Low doses of cisplatin or gemcitabine plus Photofrin/photodynamic therapy: Disjointed cell cycle phase-related activity accounts for synergistic outcome in metastatic non–small cell lung cancer cells (H1299)

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Abstract

We compared the effects of monotherapy (photodynamic therapy or chemotherapy) versus combination therapy (photodynamic therapy plus a specific drug) on the non–small cell lung cancer cell line H1299. Our aim was to evaluate whether the additive/synergistic effects of combination treatment were such that the cytostatic dose could be reduced without affecting treatment efficacy. Photodynamic therapy was done by irradiating Photofrin-preloaded H1299 p53/p16-null cells with a halogen lamp equipped with a bandpass filter. The cytotoxic drugs used were *cis*-diammine-dichloroplatinum [II] (CDDP or cisplatin) and 2′,2′-difluoro-2′-deoxycytidine (gemcitabine). Various treatment combinations yielded therapeutic effects (trypan blue dye exclusion test) ranging from additive to clearly synergistic, the most effective being a combination of photodynamic therapy and CDDP. To gain insight into the cellular response mechanisms underlying favorable outcomes, we analyzed the H1299 cell cycle profiles and the expression patterns of several key proteins after monotherapy. In our conditions, we found that photodynamic therapy with Photofrin targeted G_0-G_1 cells, thereby causing cells to accumulate in S phase. In contrast, low-dose CDDP killed cells in S phase, thereby causing an accumulation of G_0-G_1 cells (and increased p21 expression). Like photodynamic therapy, low-dose gemcitabine targeted G_0-G_1 cells, which caused a massive accumulation of cells in S phase (and increased cyclin A expression). Although we observed therapeutic reinforcement with both drugs and photodynamic therapy, reinforcement was more pronounced when the drug (CDDP) and photodynamic therapy exert disjointed phase-related cytotoxic activity. Thus, if photodynamic therapy is appropriately tuned, the dose of the cytostatic drug can be reduced without compromising the therapeutic response. [Mol Cancer Ther 2006;5(3):776–85]

Introduction

Although chemotherapy is the leading treatment for most types and stages of cancers, photodynamic therapy is an effective anticancer procedure for selected tumors (1). This procedure involves administration of a tumor-localizing photosensitizing agent that when activated by light of a specific wavelength mediates cell destruction via the production of singlet oxygen. Photodynamic therapy has been shown to have few or no side effects in patients with Barrett's esophagus (high-grade dysplasia and early carcinoma) and in selected cases of early squamous cell carcinoma (2). One of the most widely used photosensitizing drugs is the hematoporphyrin-derivative Photofrin. This nontoxic substance has been used for photodynamic therapy for ~8 years and is the only one widely approved for human therapy. On exposure of Photofrin-loaded cells to visible light, intracellular toxic oxygen species (singlet oxygen, $^1O_2$) are generated that can trigger cell apoptosis or necrosis (3, 4).

Anticancer drugs target several cellular components and activate responses that go from cell repair to cell death. However, chemotherapy is effective only at high doses, because, at low doses, tumor cells may repair damage and resume their original high proliferation rate (5). *Cis*-diammine-dichloroplatinum [II] (CDDP or cisplatin) and 2′,2′-difluoro-2′-deoxycytidine (gemcitabine) are widely used in the management of various cancers. By forming adducts in DNA, CDDP inhibits DNA replication and chain elongation, which accounts for its antineoplastic activity. In clinical practice, CDDP is often combined with other drugs. Synergy between CDDP and other chemotherapeutic agents occurs by various pathways: increased intracellular drug accumulation, enhanced binding to DNA, and decreased DNA repair (6). Gemcitabine is a deoxycytidine analogue that is effective against solid tumors, including non–small cell lung cancer. After transport into the cell,

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gemcitabine requires phosphorylation by deoxycytidine kinase to exert biological activity (7, 8). The enzyme responsible for this reaction is also the rate-limiting step in gemcitabine activation (8). It has been suggested that the combination of photodynamic therapy and conventional cancer treatments may become a workable anticancer strategy (9).

The aim of our study was to identify photodynamic therapy/chemotherapy combinations in which the dose of the most toxic compound could be decreased without a loss of efficacy. To this aim, we examined the response of H1299 human lung metastatic non–small cell lung cancer cells (cell viability, cell cycle, and protein expression) to moderately toxic doses of CDDP or gemcitabine with Photofrin/photodynamic therapy, administered separately and in appropriate combinations.

Materials and Methods

Cell Cultures

The NCI-H1299 human non–small cell lung cancer cell line was obtained from American Type Culture Collection (Rockville, MD). They were cultured in RPMI 1640, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4,500 mg/L glucose, 1,500 mg/L sodium bicarbonate, 100 μg/mL streptomycin, 100 units/mL penicillin, and 10% FCS. The medium was changed every 3 days. All media and cell culture reagents were purchased from Life Technologies (San Giuliano Milanese, Italy). NCI-H1299 cells are p53−/− and p16−/−. All treatments, individual and in combination, were done in triplicate samples in tissue culture dishes (35 mm) in which 5×10^4 cells were routinely seeded. A larger number of cells (4×10^5) were seeded for cell cycle measurements and for protein extraction for Western blotting purposes. The effects of monotherapy with drugs or photodynamic therapy or combined therapy (drugs + photodynamic therapy) were evaluated (*a*) by trypan blue assays (to check residual cell viability), (*b*) by analyzing cell cycle profiles (to determine the cell distribution in the various conditions), and (*c*) by analyzing expression patterns (Western blots) of specific proteins involved in the control of cell growth and survival.

Cytotoxic Drugs

CDDP was obtained from Sigma-Aldrich Srl (Milan, Italy). A stock solution was obtained by dissolving CDDP in DMSO to obtain a final concentration of 40 mmol/L. Aliquots were stored at −20°C until used. Gemcitabine chlorhydrate (Gemzar) was supplied as dry powder by Eli Lilly SpA (Sesto Fiorentino, Italy). A stock solution (67 mmol/L) was obtained by dissolving gemcitabine in an isotonic solution (0.9% NaCl). Aliquots were stored at −20°C until used.

Photosensitizer

The Photofrin (the hematoporphyrin derivative Porfimer sodium) used in this work was supplied as freeze-dried powder (lot no. 162A6-06; Axcan Pharma, Mont-Saint-Hilaire, Quebec, Canada). Its absorption spectrum consists of various peaks within the visible region. A Photofrin stock solution was obtained by dissolving the powder in water containing 5% glucose to obtain a final concentration of 2.5 mg/mL. This solution was stored in aliquots at −20°C in the dark. Before measurements, appropriate aliquots of this solution were diluted to the desired concentration. Except for ''dark toxicity assays,'' Photofrin was always used at a concentration of 2.5 μg/mL. All treatments involving Photofrin were done on triplicate. Cells were exposed to 2.5 μg/mL Photofrin for 16 hours before irradiation.

Chemotherapy and Chemotherapy Schedule

Usually, 5×10^4 H1299 cells were seeded in 35-mm tissue culture dishes and exposed to the cytotoxic substances (individual or combined with photodynamic therapy) 24 hours later. This procedure was carried out in triplicate. Normally, 4×10^5 cells were used for cell cycle measurements and protein extraction for Western blotting. To obtain CDDP/gemcitabine dose-response curves, culture dishes were treated with CDDP (2.5–12 μmol/L) or gemcitabine (2–12 nmol/L) and kept at 37°C for 24 hours. After incubation, cells were washed and released into fresh medium. Cell viability was evaluated with the trypan blue assay (as detailed in ref. 10) 24 hours later, counting between 15 and 30 cells per field. Two sets of data were collected for each drug. In combination experiments, after incubation with the drug (0–24 hours) and Photofrin (8–24 hours), cells were washed, placed in a colorless saline solution (Hank's), and immediately irradiated. After irradiation, cells were placed in fresh complete medium, and cells negative to trypan blue staining were counted 24 hours later (see Fig. 1). Residual viability was expressed as percentage of trypan blue–negative cells versus untreated controls.

Photodynamic Therapy and Photodynamic Therapy Schedule

Cells were irradiated by a broadband light delivered with a PTL-Penta apparatus (Teclas, Sorengo, Switzerland). This apparatus consists of a halogen lamp (Osram 250 W, 24 V Osram, Munich, Germany) equipped with a bandpass filter (>80% transmittance in the 510 to 590 nm spectral region; bandwidth ~40 nm at 50% of the peak) corresponding approximately to one of the Photofrin absorption peaks. The emission spectrum was measured with a Macam SR9910 spectroradiometer (Macam Photometrics, Livingston, Scotland, United Kingdom). The light was delivered through an 8-mm bundle of optical fibers placed at a distance from the cell plates that ensures uniform illumination of the entire cell monolayer. The fluence rate at the level of the cell monolayer was fixed at 6 mW/cm^2 and irradiations were done with light doses of up to 1.8 J/cm^2. We invariably used a Photofrin concentration of 2.5 μg/mL. At a fluence of 0.54 J/cm^2, photodynamic therapy monotherapy reduced H1299 cell viability to ~50% that of treated but not irradiated cells (controls). Doses between 0.18 and 0.54 J/cm^2 were used in the combination experiments. Because only a few cells survived combined therapy with fluences >0.54 J/cm^2, higher fluences were not considered. In brief, after incubation, cells were washed

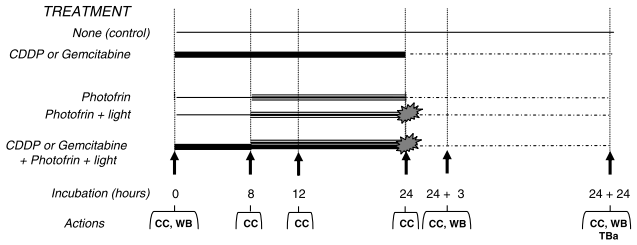

Figure 1. Single-agent and combination treatment schedule. For single-agent CDDP or gemcitabine treatments, cells were incubated with chemotherapeutic drugs for 8, 12, or 24 h, washed, and analyzed immediately and after 3 h. Cells exposed to drugs for 24 h were also analyzed 24 h after washing and release into fresh medium. Before photodynamic therapy, cells were incubated for 16 h with Photofrin in the dark. For combination treatments, cells were treated with CDDP or gemcitabine at prefixed concentrations, and Photofrin was added 8 h later. The samples were washed and analyzed immediately or released in fresh medium and analyzed 24 h later. *CC,* cell cycle; *WB,* Western blot; *TBa,* trypan blue assay; *star,* light turned on.

extensively, placed in colorless Hank's solution, exposed to light, and analyzed by cytofluorimetry either immediately or after exposure to fresh medium for a further 24 hours. Similarly, cell extracts obtained ~3 or 24 hours after irradiation were used to obtain protein profiles (Western blots). Residual cell viability was assessed by trypan blue assay after cells have been placed in fresh medium for 24 hours (Fig. 1).

Flow Cytometry

Dishes (10 cm) containing 4×10^5 H1299 cells were incubated for 24 hours at 37°C in 7 mL complete medium (controls) or in medium supplemented with Photofrin (2.5 μg/mL) alone or associated with CDDP (2.5 μmol/L) or gemcitabine (4 nmol/L). Cells were exposed to photodynamic therapy as described above and were then detached from the dishes by trypsinization, suspended in serum-rich medium, centrifuged, washed twice with 1 mL PBS, and resuspended for storage (−20°C) in 95% ethanol. Before analysis, fixed cells were washed twice, centrifuged, and resuspended in 1 mL PBS containing 1 μg RNase and 100 μg propidium iodide (11). Samples were stored in the dark for 20 minutes at room temperature before final readings. The cellular orange fluorescence of propidium iodide was detected in a linear scale using a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA) equipped with an excitation laser line at 488 nm. About 30,000 events (i.e., fluorescence readings, corresponding to not less than 20,000 cells) were recorded for each sample. The cell cycle was examined after monotherapy and combined treatment at the indicated times (see Fig. 1). Data were analyzed with ModFit/LT (Verity Software, Topsham, ME).

Median Effect Analysis

We used the median effect analysis (12) together with the combination index (CI) to ascertain synergy and the additivity/antagonism of photodynamic therapy and chemotherapy. The median effect analysis derives directly from the mass action law and does not depend on a reaction mechanism. The reference equation is $F_a / F_u = (D / Dm) * m$, where F_a is the affected fraction, F_u is the unaffected fraction, D is the dose, Dm is the median dose (50%, for instance), and m is a coefficient related to the shape of the dose-response curve ($m = 1$, hyperbolic; $m > 1$, sigmoidal; $m < 1$, negatively sigmoidal). The shape is easily determined from the dose-effect curve from which Dm is directly estimated. The CI for treatments that are not mutually exclusive (i.e., that unequivocally have different modes of action) can be calculated by (13): $CI = (D)_1 / (D_x)_1 + (D)_2 / (D_x)_2 + [(D)_1(D)_2] / [(D_x)_1(D_x)_2]$, in which $(D)_n$ is the dose of the nth drug (or of light) needed to obtain a given effect in the combination and $(D_x)_n$ is the dose of the same drug (or light) necessary to obtain the same effect when used alone. Chou and Talalay (12) defined synergism as a more than expected additive effect, where the additive effect is designated CI equal to unity. Thus, for mutually exclusive agents that have totally independent modes of action, $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergy, additive effect, and antagonism, respectively. We thus constructed several dose-response curves at a constant fluence and at a constant drug concentration.

Western Blot Analysis

Total cell protein preparations were obtained by lysing cells in 50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1% NP40, 0.1% Triton, 2 mmol/L EDTA, 10 μg/mL aprotinin, and 100 μg/mL phenylmethylsulfonyl fluoride. Protein concentration was routinely measured with the Bio-Rad protein assay (14). Polyacrylamide gels (10–15%) were prepared essentially as described by Laemmli (15). Molecular weight standards were from New England Biolabs (Beverly, MA). Proteins separated on the polyacrylamide gels were blotted onto nitrocellulose filters (Hybond-C pure, Amersham Italia, Milan, Italy). Filters were washed and stained with specific primary antibodies and then with secondary antisera conjugated with horseradish peroxidase (Bio-Rad; diluted 1:2,000). Filters were developed using an electrochemiluminescent Western blotting detection reagent (Amersham Italia) and quantified by scanning with a Discover Pharmacia scanner equipped with a Sun Spark Classic Workstation. The anti-Bcl-2 (100), Bcl-XL (S-18), Bax (N-20), p21 Cip1 (C-19), cyclin A (C-19), Cdk2 (M2), HSP60 (N-20), HSP70 (K-20), and actin (C-2) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-3 (Ab-3), human (mouse), was from Calbiochem (San Diego, CA).

Results and Discussion

The metastatic non–small cell lung cancer H1299 carcinoma cell line does not express p53 or p16 INK4a. Although sensitivity to photodynamic therapy is not altered in human tumor cells after p53 abrogation (16), inactivation of the p53 and p16 INK4a pathways does not completely abrogate stress responses in carcinoma cells (17).

We evaluated if Photofrin/photodynamic therapy plus CDDP or gemcitabine induces an additive or even a synergistic effect, our aim being to determine whether the

dose of the cytostatic drug can be reduced. To identify the most effective doses of the components of combination therapy, we analyzed (*a*) the response of Photofrin-treated H1299 cells to increasing light doses, (*b*) the response of cells to increasing concentrations of CDDP or gemcitabine, and (*c*) the response of cells to combined therapy in selected conditions.

We first tested low CDDP (≤2.5 μmol/L) and gemcitabine (between 2 and 8 nmol/L) concentrations and mild photodynamic therapy conditions (Photofrin concentration, 2.5 μg/mL; light fluence, 0.18–0.54 J/cm^2). However, we focused on a concentration of 2.5 μmol/L for CDDP and 4 nmol/L for gemcitabine, and the fluence of choice was 0.54 J/cm^2. These drug concentrations caused a near 50% mortality when used alone. Higher concentrations (>5 μmol/L CDDP or >8 nmol/L gemcitabine) were not compatible with photodynamic therapy at a fluence of 0.54 J/cm^2, because no (CDDP) or only a few cells (gemcitabine) survived this combined treatment. Fluctuations of the lamp, which make it difficult to measure fluence accurately within the short irradiation time (<30 seconds), precluded the use of lower fluences (<0.2 J/cm^2).

We evaluated Photofrin-induced toxicity in the absence of light (''dark toxicity'') in 5×10^4 cells seeded in 35-mm tissue culture dishes and treated 24 hours later with 0, 2.5, 4, 8, 10, and 25 μg/mL Photofrin. Sixteen hours later, cell viability was evaluated by the trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (data not shown). We compared the viability of normally growing cells (100%) versus Photofrin-treated but not light-sensitized cells. Cell viability decreased in a dose-dependent fashion starting with 3.0 μg/mL Photofrin, and 25 μg/mL Photofrin reduced cell viability by a factor >5. With 2.5 μg/mL Photofrin, toxicity did not exceed 5%; hence, we used this dose in the photodynamic therapy experiments.

Individual Treatments

Because photodynamic therapy was applied to cells treated for 24 hours with CDDP or gemcitabine, we first determined the cycle stage of cells on irradiation. To this end, we incubated H1299 cells with 4 nmol/L gemcitabine or 2.5 μmol/L CDDP for 0 (controls), 8, 12, and 24 hours. The cells were then washed and immediately fixed for cytofluorimetry. Gemcitabine rapidly induced depletion of G_2-M cells and hence an accumulation of G_1 cells; 12 hours later, almost all cells were in S phase (Table 1). Therefore, when photodynamic therapy was applied, gemcitabine-exposed (4 nmol/L) cells were mostly in S phase. CDDP, on the contrary, caused cell accumulation in S phase followed by cell synchronization in G_1. Therefore, on photodynamic therapy, the S and G_2-M phases were partially emptied. After cells were withdrawn from CDDP- or gemcitabine-containing medium and placed in complete medium, they reentered the normal cycle within 24 hours (see also Fig. 1).

The two cytostatic drugs dose-dependently affected cell viability (Fig. 2). At 2.5 μmol/L, CDDP reduced cell viability by ~50% versus controls, and with 12 μmol/L CDDP, cell viability approached 0. Similarly, 4 nmol/L gemcitabine reduced cell viability by ~50%, and 10 nmol/L gemcitabine reduced it to <20% of control values. Cell viability was not completely abolished even at much higher gemcitabine concentrations (data not shown).

Table 1. Cytofluorimetric profiles of H1299 cells at 0 and 8, 12, and 24 hours of treatment with CDDP (2.5 μmol/L) or gemcitabine (4 nmol/L)

Phase	Control	CDDP (2.5 μmol/L)			Gemcitabine (4 nmol/L)		
	0 h	8 h	12 h	24 h	8 h	12 h	24 h
G_0-G_1	47.3	40.2	39.0	**64.9**	57.6	56.5	18.1
S	39.4	51.0	51.3	28.5	42.4	43.4	**81.9**
G_2-M	13.3	8.8	9.7	6.6	0	0.1	0

NOTE: Values are expressed as percent. Note the specific effects at 24 hours (bold).

The photodynamic therapy experiments were done in triplicate with 5×10^4 H1299 cells seeded in 35-mm tissue culture dishes. Twenty-four hours after seeding, the cells were incubated with Photofrin (2.5 μg/mL) for 16 hours, washed thrice with HBSS, and irradiated with increasing doses of light (see below). Cells were released into drug-free complete medium and left to grow for ~3 or 24 hours. At nontoxic Photofrin concentrations (i.e., 2.5 μg/mL), photodynamic therapy dose-dependently affected the magnitude of cell injury (Fig. 3). When light fluence increased from 0 to ~1.40 J/cm^2, cell viability decreased proportionally. No cells survived a fluence of 1.80 J/cm^2 (data not shown). At 0.54 J/cm^2, cell viability was reduced by ~50%. We used this sublethal dose in combination experiments with cytotoxic drugs. Unless indicated otherwise, all experiments reported hereafter were conducted with cells incubated for 16 hours with 2.5 μg/mL Photofrin and irradiated with a light fluence of 0.54 J/cm^2.

Combined Effects

Because we aimed at using the lowest drug concentration possible, we tested CDDP concentrations between 0.5 and 2.5 μmol/L and gemcitabine concentrations between 2 and 8 nmol/L while maintaining fluence at 0.54 J/cm^2. Very few cells treated with low concentrations of CDDP or gemcitabine survived fluences above 0.54 J/cm^2. However, within the concentration range tested, gemcitabine did not kill all H1299 cells (Fig. 2). Thus, for gemcitabine, we evaluated the effects caused by a fixed fluence on cells incubated with drug concentrations between 2 and 8 nmol/L and the effects caused by fixed drug concentrations (i.e., 4 and 8 nmol/L) and light fluences of 0.18, 0.28, 0.36, and 0.54 J/cm^2. We evaluated the efficacy of combined therapy by calculating the CI as detailed in the next paragraph and as reported in Table 2A (fixed fluence, 0.54 J/cm^2; CDDP between 0.25 and 2.50 μmol/L), Table 2B (fixed fluence, 0.28 J/cm^2; gemcitabine between 2 and 8 nmol/L), and Table 2C (fluence between 0.18 and 0.54 J/cm^2, gemcitabine 4 nmol/L). The most striking results were obtained with a light fluence of 0.54 J/cm^2 combined

with 2.5 μmol/L CDDP or 4 nmol/L gemcitabine. Individually, photodynamic therapy, CDDP, and gemcitabine reduced cell viability to 50 ± 7%, whereas photodynamic therapy combined with CDDP or gemcitabine reduced cell viability to 8 ± 2% and 14 ± 4%, respectively (Fig. 4).

Median Effect Analysis

Using the Chou and Talalay method (12) and the equations developed by Mack et al. (13), we calculated the CI and show that the effects of combined therapy at a fixed light fluence (0.54 J/cm^2) are additive within the range of CDDP concentrations used. These effects changed from possibly synergistic to explicitly synergistic at CDDP concentrations above 2.0 μmol/L (Table 2A). Differently, the overall effect remained additive with gemcitabine plus photodynamic therapy. The CI was 1 both when the drug was increased from 2 to 8 nmol/L and the light fluence was fixed at 0.28 J/cm^2 (Table 2B) and with gemcitabine fixed at 4 nmol/L and light fluences were between 0.18 and 0.54 J/cm^2 (Table 2C).

Cell Cycle and Protein Expression

To investigate the mechanisms by which Photofrin/photodynamic therapy and the cytotoxic drugs exert their effects when used alone or in combination, we analyzed the H1299 cell cycle and the expression levels of the Bcl-2, Bcl-XL, Bax, caspase-3, p21, pRb, cyclin A, Cdk2, HSP60, and HSP70 proteins (Fig. 5), which are related to apoptosis, cell cycle, and stress. Figure 1 shows the schedules of single-agent and combination treatments. The cytofluorimetric profiles obtained after the release of cells in drug-free medium for an additional 24 hours represents the distribution that would be found in the event of ineffective photodynamic therapy.

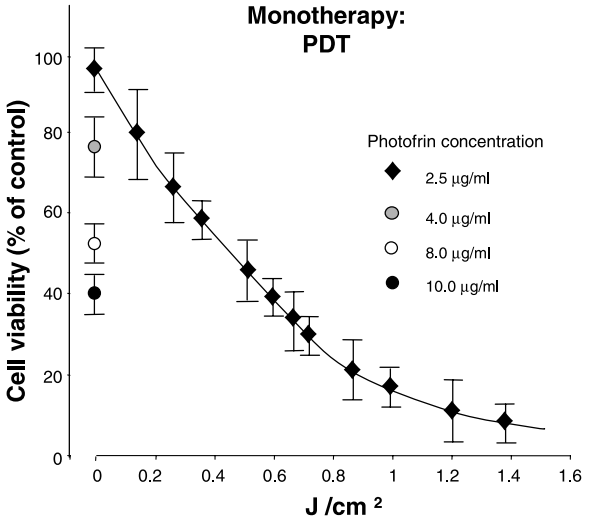

Figure 3. H1299 cells were incubated in the dark for 16 h with Photofrin (2.5, 4.0, 8, or 10 μg/mL). Dark toxicity was low (<5%) with 2.5 μg/mL Photofrin and high with 4 to 10 μg/mL (*circles*). Cell viability on irradiation was measured by the trypan blue exclusion test with a Photofrin concentration of 2.5 μg/mL. *Points,* average of three determinations; *bars,* SD. *PDT,* photodynamic therapy.

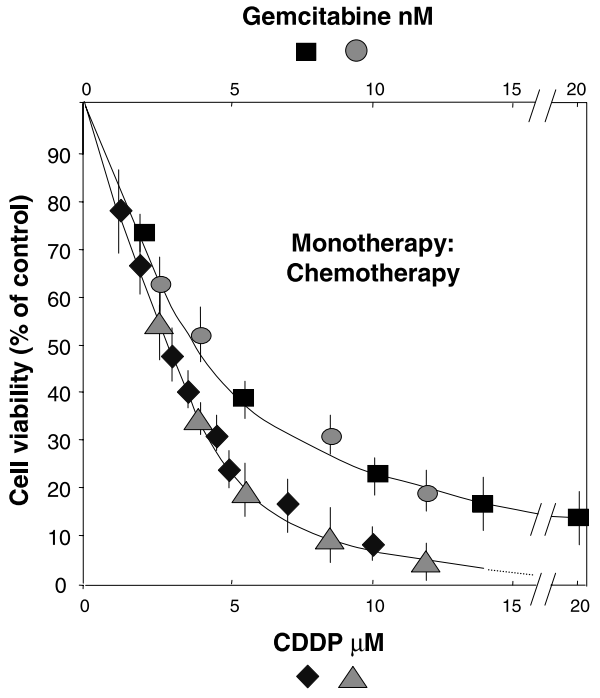

Figure 2. Effect of increasing concentrations of CDDP (*bottom curve*; 0–15 μmol/L) and gemcitabine (*top curve*; 0–12 nmol/L) on the viability of H1299 cells. Two sets of data from separate experiments are reported. Cells were incubated at the indicated CDDP or gemcitabine concentrations for 24 h, washed, and incubated for 24 h in fresh medium. Cell viability was measured by counting cells negative to trypan staining. Residual viability was expressed as percentage of untreated cells (controls). *Triangles* and *diamonds,* CDDP; *squares* and *circles,* gemcitabine. *Points,* average of three determinations; *bars,* SD.

CDDP. Using cytofluorimetry, we examined the H1299 cell cycle after 0, 8, 12, and 24 hours of incubation with 2.5 μmol/L CDDP (Table 1). After 24 hours, cells have accumulated in G_0-G_1 phase (see Table 1; Fig. 6) with a consequent loss of cells (~50%; trypan blue assay) in S phase. After exposure to CDDP for 24 hours, cells were released into fresh medium for ~3 and 24 hours. Synchronization in S phase was sustained for the first 3 hours, whereas cells approached the normal cycle at 24 hours (data not shown). This observation implies that the arrest in S phase was reversible. Protein expression studies (Western blot) support this notion. In fact, p21 protein expression was enhanced in H1299 cells exposed to 2.5 μmol/L CDDP for 24 hours (Fig. 5A, *lane 2*). However, p21 expression returned to basal levels within 24 hours (Fig. 5A, *lane 5*).

Surprisingly, although H1299 cells are p53 mutated (−/−), p21 protein expression seemed to be up-regulated at transcriptional level in the presence of CDDP. However, the p21 gene is also regulated by p53-independent factors, including growth promotion factors and differentiation-associated transcription factors (18). Moreover, CDDP concentration and incubation conditions may account for cytofluorimetric profile changes in various cells. In fact, whereas high CDDP concentrations cause cells to accumulate in the S-G_2 phases (13, 19–21), low CDDP doses induce

Table 2. Monotherapy versus combined therapy: median effect analysis and CI

A. Comparison of monotherapy (photodynamic therapy or CDDP) versus combined therapy (photodynamic therapy and CDDP) at a constant light fluence (CI < 1 indicates synergy)

Monotherapy			Combined therapy			
1	2	3	4	5	6	7
$(D_x)_1$ Fluence (J/cm2)	$(D_x)_2$ CDDP (μmol/L)	Observed effect cell viability (%)	D_1 fluence (J/cm^2)	D_2 CDDP (μmol/L)	CI	Combined effect
0	0	100	0.54	—	—	—
0.18	ND	82		—	—	—
0.36	1.5	68.5		—	—	—
0.54	2.5	50		0	1	Additive
0.61	3.0	45		0.25	1.02	Additive
0.73	4.0	36		0.50	0.96	Additive
0.81	1.5	33		1.00	1.04	Additive
0.99	5.0	25		1.50	0.90	Additive
1.08	6.0	20		2.00	0.85	Additive/synergistic
1.25	9.0	12		2.25	0.76	Synergistic
1.44	10.0	7		2.50	0.74	Synergistic

B. Comparison of monotherapy (photodynamic therapy or gemcitabine) versus combined therapy (photodynamic therapy and gemcitabine) at a constant light fluence (CI = 1 indicates an additive effect)

Monotherapy			Combined therapy			
1	2	3	4	5	6	7
$(D_x)_1$ Fluence (J/cm^2)	$(D_x)_2$ Gemcitabine (nmol/L)	Observed effect cell viability (%)	D_1 Fluence (J/cm^2)	D_2 Gemcitabine (nmol/L)	CI	Combined effect
0.28	1.5	80	0.28	0	1	—
0.38	2.0	72		2.0	1.03	Additive
0.60	5.5	44		4.0	1.06	Additive
0.80	8.0	30		6.0	0.97	Additive
0.92	12.5	22		8.0	0.99	Additive

C. Comparison of monotherapy (photodynamic therapy or gemcitabine) versus combined therapy (photodynamic therapy and gemcitabine) at a constant gemcitabine concentration (CI = 1 indicates additivity)

Monotherapy			Combined therapy			
1	2	3	4	5	6	7
$(D_x)_1$ Fluence (J/cm^2)	$(D_x)_2$ Gemcitabine (nmol/L)	Observed effect cell viability (%)	D_1 Fluence (J/cm^2)	D_2 Gemcitabine (nmol/L)	CI	Combined effect
0.54	4.0	50	0	4.0	1	—
0.65	6.0	41	0.18		1.05	Additive
0.72	6.5	37	0.28		1.01	Additive
0.98	11.0	21	0.36		0.99	Additive
1.2	14.5	15	0.54		0.98	Additive

NOTE: $(D_x)_1$ and $(D_x)_2$ are the doses of light or drug necessary to obtain a given effect when used alone; (D_1) and (D_2) are the doses of light and drug needed to obtain the same effect when used in the combination. Column 3 shows the residual cell viability on irradiation at the indicated light fluences [column 1, $(D_x)_1$] or drug treatment at the indicated concentrations [column 2, $(D_x)_2$], respectively. Columns 4 and 5 (D_1 and D_2) report the light fluences and drug concentrations used in combination. Columns 6 and 7 show the CI and the type of effect ensuing from combination. Monotherapy data in Table 2A–C are taken from Figs. 2 and 3, which provide the relative SD. Combined therapy data were obtained in separate experiments (data not shown).

an increase in p21 expression unaccompanied by bromodeoxyuridine incorporation (21), which suggests an arrest in G_1. A G_1 arrest has also been reported in other cell lines at high CDDP concentrations (50 μmol/L) but with short-term (2 hours) exposure (22). Also in our hands, 24-hour incubation with CDDP concentrations as high as 8 μmol/L caused cells to accumulate in G_2 (from <14% at 2.5 μmol/L to >31% at 8 μmol/L; data not shown). In conclusion,

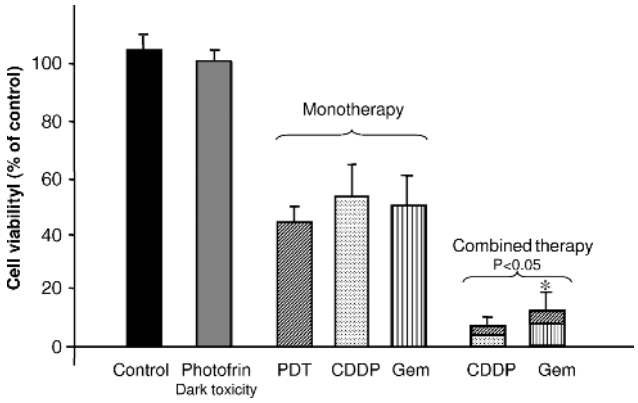

Figure 4. Effect of single versus combined treatment on the viability of H1299 cells. Viability was evaluated by the trypan blue assay in control cells, and cells incubated in the dark with Photofrin (2.5 μg/mL), after monotherapy with CDDP (2.5 μmol/L), gemcitabine (*Gem*; 4 nmol/L), or photodynamic therapy (0.54 J/cm^2), or combined therapy with 2.5 μmol/L CDDP and photodynamic therapy (0.54 J/cm^2) or 4 nmol/L gemcitabine and photodynamic therapy (0.54 J/cm^2). *Columns,* average of four determinations (trypan blue exclusion assay); *bars,* SD.

it seems that exposure of H1229 cells to sublethal doses of CDDP for 24 hours was detrimental for cells in S phase and caused cells to accumulate in G_0-G_1. This cytofluorimetric scenario is mirrored by the results of Western blot experiments.

Gemcitabine. The cytofluorimetric profile of H1299 cells after incubation for 24 hours with low doses of gemcitabine indicates a significant accumulation of cells in S phase (Table 1; Fig. 6), resulting from a massive (50%; trypan blue assay) loss of cells in the G_0-G_1 phase. The cell cycle was profoundly altered when cells were exposed to gemcitabine (4 nmol/L) for 8, 12, and 24 hours. At 24 hours, most cells were synchronized in S phase (Table 1). Synchronization in S phase was sustained for at least 3 hours after drug removal and the release of cells into fresh medium (data not shown). The arrest in S phase was not permanent as indicated by the fact that surviving cells reverted to the original (control) profile within 24 hours (data not shown). Cell accumulation in S phase after 24-hour incubation with gemcitabine, as shown by cytofluorimetry, was also consistent with an increase in cyclin A expression (Fig. 5B, *lane* 2) and in line with previous observations (23, 24). In conclusion, treatment for 24 hours of H1299 with sublethal gemcitabine doses was detrimental for cells in the G_0-G_1 phase and induced an accumulation of cells in S phase. This interpretation is supported by both Western blot experiments and cytofluorimetric profiles. The altered expression profiles were totally reverted 24 hours after drug removal.

Photodynamic Therapy. Photodynamic therapy with 2.5 μg/mL Photofrin (i.e., at a concentration that does not present ''dark toxicity'') induced cellular damage proportional to light fluence. Cell injury went from reversible, with rapid recovery of cellular functions and growth, to profound and irreversible. At concentrations ~10 μg/mL Photofrin, photodynamic therapy causes cell death, apoptosis, or necrosis depending on such factors as light

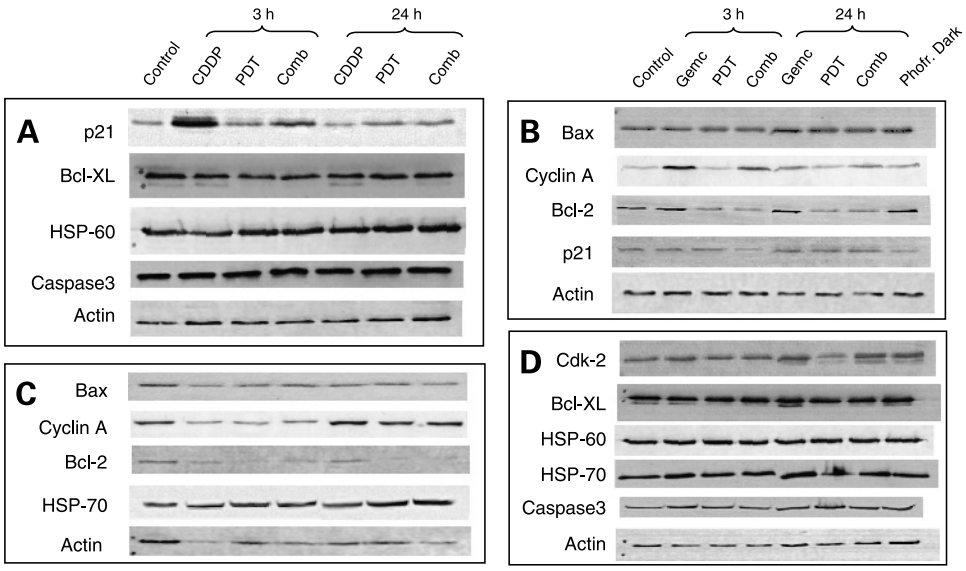

Figure 5. Protein expression in cells treated with monotherapy or combined therapy. Protein extracts were analyzed 3 or 24 h after cells were placed in fresh medium after individual or combined treatments (see Fig. 4). **A** and **C**, CDDP; **B** and **D**, gemcitabine. Actin was always used as loading control. Further observations: photodynamic therapy increased the expression of p21 (1 – 3 h), split the Bcl-XL electrophoretic band in a doublet (see also **D**), and seemed to destroy Bcl-2 (**B**). Photodynamic therapy also caused a detectable increase in HSP60 and HSP70 expression. Indeed, although the expression of actin in **C** is highly variable, the expression of HSP70 on photodynamic therapy (alone or in combination) is clearly increased, whereas the questionable fading of Bcl-2 is confirmed by inspection of **B**. Gemcitabine significantly affected cyclin A expression (**B**). Treatment did not affect Bax and caspase-3 expression (**C, B** and **A, D**). Photofrin in the absence of light (dark effect) did not affect cells as shown by two experiments shown in **B** and **D**.

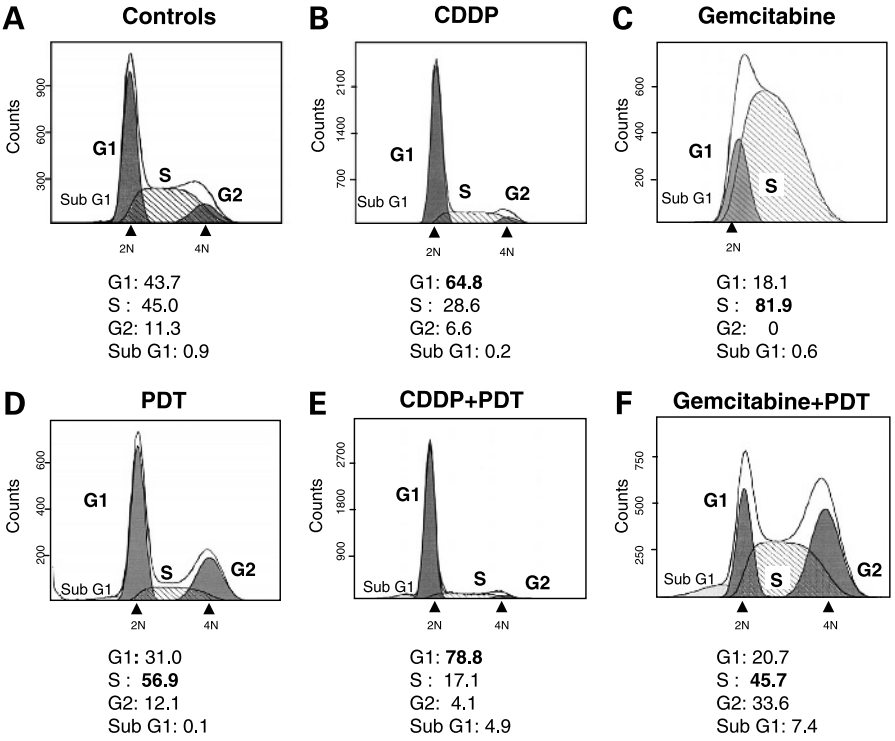

Figure 6. Representative experiments depicting the cell cycle distribution of H1299 cells as measured by flow cytometric analysis of DNA content before (**A**, controls) and after treatment with CDDP (**B**), gemcitabine (**C**), photodynamic therapy alone (**D**), or in sequential combination (**E** and **F**). In these cases, cells were analyzed 24 h after photodynamic therapy. The FACScan software assigns apoptotic cells and large cell debris to sub-G_1 phase. *Gray histograms,* G_0-G_1 and G_2-M phases; *dashed histograms,* S phase.

fluence, photosensitizer concentration, and cell type (25). The Photofrin incubation protocol has also been implicated in the cell death pathway (26). Although we focused on the effects of a single light dose (0.54 J/cm^2), after 16 hours of incubation with 2.5 μg/L Photofrin, few H1299 cells underwent apoptosis (sub-G_1; Fig. 6). Indeed, the expression of Bax (Fig. 5B and C) and caspase-3 (Fig. 5A and D) was unchanged 3 and 24 hours after photodynamic therapy. Differently, Bcl-2 expression was significantly reduced. This coincides with the finding that some photosensitizers induce Bcl-2 degradation (27). Moreover, Hypericin/photodynamic therapy alters the expression and function of Bcl-2 and delays the onset of apoptosis (28). Here, we show that also Photofrin/photodynamic therapy causes Bcl-2 degradation. This degradation, which can only be inferred from Fig. 5C (*lane 3*; because of the large actin control variability), clearly emerges in Fig. 5B (i.e., *lane 3*). Finally, Photofrin photodynamic therapy caused changes in the electrophoretic profile of Bcl-XL as indicated by the disappearance of the faster-migrating Bcl-XL isoform (Fig. 5A, *lane 3*, and D, *lane 3*). Pc 4 photodynamic therapy has been shown to damage Bcl-XL isoforms in several other human cancer cells (29). Although Hanlon et al. (30) reported a very important increase in the expressions of HSP60 and HSP70 proteins, they were only modestly, albeit clearly, enhanced by photodynamic therapy in our conditions (Fig. 5A, B and C, D, respectively). However, Hanlon et al. used different cell lines as well as much higher Photofrin concentrations. Indeed, Photofrin concentrations higher than 5 μg/mL are per se highly toxic for H1299 cells even in the dark.

We used Western blotting to evaluate changes in protein expression after monotherapy (i.e., individual drugs or photodynamic therapy) and after combined treatments (i.e., each drug + photodynamic therapy). It is noteworthy that with time most protein expression profiles tended to revert to their original qualitative and quantitative patterns (Fig. 5A–D). The apparent ''increase'' in cyclin A expression (Fig. 5C, *lanes 5–7*) may represent an experimental artifact given the large variability of the actin control. As stated, the expression of HSP60 and HSP70 proteins was enhanced after 24 hours albeit to a lesser extent versus cyclin A (Fig. 5A, C, and D). The cell cycle patterns after combined therapy (Fig. 6E and F) are not in disagreement with the protein expression profiling data.

Figure 6 is representative of various experiments concerning the effects of combined treatment. Within 24 hours, 2.5 μmol/L CDDP killed ~50% of cells, most of which were in S phase (Fig. 6B). At variance, photodynamic therapy (0.54 J/cm^2) eliminated mainly G_0-G_1 cells (Fig. 6D), and most of the remaining cells were in S phase. In conclusion, CDDP, at a low concentration, killed cells in S phase, and surviving cells occurred mainly in G_0-G_1, a phase in which cells are susceptible to photodynamic therapy (Table 2A).

Gemcitabine alone, at a concentration as low as 4 nmol/L, killed ~50% of cells (most of which were in G_0-G_1 phase) and induced a pronounced accumulation of cells in S phase (Fig. 6C). It seems that gemcitabine at this concentration enhances photodynamic therapy cytotoxicity, but to a much lesser extent than CDDP, and the effect is essentially additive (Table 2B and C). In controls, 44.2% were in G_0-G_1, 44.6% were in S, and 11.2% were in G_2-M. After photodynamic

therapy (0.54 J/cm^2) or 24 hours of incubation with CDDP (2.5 μmol/L) or gemcitabine (4 nmol/L; all conditions that kill ~50% of cells), 31.2%, 76.1%, and 18.0% of cells were in G_0-G_1, 56.7%, 10.5%, and 82.0% were in S, and 12.1%, 13.4%, and ~0% were in G_2-M, respectively. Hence, photodynamic therapy and gemcitabine preferentially damage cells in G_0-G_1, whereas CDDP targets cells in S phase. In conclusion, it seems that the targeting of cells in different phases results in an accumulation of death signals caused separately and independently by photodynamic therapy and a suitable cytotoxic drug.

Photodynamic therapy is a well-established stand-alone therapy (9) in which the photosensitizer can be administered by i.v. injection or applied on the skin and selectively targets cancer cells. Its effectiveness increases when combined with other therapeutic measures. Here, we describe a way to exploit mutual reinforcement of two independent therapeutic modalities: Photofrin/photodynamic therapy and chemotherapy. Photofrin and porphyrin derivatives, in general, are the photosensitizers most widely used in photodynamic therapy. At variance with gemcitabine, which to our knowledge has never been used with Photofrin/photodynamic therapy, CDDP has been used in combination with various photosensitizers, including porphyrin derivatives (11, 31–35). However, previous studies were mainly descriptive and were limited to observations about the effects exerted on cell mortality. No reasons were advanced to account for the greater efficacy of combined treatment, nor did those studies report changes in protein expression and related cell cycle changes. Our study shows that doses of CDDP or gemcitabine and light/Photofrin that were partially effective in killing H1299 cells when given singly were remarkably more effective when used in combination. These effects span from additive to synergistic. Additivity occurred with both drugs, but the effect was synergistic when the drug (CDDP) exerted its activity disjointed, in terms of cell cycle, from those of photodynamic therapy. Under the conditions we used, not all metastatic cells were killed. However, preincubation of H1299 cells with the low CDDP dose (2.5 μmol/L) followed by their exposure to a light fluence only 1.5-fold higher (i.e., ~0.80 J/cm^2) than that used in most experiments described herein (~0.54 J/cm^2) resulted in the rapid death of all cells.

In conclusion, selectively localized photosensitizers and appropriate doses of light combined with low doses of chemotherapeutic drugs represent a promising treatment strategy for cancer. In principle, combinations of photodynamic therapy and drugs would not only destroy cancer cells more efficiently but would also reduce the noxious side effects of chemotherapy.

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References

1. Dougherty TJ, Gomer CJ, Henderson BW, et al. Photodynamic therapy. J Natl Cancer Inst 1998;90:889–905.

2. Gossner L. Photodynamic therapy: esophagus [review]. Can J Gastroenterol 2002;16:642–4.

3. Gomer TJ, Dougherty TJ. Determination of ^{3}H and ^{14}C hematoporphyrin derivative distribution in malignant and normal tissues. Cancer Res 1979;39:146–51.

4. Oleinick NL, Morris RL, Belichenko I. The role of apoptosis in response to photodynamic therapy: what, where, why, and how. Photochem Photobiol Sci 2002;1:1–21.

5. Roninson IB, Broude EV, Chang BD. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells [review]. Drug Resist Updat 2001;4:303–13.

6. Crul M, van Waardenburg RCA M, Beijnen JH, Schellens JHM. DNA-based drug interactions of cis-platin. Cancer 2002;28:291–303.

7. Mackey JR, Mani RS, Selner M, et al. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res 1998;58:4349–57.

8. Shewach DS, Reynolds KK, Hertel L. Nucleotide specificity of human deoxycytidine kinase. Mol Pharmacol 1992;42:518–24.

9. Dolmans DE, Fukumura D, Jain RK. Photodynamic therapy for cancer [review]. Nat Rev Cancer 2003;3:380–7.

10. Doyle A, Griffiths JB, Newell DG. Trypan blue exclusion method for cell viability estimation. In: Cell and tissue culture: laboratory procedures. London: Wiley; 1993; 4:5.2.

11. Crescenzi E, Varriale L, Iovino M, Chiaviello A, Veneziani BM, Palumbo G. Photodynamic therapy with indocyanine green complements and enhances low-dose cisplatin cytotoxicity in MCF-7 breast cancer cells. Mol Cancer Ther 2004;3:537–44.

12. Chou TC, Talalay P. A Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22:27–55.

13. Mack PC, Gandara DR, Lau AH, Lara PN, Jr., Edelman MJ, Gumerlock PH. Cell cycle-dependent potentiation of cisplatin by UCN-01 in non-small-cell lung carcinoma. Cancer Chemother Pharmacol 2003;51:337–48.

14. 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;72:248–54.

15. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1971;227:680–5.

16. Fisher AM, Ferrario A, Rucker N, Zhang S, Gomer CJ. Photodynamic therapy sensitivity is not altered in human tumor cells after abrogation of p53 function. Cancer Res 1999;59:331–5.

17. Itahana K, Dimri G, Campisi J. Regulation of cellular senescence by p53. Eur J Biochem 2001;268:2784–91.

18. Agami R, Bernards R. Distinct initiation and maintenance mechanisms cooperate to induce G_1 cell cycle arrest in response to DNA damage. Cell 2000;102:55–66.

19. Chang BD, Swift ME, Shen M, Fang J, Broude EV, Roninson IB. Molecular determinants of terminal growth arrest induced in tumor cells by a chemotherapeutic agent. Proc Natl Acad Sci U S A 2002;99:389–94.

20. Sekiguchi I, Suzuki M, Tamada T, Shinomiya N, Tsuru S, Murata M. Effects of cisplatin on cell cycle kinetics, morphological change, and cleavage pattern of DNA in two human ovarian carcinoma cell lines. Oncology 1996;53:19–26.

21. Lanzi C, Perego P, Supino R, et al. Decreased drug accumulation and increased tolerance to DNA damage in tumor cells with a low level of cisplatin resistance. Biochem Pharmacol 1998;55:1247–54.

22. Miyaji T, Kato A, Yasuda H, Fujigaki Y, Hishida A. Role of the increase in p21 in cisplatin-induced acute renal failure in rats. J Am Soc Nephrol 2001;12:900–8.

23. Erlandsson F, Wählby C, Ekholm S, Bengtsson E, Zetterberg A. A detailed analysis of cyclin A accumulation at the G([1])/S border in normal and transformed cells. Exp Cell Res 2000;259:86–95.

24. Shi Z, Azuma A, Sampath D, Li YX, Huang P, Plunkett W. S-Phase arrest by nucleoside analogues and abrogation of survival without cell cycle progression by 7-hydroxy-stauro-sporine. Cancer Res 2001;61:1065–72.

25. Marcinkowska A, Malarska A, Saczko J, et al. Photofrin-factor of photodynamic therapy induces apoptosis and necrosis. Folia Histochem Cytobiol 2001;Suppl 2:177–8.

26. Dellinger M. Apoptosis or necrosis following Photofrin photosensitization: influence of the incubation protocol. Photochem Photobiol 1996; 64:182 – 7.

27. Xue LY, Chiu SM, Oleinick NL. Photochemical destruction of the Bcl-2 oncoprotein during photodynamic therapy with the phthalocyanine photosensitizer Pc 4. Oncogene 2000;20:3420 – 7.

28. Vantieghem A, Xu Y, Assefa Z, et al. Phosphorylation of Bcl-2 in G_2-M phase-arrested cells following photodynamic therapy with hypericin involves a CDK1-mediated signal. J Biol Chem 2002;277:37718 – 31.

29. Xue LY, Chiu SM, Fiebig A, Andrews DW, Oleinick NL. Photodamage to multiple Bcl-XL isoforms by photodynamic therapy with the phthalocyanine photosensitizer Pc-4. Oncogene 2003;22:9197 – 204.

30. Hanlon JG, Adams K, Rainbow AJ, Gupta RS, Singh G. Induction of Hsp60 by Photofrin-mediated photodynamic therapy. J Photochem Photobiol B 2001;64:55 – 61.

31. Zimmermann A, Walt H, Haller U, Baas P, Klein SD. Effects of chlorin-mediated photodynamic therapy combined with fluoropyrimidines *in vitro* and in a patient. Cancer Chemother Pharmacol 2003;51: 147 – 54.

32. Canti G, Nicolin A, Cubeddu R, Taroni P, Bandieramonte G, Valentini G. Antitumor efficacy of the combination of photodynamic therapy and chemotherapy in murine tumors. Cancer Lett 1998;125: 39 – 44.

33. Nahabedian MY, Cohen RA, Contino MF, et al. Combination cytotoxic chemotherapy with cisplatin or doxorubicin and photodynamic therapy in murine tumors. J Natl Cancer Inst 1988;80:739 – 43.

34. Sharkey SM, Wilson BC, Moorehead R, Singh G. Mitochondrial alterations in photodynamic therapy-resistant cells. Cancer Res 1993;53: 4994 – 9.

35. Ma LW, Moan J, Berg K, Peng Q, Steen HB. Potentiation of photodynamic therapy by mitomycin C in cultured human colon adenocarcinoma cells. Radiat Res 1993;134:22 – 8.