells, whose cell-cycle profiles are modestly affected by PDT, remained completely unchanged. In turn, in H1299 and HCT-116 p53 knockouts, cells that presented the largest S-phase accumulation after PDT, a barely detectable increase of p21 protein levels, were noticed (data not shown).

Since hydrophobic PpIX is synthesized in mitochondria and relocated in the cytoplasm,⁴⁵ it is conceivable that in p53-positive A549 and in p53-negative PC3 cells, the photosensitizer does not reach the nuclear compartment; thus, cell cycle and p21 remain unaffected, whereas it does in H1299 and in HCT-116 cells.

3.3 | Cell-cycle impairment correlates with γ -H2AX histone activation

We have previously shown that one of the major targets of 5-ALA/PDT is the proteasome,³⁹ i.e. the main organelle involved in intracellular protein degradation and turnover.⁴⁶ Since cyclins are proteasome substrates, the changes of cell-cycle distribution observed could be

FIGURE 1 Residual cells viability and PpIX production. Cells survival after PDT of A549, HCT-116^{+/+}, H1299, HCT-116^{-/-} and PC3 are expressed as percentage of untreated controls, with increasing light fluences. Cells viability was assessed by Trypan blue assay 24 hours after PDT treatment: cells were incubated in the presence of an excess of 5-ALA (0.5–1 mmol/L). Inserts depict the PpIX fluorescence spectra in cell extracts. Cells survival is expressed as percentage of untreated control, as function of light fluence. Each point is the average of triplicate measurements (mean±SD). Statistical significance (relative to control), **P*<.5, ***P*<.01, ****P*<.001

caused by proteasome impairment. However, they might as well be a consequence of DNA damage after PDT.

To investigate this possibility, we studied PDT-induced activation of γ -H2AX histone (a sensitive indicator of both DNA damage and DNA replication stress).^{47,48}

To this purpose cells, were treated and irradiated as above and subsequently (6 hours) analysed by cytofluorimetry, in order to detect the levels of phosphorylation on serine 139 (see Methods). It must be underlined that 5-ALA, *per se*, did not interfere with γ -H2AX

histone activation in all cell lines studied. A significant increase of histone γ -H2AX phosphorylation was observed in H1299 after irradiation (Fig. 3), as indicated by the shift of the fluorescence peak. Such shift was dependent on light fluence, suggesting a direct correlation with the extent of PDT-induced stress. At variance with the above, the phosphorylation level of γ -H2AX histone in A549 cells remained unchanged (Fig. 3). The same experiment was performed at shorter interval after irradiation (2 hours) to eliminate the possibility that DNA damage could be avoided by a prompt p53 intervention.

FIGURE 2 Cells cycle profiles. Cytofluorimetric assays were performed 6 hours after PDT (conditions as in Fig. 1). Representative cells cycle profiles are reported. Profiles were obtained using the ModFit software (Verity software House). Cell counts are reported as function of PI area. The numerical data under the various profiles are the averages of triplicate experiments (mean $\pm$ SD). Statistical significance (relative to control), * $P < .5$, ** $P < .01$, *** $P < .001$

FIGURE 3 γ -H2AX phosphorylation. Cytofluorometric evaluation of γ -H2AX phosphorylation on serine 139 at 6 hours after PDT (conditions as in Fig. 1); cell lines were grouped according to the presence/absence of p53. Phosphorylation levels are expressed as percentage of untreated control, as function of light fluence. Each bar represents the average of triplicate measures (fluorescence mean $\pm$ SD). Statistical significance (relative to control), * $P < .5$, ** $P < .01$, *** $P < .001$

However, also in the latter experimental conditions, no fluorescent shift was detected (data not shown), confirming the absence of nuclear involvement.

The most attractive hypothesis to explain the different cellular response of A549 and H1299 cell lines to ALA-PDT was that the different status of p53 protein, expressed only in A549, underlies opposite behaviours. It made sense because p53 is the genome guardian and works as the main stress sensor, and it immediately recognizes any cellular damage and then drives cell fate to death if the damage cannot be repaired, preventing (in our case) that damage reaches nucleus and involves DNA. However, further investigation proved the hypothesis unfounded. Indeed, to explore the problem, we subjected to 5-ALA/PDT two types of the human colon adenocarcinoma cells HCT-116, namely wild type (HCT-116 p53^{+/+}) and knockouts for p53 (HCT-116 p53^{-/-}).¹⁶ We could not detect significant differences in that both cell lines showed a light fluence-dependent increase in histone γ -H2AX phosphorylation levels (Fig. 3). Moreover, the observation that the status of p53 expression does not bear any major relation to DNA damage was also supported by the behaviour of the p53-negative PC3 cells, in which no major changes of γ -H2AX histone phosphorylation could be observed when exposed to PDT (Fig. 3). Thus, p53 expression does not appear to be sufficient to shield nuclear involvement and DNA damage from PDT. Indeed, HCT-116 cells and HCT-116 knockouts for p53 display nearly equal levels of damage after PDT.

3.4 | 5-ALA/PDT induces DNA damage in H1299 and HCT-116 cell lines

The occurrence of DNA damage in cell lines exposed to PDT was explored also by alkaline comet assay performed 6 hours after PDT, applying the same experimental conditions used for the evaluation of γ -H2AX histone phosphorylation. Once it became clear that exposure to PDT causes DNA damage, as shown by γ -H2AX histone activation, it became mandatory to confirm DNA injury by a different method. The single-cell gel electrophoresis, otherwise known as "the comet assay" is regarded as a sensitive, reliable method to assess DNA damage. We opted to use this method to evaluate, in greater details, the extent of DNA injury in the different cell lines used in this study. Without going into greater details, in the comet assay DNA stripped of proteins is a super coiled structure, which resists electrophoresis if it is not perturbed. Breaks in DNA loops relax the super coils, which are free to migrate. DNA fragments become "tails" to the nucleoid "heads"; hence, the formation of a "comet," whereby the proportion of DNA that moves into the tail reflects the extent of DNA breaks. Quantization of the comet assay is based on three parameters: "tail moment," "% DNA tail mean," and "olive moment mean." The "tail moment" is an index representative of both DNA migration and the relative amount of DNA in the tail. The % of DNA tail is related to the ratio between the fluorescence intensity of the head and tail (from

FIGURE 4 Comet Assay on p53-negative cell lines. Comets images collected at 6 hours after PDT (conditions as in Fig. 1) through COMET Score™ software (TriTek Corp.) for H1299, HCT116^{-/-} and PC3 cells (panels a, e and i respectively). Percentage of DNA tail (a. u.) as function of light fluence for H1299, HCT116^{-/-} and PC3 cells (panels b, f and j respectively). Tail moment (a. u.) as function of light fluence for H1299, HCT116^{-/-} and PC3 cells (panels c, g and k respectively). Olive moment (a. u.) as function of light fluence for H1299, HCT116^{-/-} and PC3 cells (panels d, h and l respectively). Each point represents the fluorescence of >100 cells (mean±SD). Statistical significance (in respect to control), *P<.5, **P<.01, ***P<.001

FIGURE 5 Comet Assay on p53-positive cell lines. Comets images collected at 6 hours after PDT (conditions as in Fig. 1) through COMET Score™ software (TriTek Corp.) for A549 and HCT116^{+/+} cells (panels a and e respectively). Percentage of DNA tail (a. u.) as function of light fluence for A549 and HCT116^{+/+} cells (panels b and f respectively). Tail moment (a. u.) as function of light fluence for A549 and HCT116^{+/+} cells (panels c and g respectively). Olive moment (a. u.) as function of light fluence for A549 and HCT116^{+/+} cells (panels d and h respectively). Each point represents the fluorescence of >100 cells (mean±SD). Statistical significance (in respect to control), **P*<.5, ***P*<.01, ****P*<.001

0 to 100%). The “olive tail moment” is the product of the tail length multiplied the % tail DNA.³⁸

All cell lines were subjected to PDT at the light fluences used in other experiments and analysed 6 hours later, like for histone γ-H2AX activation. In Fig. 4, the results obtained from p53-negative cell lines, namely H1299 HCT-116^{-/-} and PC3, are reported, whereas in Fig. 5, the results obtained from p53-positive cell lines, namely A549 and HCT-116^{+/+}, are reported.

An increase of comets number and size can be observed in H1299 and HCT-116 cell lines. The extent of DNA damage was qualitatively estimated by the appearance of comets and by the increase of the % DNA tail moment and olive moment. Different results were obtained

when the assays were performed in A549 and PC3 cells; in fact, in these cells, PDT did not induce any appreciable increase of comets number and size, nor did it alter nuclear morphology.

3.5 | DNA damage correlates with the absence of ABCG2 transporter

Recently, evidence has accumulated indicating that the ATP-binding cassette (ABC) transporter ABCG2 has a role also in regulating cellular accumulation of porphyrin derivatives in a variety of cancer cells.⁴⁹ This finding has led to the contention that the transporter could affect cellular response to photodynamic therapy. Indeed, human ABCG2

FIGURE 6 Expression profiles of ABCG2. All cell lines have been grouped according to the presence/absence of p53 and ABCG2. Western blot analyses were performed at 0.5, 1, 3 and 6 hours after PDT. Tubulin or actin were always used as loading control

was originally found in doxorubicin-resistant breast cancer cells²⁵ named breast cancer resistant protein. The same transporter has been found also in other human tissues, thus generating a great deal of interest as a target to prevent drug resistance in human cancer.

We have investigated the level of expression of ABCG2 in the lines used in this study, before and after incubation with 5-ALA, and at different intervals following PDT (i.e. at 0.5, 1, 3 and 6 hours) at the two light fluences used in the experiments described above. Figure 6 shows that the amount of ABCG2 produced by each cell lines varies quite significantly. Thus, H1299 and HCT-116 (p53^{+/+} and p53^{-/-}) produce modest amounts of ABCG2 protein (Fig. 6a,c), independent of the light fluence used for PDT and time elapsed after treatment,^{50–52} whereas A549 and PC3 cells exhibited a much greater production of ABCG2 in response to PDT (Fig. 6b,d), as previously reported.^{53–55}

Analysis of γ -H2AX histone activation data (Fig. 3) and of comet assay data (Figs. 4 and 5), in the light of the data on ABCG2 expression of Fig. 6, suggests an inverse relationship between the transporter production and DNA damage. Thus, DNA of A549 and PC3 cells, that produce greater amounts of ABCG2 after PDT, appears to be the least damaged by PDT.

3.6 | PpIX reaches nuclear compartment in the absence of ABCG2

For PpIX to cause DNA injury, it must reach the nuclear compartment and stay there at the time of irradiation. The absence of ABCG2

protein is associated with nuclear involvement, and in particular with DNA damage. It is conceivable that the extrusion systems, including ABCG2, when active, come into play and clear the cells of substances that have reached abnormally high concentration.

We have exploited the intrinsic fluorescence of PpIX and followed its distribution throughout the cell compartments after PDT, at light fluences known to be partially cytotoxic, to test this hypothesis. Figure 7 refers to H1299 and PC3 cells. These cells were chosen because they are both p53 negative (p53^{-/-}) but differ in a condition relevant in determining cell response to PDT, in that PC3 cells are very active ABCG2 producers whereas H1299 are not (Fig. 6). The two cell lines were exposed to PDT and irradiated at the highest light fluences, i.e. 12 Jcm⁻² (H1299) and 10 Jcm⁻² (PC3). PpIX reaches nuclear compartment in the absence of ABCG2.

For PpIX to cause DNA injury, it must reach the nuclear compartment and stay there at the time of irradiation. The absence of ABCG2 protein is associated with nuclear involvement, and in particular with PDT-mediated DNA damage. It is conceivable that the extrusion systems, including ABCG2, when active, come into play and clear the cells of substances that have reached abnormally high concentration.

We have exploited the intrinsic fluorescence of PpIX and followed its distribution throughout the cell compartments after PDT, at light fluences known to be partially cytotoxic, to test this hypothesis. Figure 7 refers to H1299 and PC3 cells. These cells were chosen because they are both p53 negative (p53^{-/-}), but differ in a condition relevant in determining cell response to PDT, in that PC3 cells are very

FIGURE 7 PpIX distribution in H1299 and PC3 cells, without and with Ko143 inhibitor. Fluorescence microscopy of H1299 cells (panels a through d) and PC3 cells (panels e through h) after incubation with 5-ALA (as in Fig. 1) and, when indicated, 6 hours after irradiation with the reported light fluences. PpIX distribution is revealed by the red fluorescence (see text for details). In panels c, d, g and h, Ko143 concentration is 2 μ mol/L

active ABCG2 producers, whereas H1299 are not (Fig. 6). The two cell lines were exposed to PDT and irradiated at the highest light fluences, i.e. 12 Jcm $^{-2}$ (H1299) and 10 Jcm $^{-2}$ (PC3).

The results are summarized in Fig. 7. The treatment with 5-ALA induced the synthesis and accumulation of PpIX inside H1299 cells

as indicated by the emission of red fluorescence following the incubation. In particular, most of the fluorescence was localized in the cytoplasm and in the perinuclear area. Such fluorescence did not reach the nucleus but, in addition, disappeared spontaneously within the next 6 hours, suggesting an outside diffusion of the PpIX synthesized in

(a) A549

+ Ko143

(e) PC3

+ Ko143

FIGURE 8 Comet assay for A549 and PC3 cells after treatment with Ko143. Comets images collected at 6 hours after PDT (conditions as in Fig. 1) in the presence of Ko143 (1.5 $\mu\text{mol/L}$ for A549 and 2.0 $\mu\text{mol/L}$ for PC3) through COMET Score™ software (TriTek Corp.) for A549 and PC3 cells (panels a and e respectively). Percentage of DNA tail (a. u.) as function of light fluence for A549 and PC3 cells (panels b and f respectively). Tail moment (a. u.) as function of light fluence for A549 and PC3 cells (panels c and g respectively). Olive moment (a. u.) as function of light fluence for A549 and PC3 cells (panels d and h respectively). Each point represents the fluorescence of >100 cells (mean $\pm$ SD). Statistical significance (in respect to control), * $P < .5$, ** $P < .01$, *** $P < .001$

excess (Fig. 7a). Interestingly enough, when H1299 cells were exposed to light, soon after the irradiation, the PpIX localization did not change. At variance, 6 hours after the irradiation, i.e. when DNA damage is observed, PpIX fluorescence appeared in the nucleus (Fig. 7b). As

expected, the addition of the Ko143 inhibitor to H1299 cells (which lack the ABCG2 transporter), in the same conditions as above, did not change the picture (Fig. 7c,d). PpIX showed a cytoplasmic and perinuclear localization soon after 5-ALA incubation also in PC3 cells; as

expected, PpIX is rapidly cleared showing no appreciable fluorescence after 6 hours (Fig. 7e). The difference between H1299 and PC3 cells was clearly evident at 6 hours after irradiation, exactly when DNA damage is evident in H1299 and not detectable in PC3 cells. In fact, PpIX is not detectable in ABCG2-positive cell lines at 6 hours from irradiation (Fig 7f). The major involvement of ABCG2 transporter in the response of PC3 cells to 5-ALA/PDT is demonstrated by incubating PC3 cells with Ko143, a powerful, albeit non-specific inhibitor of ABCG2.^{34,35}

Ko143 did not affect significantly PpIX localization soon after the incubation with 5-ALA or soon after the irradiation, which was confined to the cytoplasm (Fig. 7g, left and h, left). Moreover, when this inhibitor remains for all the period of release after 5-ALA incubation, PpIX signal did not disappear but still remained inside within the cell and the PpIX is less cytoplasmic and more perinuclear (Fig. 7g, right). This proved that when ABCG2 activity is blocked, the membrane transporter is unable to extrude PpIX outside the cellular compartment. At 6 hours after irradiation, PpIX signal is still clear and visible, and the nucleus became clearly compromised (Fig. 7h, right) as for H1299 (Fig. 7b). The inactivity of ABCG2 protein induced the retention of PpIX inside the cellular compartment, which can reach the nucleus within 6 hours. In addition, when PC3 cells were incubated with Ko143, Western blot assay confirmed disappearance of ABCG2 protein (data not shown).

3.7 | DNA damage occurs in ABCG2 positive cell lines in the presence of the inhibitor Ko143

We have shown that nuclear localization of PpIX is a condition for the occurrence of DNA damage after PDT and that the inhibition of ABCG2 stops the extrusion of PpIX from cell causing its repositioning in the vicinities of the nucleus. This means that inhibition of ABCG2 transporter makes vulnerable to DNA damage also cells that seem to have a shielded nucleus.

DNA damage, as evaluated by comet assay, occurs also in A549 and PC3 cells in the presence of the ABCG2 inhibitor Ko143 at 1.5 and 2.0 $\mu\text{mol/L}$ for A549 and PC3 cells, respectively. In A549 and PC3 cells exposed to PDT, especially at highest light fluences, an appreciable increase of comets number and size was detected (Fig. 8a,e, respectively). This result was confirmed also by additional parameters such as tail moment (Fig. 8b,f, respectively), percentage of DNA in tail (Fig. 8c,g, respectively) and of olive moment (Fig. 8d,h, respectively).

Taken together, these data confirm that 5-ALA/PDT induces DNA damage in cells in which ABCG2 is either absent or not functional because of an inhibitor.

4 | DISCUSSION

In a previous work,³³ we studied the effect of combining 5-ALA/PDT with Gefitinib (a powerful EGF receptor inhibitor) on the p53^{+/+} A549 and p53^{-/-} H1299 cell lines. While the combined therapy proved highly effective in both cell lines, the response to PDT alone was

remarkably different. In particular, 5-ALA/PDT caused a light-dose-dependent accumulation of H1299 cells in the S phase, but did not cause similar effects in A549 cells. This observation prompted us to further investigate this issue, as the behaviour of the two different cell lines may have therapeutic implications and impact on the clinical use of this type of therapy.

As expected, PDT caused reduction of cell viability in both A549 and H1299 cells. However, the viability of the A549 (p53^{+/+}) cells was lost at substantially lower light fluences than for the H1299 cells (p53^{-/-}). In spite of their major loss in viability, cell-cycle progression of A549 cells surviving PDT was minimally affected. In contrast, remarkable alterations were observed in H1299 cells, whereby a large proportion of cells left G₁, accumulated in S and stopped cycling. At glance, this behaviour may be explained on the basis of p53 status of these cell lines. In fact, a legitimate hypothesis would predict that p53 in A549 cells initiates and manages a programme of cell-cycle arrest, DNA repair or apoptosis, activities resulting in the protection of cells from the noxious effect of PDT at nuclear level. The same is not applicable to H1299 cells that are p53 null. To better investigate if, indeed, p53 protects cells from PDT, we extended our study to other two cell lines differing for the p53 status (namely HCT-116 p53^{+/+} and the cognate HCT-116 p53 knockout). As in the previous case, we confirmed that 5-ALA/PDT was more effective in abating cell viability of p53 positive HCT-116 cells than that of the p53 negative counterpart. However, in both HCT-116 lines, irrespective of the p53 expression, we observed profound cell-cycle alterations. An additional evidence that p53 was not primarily involved in protection from PDT-induced nuclear damage was also provided by data obtained with a fifth cell line, namely the PC3 cells, which are constitutively p53 null. When, exposed to PDT, these cells did not show significant cell-cycle alterations and behaved like A549 cells which, in contrast, are p53^{+/+}.

The sum and substance of the observations above suggests that the response of cancer cells to PDT could not be predicted simply by the level expression of p53 or its lack thereof.

Cell-cycle alterations following PDT are solid clues of nuclear DNA damage. However, so far there are no strong and definitive evidences that PDT causes nuclear damage. To probe this, we have investigated the effects of 5-ALA/PDT on nuclear DNA using different approaches, namely the levels of phosphorylation of serine 139 in γ -H2AX histone (cytofluorometry) and the formation of comets (comet assays), both recognized indicators of DNA damage and replication stress.^{47,48}

The three cell lines (H1299 and HCT-116 (p53^{+/+} and p53^{-/-}), whose cell-cycle profiles were affected by PDT, showed an increase in the phosphorylation level of histone γ -H2AX, a clue of the initiation of the assembly of DNA repair proteins at the sites containing damaged chromatin and activation of checkpoints proteins for the arrest of cell-cycle progression. In addition, in these cell lines, DNA damage, following PDT, was further confirmed by the formation of DNA comets (comet assay). At variance with these results, the absence of any significant increase of histone γ -H2AX phosphorylation levels and lack of comets formation in A549 and PC3 cell lines were in line with the absence of detectable cell-cycle alterations.

The whole body of data collected, while demonstrating that 5-ALA/PDT may affect DNA integrity in some cell lines, does not point to the absence of a functional p53 as the culprit. Possibly, DNA damage occurs in cells that allow cytoplasmic accumulation of the photosensitizer around the nuclear envelope but does not occur in cells that rapidly get rid of PpIX.

Several factors regulate PpIX accumulation in cells exposed to 5-ALA; these include uptake rate and metabolism of the precursor and the activity of the numerous enzymes involved in biosynthesis and transport of intermediates.^{56,57} With respect to precursor uptake, metabolism and intracellular distribution of 5-ALA, all cell lines studied, independent of p53 status, take up and convert the precursor into PpIX. This is clearly shown by the appearance, in all cell extracts, of a characteristic PpIX fluorescence spectrum. In addition, fluorescence detected by a confocal microscope provides detailed information of the intracellular distribution of the photosensitizer at the time of its synthesis and further outcome.

Recently, particular attention has been paid to the ATP-binding cassette (ABC) transporters, in particular ABCG2, since it may mediate the efflux of xenobiotics and several therapeutics.^{26,58} In a recent study, ABCG2 has been described also as an important factor of resistance to photodynamic treatment³⁰; indeed, facilitated efflux of PpIX may alter PDT effectiveness.^{31,49} Forcing porphyrins out of the cells,⁵⁷ the transporter supports cell survival under stress.⁵⁹ Several proteins are involved in the 5-ALA-mediated PpIX accumulation; however, according to Kobuchi et al.⁵³ only ABCG2 correlates negatively with the process.

Looking for the expression levels of ABCG2 protein in our experimental models, we found that DNA damage in our cell lines was related to the absence of the transporter. Kobuchi et al.⁵³ reported that Ko143, a strong, albeit non-specific inhibitor of ABCG2, enhanced PpIX accumulation; these authors described a reduction of PpIX accumulation in ABCG2 transfected HEK cells that could be reverted in the presence of the inhibitor.

When the expression of ABCG2 in A549 and in PC3 cell lines was inhibited by Ko143, 5-ALA/PDT caused DNA damage as demonstrated by the appearance of DNA comets. This result is in line with a study that suggested a protective role for ABCG2 on cells viability after PDT,⁶⁰ but made no mention of nuclear damage.

We compared the cellular PpIX localization in two cell lines, H1299 and PC3, both p53 null, but different with regard to ABCG2 expression. Both cell lines internalize and convert 5-ALA to PpIX; however, the distribution of the photosensitizer was somewhat different in that it appeared primarily perinuclear in the ABCG2 negative H1299,⁵⁰ and largely cytoplasmic in the ABCG2 positive PC3 cells.⁵⁵ It appears that extrusion systems for PpIX are operative in both cell lines, since the dye was cleared by both cell types with time, if not exposed to PDT. Exposure to light produced different effects in the two cell lines: PC3 cells analysed 6 hours after PDT showed a residual, diffuse, cytoplasmic fluorescence, whereas in H1299 cells, after identical treatment, a strong PpIX fluorescent signal was still present. The different behaviour could be attributed to the activity of ABCG2, since PC3 cells treated with the inhibitor Ko143 when incubated with 5-ALA, lose the ability to clear PpIX.

Overall, it appears that ABCG2 expression could be a key element for the response to 5-ALA/PDT, in that when the transporter activity is inhibited, protection of PDT-induced DNA damage no longer exists. When 5-ALA incubation is in the presence of Ko143, PpIX accumulates within the cytoplasm and appears to concentrate in the perinuclear area, and this is likely to happen because its extrusion is reduced. It is possible that the higher concentration of the photosensitizer in the perinuclear area makes the nucleus more susceptible to the action of ROS generated by PpIX photoactivation, thus facilitating DNA exposure and damage. It has to be noted that DNA damage observed in our cell lines occurred 6 hours after irradiation and that a time window of 6 hours is sufficient to change the PpIX cellular localization when ABCG2 is functionally inactivated. This may suggest that DNA damage is the final effect of ROS propagation.

When present, the PDT-induced DNA damage leads to profound effects on cells and this may have potentially positive therapeutic return.

In conclusion, the present observations not only provide deeper insight into the mechanisms of PDT but also offer potentially valuable information in predicting the effectiveness of 5-ALA/PDT and indicate a way to shift this treatment from a palliative to a more effective approach in cancer therapy.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTION

IP and FB performed all experiments including spectrofluorometric measurements, PDT, cytofluorimetric analysis, PpIX localization, Western blot analysis and comet assays; GP, who proposed the study and designed the experiments, discussed the data and wrote the manuscript with SMA who finally edited the text. All authors reviewed the results and approved the final version of the manuscript.

REFERENCES

1. Dougherty TJ. An update on photodynamic therapy applications. *J Clin Laser Med Surg*. 2002;20:3–7.
2. Foote CS. Definition of type I and type II photosensitized oxidation. *Photochem Photobiol*. 1991;54:659.
3. Hatz S, Lambert JD, Ogilby PR. Measuring the lifetime of singlet oxygen in a single cell: addressing the issue of cell viability. *Photochem Photobiol Sci*. 2007;6:1106–1116.
4. Peng Q, Moan J, Nesland JM. Correlation of subcellular and intratumoural photosensitizer localization with ultrastructural features after photodynamic therapy. *Ultrastruct Pathol*. 1996;20:109–129.
5. Almeida RD, Manadas BJ, Carvalho AP, Duarte CB. Intracellular signaling mechanisms in photodynamic therapy. *Biochim Biophys Acta*. 2004;1704:59–86.
6. Akhlynnina TV, Jans DA, Statsyuk NV, et al. Adenovirus synergize with nuclear localisation signals to enhance nuclear delivery and photodynamic action of internalizable conjugates containing chlorin e6. *Int J Cancer*. 1999;81:734–740.

7. Akhlynnina TV, Jans DA, Rosenkranz AA, et al. Nuclear targeting of chlorin e6 enhances its photosensitizing activity. *J Biol Chem*. 1997;272:328–331.
8. Kvam E, Stokke T, Moan J. The lengths of DNA fragments light-induced in the presence of a photosensitizer localized at the nuclear membrane of human cells. *Biochim Biophys Acta*. 1990;1049:33–37.
9. Oleinick NL, Evans HH. The photobiology of photodynamic therapy: cellular targets and mechanisms. *Radiat Res*. 1998;150(Suppl):S146–S156.
10. Evans HH, Horng MF, Ricanati M, Deahl JT, Oleinick NL. Mutagenicity of photodynamic therapy as compared to UVC and ionizing radiation in human and murine lymphoblast cell lines. *Photochem Photobiol*. 1997;66:690–696.
11. Noodt BB, Kvam E, Steen HB, Moan J. Primary DNA damage, HPRT mutation and cell inactivation photo-induced with various sensitizers in V79 cells. *Photochem Photobiol*. 1993;58:541–547.
12. Moan J, Kvam E, Steen HB. No correlation between DNA strand breaks and HPRT mutation induced by photochemical treatment in V79 cells. *Mutat Res*. 1994;323:75–79.
13. El-Hussein A, Harith M, Abrahamse H. Assessment of DNA Damage after Photodynamic Therapy Using a Metallophthalocyanine Photosensitizer. *Int J Photoenergy*. 2012;2012:1–10. doi: 10.1155/2012/281068.
14. Tada-Oikawa S, Oikawa S, Hirayama J, Hirakawa K, Kawanishi S. DNA damage and apoptosis induced by photosensitization of 5,10,15,20-Tetrakis (N-methyl-4-pyridyl)-21H,23H-porphyrin via singlet oxygen generation. *Photochem Photobiol*. 2009;85:1391.
15. Wiseman H, Halliwell B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem J*. 1996;313:17–29.
16. Elfsson B, Wallin I, Eksborg S, Rudaeus K, Ros AM, Ehrsson H. Stability of 5-aminolaevulinic acid in aqueous solution. *Eur J Pharm Sci*. 1998;7:87–91.
17. Peng Q, Warloe T, Berg K, et al. 5-Aminolaevulinic acid-based photodynamic therapy: clinical research and future challenges. *Cancer*. 1997;97:2282–2308.
18. El-Sharabasy MMH, El-Wassel AM, Hafez MM, Salim SA. Porphyrin metabolism in some malignant diseases. *Br J Cancer*. 1992;65:409–412.
19. van Hillegersberg R, Van den Berg JW, Kort WJ, Terpstra OT, Wilson JH. Selective accumulation of endogenously produced porphyrins in a liver metastasis model in rats. *Gastroenterology*. 1992;103:647–651.
20. Datta SN, Loh CS, MacRobert AJ, Whatley SD, Matthews PN. Quantitative studies of the kinetics of 5-aminolaevulinic acid induced fluorescence in bladder transitional cell carcinoma. *Br J Cancer*. 1998;78:1113–1118.
21. Fritsch C, Goerz G, Ruzicka T. Photodynamic therapy in dermatology. *Arch Dermatol*. 1998;134:207–214.
22. Uehlinger P, Zellweger M, Wagnier G, Juillerat-Jeanneret L, van den Bergh H, Lange N. 5-Aminolaevulinic acid and its derivatives: physical chemical properties and protoporphyrin IX formation in cultured cells. *J Photochem Photobiol, B*. 2000;54:72–80.
23. Bunz F, Hwang PM, Torrance C, et al. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest*. 1999;104:263–269.
24. Vousden KH, Prives C. Blinded by the light: the growing complexity of p53. *Cell*. 2009;137:413–431. doi:10.1016/j.cell.2009.04.037.
25. Doyle LA, Yang W, Abruzzo LV, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci USA*. 1998;95:15665–15670.
26. Allen JD, Brinkhuis RF, Wijnholds J, Schinkel AH. The mouse Bcrp1/Mxr/Abcp gene: amplification and overexpression in cell lines selected for resistance to topotecan, mitoxantrone, or doxorubicin. *Cancer Res*. 1999;59:4237–4241.
27. Knutsen T, Rao VK, Ried T, et al. Amplification of 4q21-q22 and the MXR gene in independently derived mitoxantrone-resistant cell lines. *Genes Chromosom Cancer*. 2000;27:110–116.
28. Robey RW, Medina-Perez WY, Nishiyama K, et al. Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. *Clin Cancer Res*. 2001;7:145–152.
29. Zhou S, Zong Y, Ney PA, Nair G, Stewart CF, Sorrentino BP. Increased expression of the Abcg2 transporter during erythroid maturation plays a role in decreasing cellular protoporphyrin IX levels. *Blood*. 2005;105:2571–2576.
30. Kim JH, Park JM, Roh YJ, Kim IW, Hasan T, Choi MG. Enhanced efficacy of photodynamic therapy by inhibiting ABCG2 in colon cancers. *BMC Cancer*. 2015;15:504. doi:10.1186/s12885-015-1514-4.
31. Robey RW, Steadman K, Polgar O, Bates SE. ABCG2-mediated transport of photosensitizers: potential impact on photodynamic therapy. *Cancer Biol Ther*. 2005;4:187–194.
32. Jonker JW, Buitelaar M, Wagenaar E, et al. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc Natl Acad Sci USA*. 2002;99:15649–15654.
33. Postiglione I, Chiaviello A, Aloj SM, Palumbo G. 5-Aminolaevulinic acid/photo-dynamic therapy and gefitinib in non-small cell lung cancer cell lines: a potential strategy to improve gefitinib therapeutic efficacy. *Cell Prolif*. 2013;46:382–395.
34. Barron GA, Moseley H, Woods JA. Differential sensitivity in cell lines to photodynamic therapy in combination with ABCG2 inhibition. *J Photochem Photobiol, B*. 2013;126:87–96.
35. Weidner LD, Zoghbi SS, Lu S, et al. The inhibitor Ko143 is not specific for ABCG2. *J Pharmacol Exp Ther*. 2015;354:384–393. doi:10.1124/jpet.115.225482.
36. Egli RJ, Di Criscio A, Hempfing A, et al. In vitro resistance of articular chondrocytes to 5-Aminolaevulinic acid based photodynamic therapy. *Lasers Surg Med*. 2008;40:282–290.
37. Wild L, Burn JL, Reed MW, Brown NJ. Factors affecting amino-laevulinic acid-induced generation of protoporphyrin IX. *Br J Cancer*. 1997;76:705–712.
38. Lovell DP, Omori T. Statistical issues in the use of the comet assay. *Mutagenesis*. 2008;23:171–182.
39. Chiaviello A, Paciello I, Postiglione I, Crescenzi E, Palumbo G. Combination of photodynamic therapy with aspirin in human-derived lung adenocarcinoma cells affects proteasome activity and induces apoptosis. *Cell Prolif*. 2010;43:480–493.
40. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem*. 1976;72:248–254.
41. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1971;227:680–685.
42. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G₁-phase progression. *Genes Dev*. 1999;13:1501–1512.
43. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev*. 2001;15:2177–2196.
44. Li R, Hannon GJ, Beach D, Stillman B. Subcellular distribution of p21 and PCNA in normal and repair-deficient cells following DNA damage. *Curr Biol*. 1996;6:189–199.
45. Calzavara-Pinton PG, Venturini M, Sala R. Photodynamic therapy: update 2006. Part 1: Photochemistry and photobiology. *J Eur Acad Dermatol Venereol*. 2006;21:293–302.
46. Adams J. The proteasome: a suitable antineoplastic target. *Nat Rev Cancer*. 2004;4:349–360.
47. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem*. 1998;273:5858–5868.
48. Redon C, Pilch D, Rogakou E, Sedelnikova O, Newrock K, Bonner W. Histone H2A variants H2AX and H2AZ. *Curr Opin Genet Dev*. 2002;12:162–169.

49. Ishikawa T, Nakagawa H, Hagiya Y, Nonoguchi N, Miyatake S, Kuroiwa T. Key Role of Human ABC Transporter ABCG2 in Photodynamic Therapy and Photodynamic Diagnosis. *Adv Pharmacol Sci*. 2010;2010:587306. doi:10.1155/2010/587306.
50. Hsieh JL, Lu CS, Huang CL, et al. Acquisition of an enhanced aggressive phenotype in human lung cancer cells selected by suboptimal doses of cisplatin following cell detachment and reattachment. *Cancer Lett*. 2012;321:36–44.
51. Westover D, Ling X, Lam H, et al. FL118, a novel camptothecin derivative, is insensitive to ABCG2 expression and shows improved efficacy in comparison with irinotecan in colon and lung cancer models with ABCG2-induced resistance. *Mol Cancer*. 2015;14:92.
52. Candeil L, Gourdier I, Peyron D, et al. ABCG2 overexpression in colon cancer cells resistant to SN38 and in irinotecan-treated metastases. *Int J Cancer*. 2004;109:848–854.
53. Kobuchi H, Moriya K, Ogino T, et al. Mitochondrial localization of ABC transporter ABCG2 and its function in 5-aminolaevulinic acid-mediated protoporphyrin IX accumulation. *PLoS ONE*. 2012;7:e50082.
54. Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood*. 2002;99:507–512.
55. Ma Y, Liang D, Liu J, et al. Prostate cancer cell lines under hypoxia exhibit greater stem-like properties. *PLoS ONE*. 2011;6:e29170.
56. Frank J, Lornejad-Schäfer MR, Schöffl H, Flaccus A, Lambert C, Biesalski HK. Inhibition of heme oxygenase-1 increases responsiveness of melanoma cells to ALA-based photodynamic therapy. *Int J Oncol*. 2007;31:1539–1545.
57. Krishnamurthy P, Xie T, Schuetz JD. The role of transporters in cellular heme and porphyrin homeostasis. *Pharmacol Ther*. 2007;114:345–358.
58. Bakhsheshian J, Hall MD, Robey RW, et al. Overlapping substrate and inhibitor specificity of human and murine ABCG2. *Drug Metab Dispos*. 2013;41:1805–1812. doi:10.1124/dmd.113.053140).
59. Krishnamurthy P, Ross DD, Nakanishi T, Bailey-Dell K, Zhou S, Mercer KE. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. *Biol Chem*. 2004;279:24218–24225. Epub 2004.
60. Palasuberniam P, Yang X, Kraus D, Jones P, Myers KA, Chen B. ABCG2 transporter inhibitor restores the sensitivity of triple negative breast cancer cells to aminolevulinic acid-mediated photodynamic therapy. *Sci Rep*. 2015;5:13298. doi:10.1038/srep13298.