

Photodynamic therapy with indocyanine green complements and enhances low-dose cisplatin cytotoxicity in MCF-7 breast cancer cells

Elvira Crescenzi, Linda Varriale, Mariangela Iovino, Angela Chiaviello, Bianca Maria Veneziani, and Giuseppe Palumbo

Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano," University of Naples, Federico II, Naples, Italy

Abstract

Objective: We investigated the effects of photodynamic therapy (PDT) combined with low-dose chemotherapy on breast cancer cells. Photodynamic treatment was administered by irradiating indocyanine green-preloaded MCF-7 cells with an IR diode laser source at 805 nm; cisplatin was used for chemotherapy. **Methods:** The dose-response phenomena associated with the two treatments administered individually and together were evaluated with the following tests: trypan blue dye exclusion, 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) assay, clonogenic survival, thymidine and methionine incorporation, and insulin-dependent and insulin-independent glucose transport. **Results:** Viability and metabolic data demonstrated mutual reinforcement of therapeutic efficacy. However, isobolographic analysis of quantal and variable data indicated that reinforcement was additive according to trypan blue data and synergistic according to MTT data. To investigate the molecular mechanisms underlying alterations in cell proliferation and apoptosis, we evaluated (by Western blotting) the expression of proteins Bcl-2, Bax, Bcl-X_L, p21, p53, and poly(ADP-ribose) polymerase. Photodynamic treatment caused transient selective destruction of Bcl-2 and up-regulation of Bax. It also induced apoptosis in a limited fraction of cells (10–12%). Flow cytometry data showed that PDT killed mostly G₁-phase cells, whereas cisplatin killed mostly S-phase cells. This disjointed phase-related effect may account for the favorable effects exerted by combined treatment. **Conclusions:** Our findings imply that low doses of cytostatic drugs may be as effective or even more effective

than currently used doses if appropriately combined with PDT. [Mol Cancer Ther 2004;3(5):537–44]

Introduction

Photodynamic therapy (PDT) is a relatively new and effective treatment for human tumors (1). It entails local or systemic administration of a photosensitizer and subsequent exposure of the neoplastic area to a light of suitable wavelength. The rationale is that, on exposure to light, the photosensitizer, which is absorbed by all cells and selectively retained by malignant tissue (2, 3), is promoted to an excited state and then, as it decays, induces local release of cytotoxic reactive oxygen species. Depending on the experimental conditions and cell sensitivity, the cytotoxic molecular species resulting from PDT may trigger cell apoptosis or induce necrosis (4).

The near-IR spectral region is particularly suitable for PDT because it penetrates deep into tissues without causing significant heating. To date, the most suitable IR-photosensitizable molecule is indocyanine green dye (ICG). In fact, ICG is not toxic, is widely used in humans as a diagnostic tool in hemodynamics (5, 6), has an absorption peak between 775 and 810 nm (7, 8), and is a photosensitizer.

Cisplatin [*cis*-diammine-dichloroplatinum (II)] is used in the management of various cancers. By forming adducts to DNA, cisplatin inhibits DNA replication and chain elongation, which accounts for its antineoplastic activity. In clinical practice, cisplatin is often combined with other chemotherapeutic agents. Synergy between cisplatin and other chemotherapeutic agents occurs by various pathways: increased intracellular drug accumulation, enhanced binding to DNA, and decreased DNA repair (9).

The MCF-7 cell line was established 30 years ago from the pleural effusion of a patient with a breast carcinoma (10). These malignant cells have functional p53, lack caspase-3 activity, maintain a functionally intact estrogen receptor, and have constitutively high levels of Bcl-2, a protein endowed with cytostatic and antiapoptotic activities (11, 12). The latter two features are probably closely related because, as we have recently shown, the coding sequence of the *bcl-2* gene includes a functioning estrogen-responsive element (13).

MCF-7 cells are remarkably resistant to various treatments including cisplatin and some types of PDT but not to tumor necrosis factor or staurosporin (14). However, the cells become sensitive to cisplatin on p53 disruption (15). In contrast, parental MCF-7 cells and p53-abrogated cells are equally sensitive to photofrin/PDT (16).

In an attempt to identify treatment combinations in which the dose of the toxic compound could be decreased without reducing efficacy, we examined the effects of

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Note: E. Crescenzi and L. Varriale contributed equally to this study.

Requests for Reprints: Giuseppe Palumbo, Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano," University of Naples, Federico II, Via S. Pansini 5, 80131 Naples, Italy. Phone: 39-81-7463249; Fax: 39-81-7701016. E-mail: palumbo@unina.it

moderately toxic doses of cisplatin and ICG/PDT, administered separately and together, on various metabolic and proliferative parameters of MCF-7 cells. We also describe changes in the expression profile of some proteins involved in the control of the cellular proliferative status and apoptosis.

Materials and Methods

Cells

Human breast cancer MCF-7 cells were grown at 37°C in a humidified atmosphere (95% air and 5% CO₂), DMEM supplemented with phenol red, L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), and 10% FCS. Fresh medium was provided every 3 days.

Laser Source

The laser source was a diode array laser from Quanta System (Milan, Italy) emitting at 805 nm. The nominal output energy (continuous wave) was 0.65 W. The laser was equipped with an adjustable (0.1 s) time shutter.

Photosensitizer and Photosensitization

The ICG ($M_r = 775.0$) preparation (Sigma Chemical Co., St. Louis, MO) was iodide free, unlike the ICG preparations used for most previous studies (7, 17). The ICG stock solution was obtained by dissolving the deep green powder (~1 mg/ml) in 100 mM sodium phosphate (pH 7.2). Before measurements, appropriate aliquots of this solution were diluted to the desired concentration. The purity of the commercial product was checked by isocratic high-performance liquid chromatography in 10 mM sodium phosphate in 47% acetonitrile, 3% methanol using a C18 reverse-phase column equipped with a UV detector set at 230 nm. Aqueous ICG samples at various concentrations (between 10 and 400 µM) were analyzed by high-performance liquid chromatography immediately after their preparation and after extensive irradiation (>1000 J cm⁻²). The resulting chromatograms did not differ from that of nonirradiated ICG, except for a significant decrease in the relative height (and areas) of the ICG peaks, which indicates extensive photobleaching of the dye.

Photosensitization experiments were performed with MCF-7 cells incubated in 20 µM ICG for 24 h. Because of the low concentration of ICG used, we did not measure uptake of the dye by MCF-7 cells in these conditions. However, the ICG uptake in keratinocytes under similar experimental conditions was about 3–5 nmol/10⁵ cells (17). Plates were washed twice with ICG-free medium and positioned under the laser beam at the appropriate distance. The temperature of plates during irradiation was controlled by their partial immersion in a water bath heated at 35°C. Except for the dose-response curves shown in Fig. 1, cells were routinely irradiated with light doses of 25 J cm⁻². After exposure(s), cells (normally quadruplicates) were incubated in the dark at 37°C for an additional 5 h (or >10 h in some cases) before further analysis.

Sample Treatment Schedules

All experiments reported herein included several controls (*i.e.*, untreated cells, ICG-loaded but not light-

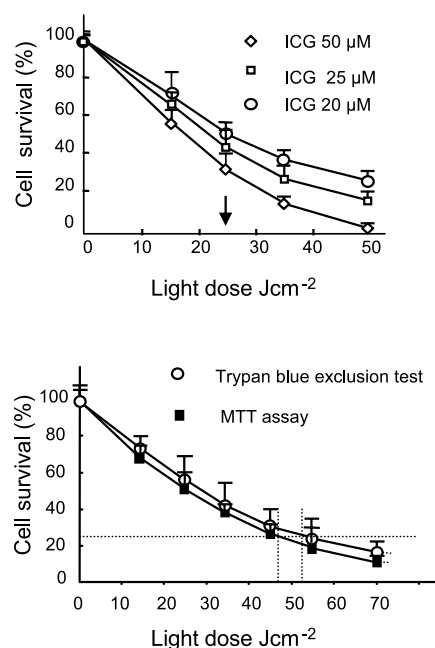


Figure 1. Effect of three doses of ICG photosensitization at 805 nm on MCF-7 cells. Five hours after each irradiation, cell viability was assessed by the trypan blue exclusion test. *Points*, average of four determinations; *bars*, SD. Combination treatments were performed at a light fluence of 25 J cm⁻² and an ICG concentration of 20 µM (*hatched line*).

sensitized cells, and cells exposed to light in the absence of ICG). The various parameters evaluated under these conditions did not differ and are not reported herein.

Cell Viability, Cytotoxicity, and Survival Assays

Trypan Blue Dye Exclusion Assay. Cell viability after treatment (irradiation, cisplatin, or both) was determined by the trypan blue dye exclusion test 5 h after each treatment (unless indicated otherwise). All assays were performed in quadruplicate and results are reported as the mean ± SD.

3-(4,5-Dimethylthiazol)-2,5-Diphenyltetrazolium Bromide Assay. Cell viability after each treatment (irradiation, cisplatin, or both) was determined by the 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) assay (18). MCF-7 cells (2×10^4 cells/0.2 ml) were seeded in 96-well plates and kept in the incubator at 37°C overnight. Cells were then treated with ICG alone, cisplatin alone, or ICG plus cisplatin and incubated for a further 24 h. After incubation, cells containing ICG were irradiated with a fluence of 25 J cm⁻². Five hours later, cells were washed, collected in tubes, and centrifuged at 1000 rpm for 5 min. Supernatants were removed and the cells were resuspended in ICG-free medium. Control cells were processed in the same way. A MTT stock solution was made by dissolving the crystals in a phenol red-free culture medium to a final concentration of 0.5 mg/ml. Typically, 50 µl of this solution were added to each well containing suspended cells in 200 µl of medium. After an additional 4 h incubation at 37°C, cells were collected by aspiration and

centrifuged at 1500 rpm for 7 min. The converted dye was extracted from the supernatant obtained by adding acidic (0.1 M HCl) isopropanol to the pelleted cells. Absorbance of the samples, read with a Titertek-Multiscan photometer (Huntsville, AL) equipped with a 532 nm filter, of converted dye in stimulated cells *versus* untreated controls is called MCF-7 viability. Quadruplicate samples were used in each experiment.

Clonogenic Survival Assay. The assay was performed exactly as described by Theodossiu *et al.* (19). Cells were seeded in 100 mm Petri dishes at low density ($\sim 3 \times 10^4$ /dish) and left to adhere for 24 h in a standard medium. ICG (20 μM) and/or cisplatin (8 μM) were added where appropriate. Twenty-four hours later, samples intended for PDT, alone or in combination, were irradiated (fluence of 25 J cm^{-2}).

After irradiation, cells were washed, immediately trypsinized, resuspended in single-cell suspension, and plated for the determination of macroscopic colony formation. After 6 days of growth, colonies were fixed with a 3:1 mixture of methanol/acetic acid and stained with crystal violet. Only colonies constituted by more than 50 cells were scored (19). Two separate experiments were performed using duplicate samples.

Flow Cytometry

Plates containing 5×10^5 MCF-7 cells were incubated for 24 h at 37°C in 2 ml complete medium (controls) or medium supplemented with ICG (20 μM), cisplatin (8 μM), or both (ICG and cisplatin). Photodynamic treatment of cells was performed as reported under Photosensitizer and Photosensitization. Cells were detached by trypsinization from 10 cm plates, suspended in serum-rich medium, centrifuged, washed twice with 1 ml PBS, and resuspended for storage (4°C) in absolute ethanol. Before analysis, fixed cells were washed twice, centrifuged, and resuspended in 1 ml PBS containing 5 μg RNase and 100 μg propidium iodide. Samples were stored in the dark for 20 min at room temperature before final readings. The cellular orange fluorescence of propidium iodide was detected on a linear scale using a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA) equipped with an excitation laser line at 488 nm. At least 20,000 events were collected for each sample. The cell cycle was examined 5 h after each single or combined treatment. Four separate experiments were performed.

Thymidine Incorporation

Thymidine incorporation experiments were performed in 24-well plates according to Love-Schimenti *et al.* (20) with minor modifications. In brief, cells (3×10^4 cells/well) were incubated at 37°C in 2 ml complete medium (controls) or medium supplement with ICG (20 μM), cisplatin (8 μM), or both. All samples were run in triplicate. After 24 h incubation, each well was washed with 1 ml warm medium. ICG-loaded cells were then irradiated at 25 J cm^{-2} . After irradiation, the medium was replaced (all samples) with fresh culture medium containing labeled [^3H]thymidine (0.5 $\mu\text{Ci}/\text{ml}$; Amersham, Buckinghamshire, UK). After 5 h at 37°C, incorporation was blocked by extensive washing with warm serum-free medium. NaOH

(0.1 M) was added to all wells (1 ml) and the plates were left at 37°C for 1 h under constant agitation. Solutions (100 μl) were used to measure the protein content (21) and the remainder was used to measure the incorporated thymidine with the semiautomatic Harvester 96 (Skatron Instruments, Lier, Norway). Thymidine counts were expressed as a fraction of counts found in controls.

Methionine Incorporation

We used six-well plates for methionine incorporation experiments as described by Consiglio *et al.* (22). Cells (10^5 cell/well) were incubated at 37°C in 2 ml complete medium (controls) or medium supplemented with ICG (20 μM), cisplatin (8 μM), or both (ICG and cisplatin). All samples were run in triplicate. After 24 h incubation under these conditions, each well was washed with 1 ml warm medium and irradiated at 25 J cm^{-2} (ICG-loaded cells only). Four hours later, the medium of each well was replaced with 1 ml medium containing 30 μCi [^{35}S]methionine (specific activity 1000 Ci/mmol; Amersham, United Kingdom). After 60 min, labeling was terminated by three consecutive washings with 1 ml cold PBS. Cells were finally scraped and lysed by keeping them for 15 min on ice with 0.45 ml lysis buffer consisting of 137 mM NaCl, 4 mM KCl, 1 mM MgCl_2 , 1% Triton X-100, 0.1% BSA and protease inhibitors, 50 $\mu\text{g}/\text{ml}$ leupeptin, and 50 $\mu\text{g}/\text{ml}$ aprotinin. Aliquots (100 μl) of cell lysates were precipitated in triplicate on GFC Whatman (Springfield Mill, UK) filters by soaking them in 10% trichloroacetic acid (TCA) on ice for 10 min. Filters were washed thrice for 10 min in 5% TCA and then rinsed twice in 95% ethanol and acetone. Finally, filters were dried at 60°C and counted in a Beckman (Fullerton, CA) liquid scintillator.

Increasing the time of lysis from 30 min to 2 h did not affect the TCA-precipitable counts. Aliquots of 100 μl were used to measure protein contents according to the procedure of Bradford (21).

Deoxyglucose Uptake

Glucose uptake was determined according to Singh *et al.* (23). Twenty-four plates (3.5 cm) containing MCF-7 cells (2×10^5 cells/well) were incubated for 24 h at 37°C in 2 ml complete medium (controls) or medium supplemented with ICG (20 μM), cisplatin (8 μM), or both. The cells were then washed with fresh medium. The plates intended for PDT (alone or in combination) were irradiated as usual. Five hours later, all cells were washed thrice again with PBS and divided into two groups of 12 plates each (four samples, each sample in triplicate). The two groups of 12 plates each (*i.e.*, three controls, three plates treated with cisplatin alone, three plates treated with ICG/PDT alone, and three plates treated with cisplatin and ICG/PDT) were incubated for 30 min at 37°C in a buffer containing 1 μCi [^{14}C]deoxyglucose (specific activity 200 $\mu\text{Ci}/\text{ml}$) in the absence or presence of 1 μM insulin. Uptake was terminated in all plates by extensively washing cells with ice-cold PBS. Cell monolayers were finally solubilized with 1 ml 0.01% Triton X-100 in 0.01 M NaOH. Aliquots of 100 μl served for protein determination (21) and the rest was used for liquid scintillation counting.

Isobolographic Analysis

Isobolograms were constructed according to a "fixed dose method" (24, 25). Firstly, the responses in a fraction x of the cells were evaluated after treatment with PDT or cisplatin. A fraction corresponding to 75% was found to be suitable and is called ED_{75} . Cells were then assigned to receive treatment consisting of a fixed dose of cisplatin (8 μM) combined with PDT of increasing potency by varying light fluence from 0 to 60 J cm^{-2} , the ICG concentration remaining fixed at 20 μM . The aim of this analysis was to ascertain experimentally the light dose necessary to attain the prefixed effect in the presence of the prefixed concentration of cisplatin.

In a typical isobologram, the ED_x , computed by the individual dose-response curves, was plotted on the vertical and horizontal axes, respectively. The theoretical dose-additive line, including its 95% fiducial limits (calculated by the SE multiplied by 1.96), was attained by joining the two ED_x of choice. If the experimental ED_x of the combination is within the boundary of the dose-additive line and the confidence interval (*i.e.*, the experimental point is nearly coincident with the theoretical point), then the specific combination of PDT with a fixed concentration of cisplatin exerts a dose-additive effect. The effect is synergistic if the experimental point and theoretical point are below the boundaries and antagonistic if they are above the boundaries.

PAGE and Western Blotting

Polyacrylamide gels (15% or 7.5%) were prepared essentially as described by Laemmli (26). Proteins separated on the polyacrylamide gels were blotted onto nitrocellulose filters (Hybond-C pure; Amersham, Milan, Italy) and immunorevealed with specific primary monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Filters were washed and treated with horseradish peroxidase-linked antibodies and the blots were revealed autoradiographically with enhanced chemiluminescence detection reagents (Amersham, Milan, Italy). The entire procedure has been described elsewhere (27). Blots were reprobed with β -actin antibody as loading control. Autoradiographs were scanned with a Discover Pharmacia Scanner equipped with a Sun Spark Classic Workstation and appropriate software (*pdi*, RFL print, version 2; Oakwood, Huntington Station, NY).

Results and Discussion

We have evaluated if treatment with ICG/PDT plus cisplatin results in an effective outcome (additive or even synergistic) so that the dose of the two components or at least of the most toxic one (*i.e.*, cisplatin) can be reduced. We analyzed (a) the response of ICG-treated cells to increasing light doses and (b) the response of MCF-7 cells to cisplatin administered alone (at increasing concentrations) or combined with ICG/PDT. In all the combined treatment experiments, we used a low cisplatin concentration (8 μM) and rather mild PDT conditions (20 μM ICG, light fluence of 25 J cm^{-2}).

Individual Treatments

Dose-Response Effects of ICG/PDT on Cell Viability. As shown in Fig. 1 (*upper panel*), both ICG concentration and light dose affected the magnitude of cell injury. At a fluence of 50 J cm^{-2} , about 30% of cells survived exposure to 20 μM ICG, much less survived exposure to 25 μM ICG, and no cells survived exposure to 50 μM ICG. To ascertain the effect of combined treatment, we administered PDT at sublethal doses using an ICG concentration of 20 μM . Figure 1 (*lower panel*) shows the dose-response curves obtained with this ICG concentration. Apparently, the ED_{75} as measured by MTT assay and the trypan blue exclusion test are not superimposable because they span from ~ 47 to $\sim 52 \text{ J cm}^{-2}$, respectively. Except where specifically indicated, all experiments reported hereafter were conducted with cells preloaded with 20 μM ICG and a light fluence of 25 J cm^{-2} .

Dose-Response Effects of ICG/PDT on Protein Expression. To test whether ICG/PDT altered the expression of proteins related to cell death, we measured the expression of proteins Bcl-2, Bax, Bcl-X_L, p21, poly(ADP-ribose) polymerase (PARP), and p53 by Western blot analysis. The analyses were performed 5 and >10 h after irradiation with either 15 or 25 μM ICG and two different fluences (*i.e.*, 15 or 25 J cm^{-2}). Figure 2 shows that Bcl-2 expression decreased while Bax expression increased as the fluence increased from 15 to 25 J cm^{-2} . PARP cleavage is clearly visible only at most effective conditions. Such increase in PDT efficacy caused also an increase in the expression of p21 and p53 proteins; at variance, no changes were observed in the expression of Bcl-X_L. Indeed, time course experiments demonstrated that the expression profiles of Bcl-2, Bax, and other proteins analyzed returned to basal levels in about 10 h (data not shown). Possibly, the PDT-driven Bcl-2 disruption transiently deranged the equilibrium between

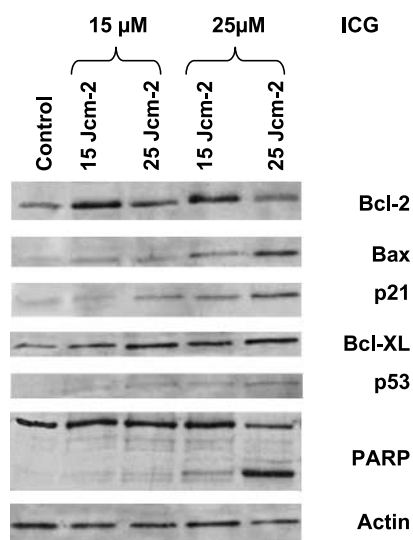


Figure 2. Expression of various proteins at different ICG concentrations and light fluence rates. Proteins were extracted 5 h after PDT. Note the effects on Bcl-2 (fading), Bax (increase), and PARP (fragmentation) at most drastic conditions.

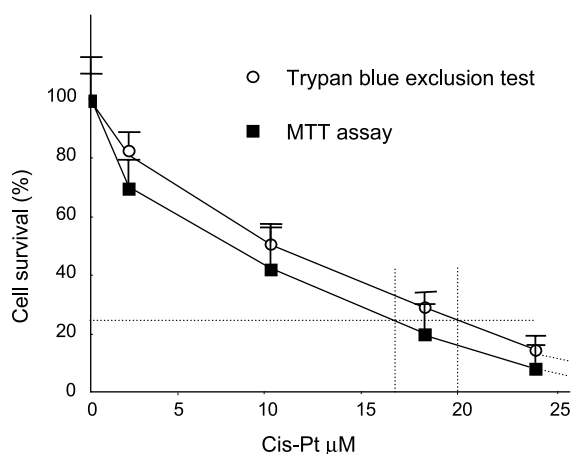


Figure 3. Effect of increasing concentrations of cisplatin (15–25 μM) on MCF-7 viability. Cells were incubated at the indicated cisplatin concentrations for 24 h. Cells were then washed with complete medium and left to stand for 5 h. Cell viability was measured by the trypan blue exclusion test (open circles) and MTT assay (filled squares). Points, average of four determinations; bars, SD.

proapoptotic and antiapoptotic proteins, thereby resulting in apoptosis of a small fraction of cells. The PARP fragmentation shown in Fig. 2 was unexpected because MCF-7 cells do not express caspase-3, the activation of which is a prerequisite for PARP degradation. However, apoptotic pathways that involve caspases (caspase-6 and/or caspase-7) other than caspase-3 have been described in MCF-7 cells (28, 29). MCF-7 human breast cancer cells are p53 positive and highly resistant to many harmful agents. The scarce sensitivity of MCF-7 cells to cisplatin-induced apoptosis has been ascribed to a functioning p53 because apoptosis was greater in cisplatin-treated cells in which p53 was abrogated (15). The effect of the antiapoptotic protein Bcl-2, the transcription of which is up-regulated in MCF-7 cells via two estrogen-responsive elements (13), may be an additional protective factor. However, apoptosis could not be elicited in these cells by photofrin/PDT even on p53 abrogation (16). Here, we report a similar observation with ICG/PDT. Although p53 and p21 expression was enhanced in relation to photosensitizer concentration and light dose (Fig. 2), only a limited fraction of cells may undergo apoptosis. The photodynamic destruction of the Bcl-2 protein, accompanied by Bax up-regulation, has already been reported in MCF-7 cells (30) although with a different photosensitizer (phthalocyanine Pc 4). Thus, Bcl-2 may also be a preferential target of ICG/PDT, and PDT-induced damage to Bcl-2 may explain the death of a fraction (10–12%) of MCF-7 cells. It is noteworthy that Bax expression increased as Bcl-2 expression declined. At present, we are unable to explain this increase. It was interesting to observe PARP fragmentation in the MCF-7 cells that lacked caspase-3; it is conceivable that ICG/PDT would trigger greater apoptosis in caspase-3-positive cells. This has indeed been shown in human retinal pigment epithelial cells (31) and in a human leukemic cell strain from a histiocytic lymphoma (8).

Dose-Response Effects of Cisplatin. Figure 3 shows the results of the trypan blue dye exclusion and MTT assays of cells treated with increasing concentrations of cisplatin (1.5–20.0 μM). The trypan blue assay reveals cells with intact membrane; the MTT assay is a measure of the reductive potential of mitochondrial enzymes. The cisplatin dose required to kill 75% of cells (ED_{75}) was near 20 μM according to the trypan blue exclusion test and 17 μM according to MTT data. With 8.0 μM cisplatin, almost 50–60% of cells were viable 5 h after withdrawal of the chemotherapeutic agent. Consequently, we used this sublethal cisplatin dose in the combined experiments reported hereafter.

Combined Treatments

In the experiments of combined treatment, we used 8 μM cisplatin and 20 μM ICG at a light fluence of 25 J cm^{-2} . Administered singly, these treatments were not lethal for MCF-7 cells. As shown in Fig. 4, cell viability as assessed by trypan blue assay (membrane integrity) was reduced by a factor of ~ 1.6 with 8 μM cisplatin, ~ 2.0 with ICG/PDT (20 $\mu\text{M}/25 \text{ J cm}^{-2}$), and ~ 4 with combined treatment (cisplatin plus ICG/PDT) as compared with untreated controls. Similar results were obtained with the clonogenic assay (same figure), which measures the ability of each cell in the culture to maintain all the functions needed to divide and form a colony. The MTT assay gave somewhat different results: the reduction in cell viability was much greater with cisplatin plus PDT (~ 10 -fold) than with the two treatments administered separately (~ 1.5 - and ~ 2.0 -fold, respectively).

Finally, we found that ICG/PDT and cisplatin target different phases of the cell cycle. In fact, only about 40% of the G_1 cell population survived ICG/PDT versus 65% of control cells. Differently, after cisplatin treatment, 85% of the surviving cells accumulated in the G_1 phase. This finding coincides with the observation that cisplatin induces cell cycle arrest and death in the S- G_2 phase (32). The cell cycle-related effect of ICG/PDT and cisplatin on MCF-7 cells ensures continuous apoptotic coverage throughout the cell cycle, thereby potentiating the overall lethal output.

Individual and Combined Treatments: Effects on Cell Metabolism

DNA Synthesis. The impairment of cellular function may be also measured by evaluating the ability of cells to incorporate thymidine into nuclear DNA. We assessed DNA synthesis in control and treated cells by measuring incorporation of thymidine into DNA. As expected, both chemotherapy and PDT significantly inhibited DNA synthesis. The residual capacity of cells to incorporate thymidine (Fig. 5, hatched bars) into DNA after individual treatments was reduced by a factor of ~ 1.3 in the presence of 8 μM cisplatin and by a factor of ~ 2 with ICG/PDT. As compared with untreated controls, DNA synthesis was reduced by ~ 10 -fold with ICG/PDT plus cisplatin.

De Novo Synthesis of Proteins. To evaluate whether protein synthesis was altered in the cells treated with ICG/PDT and cisplatin, we measured the percentage change in

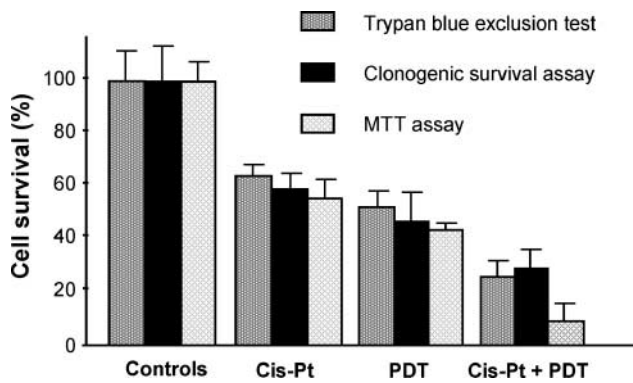


Figure 4. Viability of MCF-7 cells was estimated after single ($8 \mu\text{M}$ cisplatin or $20 \mu\text{M}$ ICG/ 25 J cm^{-2}) or combined ($8 \mu\text{M}$ cisplatin and $20 \mu\text{M}$ ICG/ 25 J cm^{-2}) treatments. Viability was measured with the trypan blue assay exclusion test, colony-forming assay (quantal responses), or MTT assay (variable response). Columns, average of four (trypan blue exclusion test and MTT assay) or two (colony-forming assay) determinations; bars, SD.

labeled methionine incorporated into neosynthesized proteins in normally growing cells (controls), in cells exposed for 24 h to cisplatin ($8 \mu\text{M}$), in cells irradiated at a fluence of 25 J cm^{-2} after 24 h incubation in a medium containing ICG ($20 \mu\text{M}$), and in cells exposed to both treatments (Fig. 5, filled bars). As expected, both chemotherapy and PDT significantly inhibited protein synthesis. The residual capacity of viable cells to incorporate methionine into their proteins after individual treatments was reduced by factors of ~ 1.6 and ~ 2.7 , respectively, with respect to controls. On combined treatment, the capacity of cells to incorporate methionine approached 0.

Glucose Uptake. Glucose uptake and metabolism are increased in most cancer cells. It is well known that malignant cancer cells are unable to store glucose and depend on constant refurbishment to meet their energy requirements (33). It is also known that GLUT-1 expression is enhanced in malignant cells (34). Therefore, inhibition of deoxyglucose uptake, either basal or insulin stimulated,

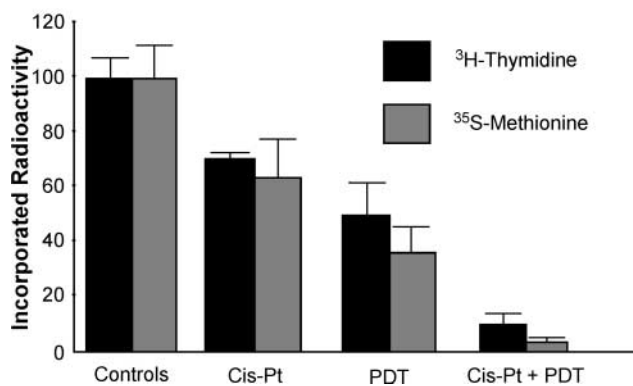


Figure 5. [^3H]Thymidine (black bars) and [^{35}S]methionine (gray bars) incorporation into MCF-7 cells after single ($8 \mu\text{M}$ cisplatin or $20 \mu\text{M}$ ICG/ 25 J cm^{-2}) and combined ($8 \mu\text{M}$ cisplatin and $20 \mu\text{M}$ ICG/ 25 J cm^{-2}) treatments. Columns, average of three determinations; bars, SD.

may give a measure of cell fitness. We found that single and combined treatments caused a significant reduction in (deoxy)glucose uptake (Fig. 6). The decrease paralleled the decrease in thymidine and methionine incorporation on similar treatments. Glucose transport was increased by 25–30% in control insulin-treated MCF-7 cells (Fig. 6). Both individual and combined treatment inhibited this response to insulin. The changes in DNA synthesis, protein synthesis, and glucose transport in surviving cells evaluated 5 h after treatment reflected the general trend of changes revealed by cell viability assays; moreover, they were very similar to the changes revealed by the MTT assay.

Isobolographic Assessment of the Effects of Combinations of PDT and Cisplatin

The effects of combined treatments may be evaluated by analyzing dose-response experimental data isoblographically. Dose-response phenomena can have either quantal or measurement values. Quantal responses are framed by questions about what fraction of exposed organisms showed the index response. Measurement responses are those with magnitude that usually increases or decreases as a continuous variable in response to mounting stimuli. In this regard, we constructed isobolograms with quantal data (trypan blue assay; Fig. 7, upper panel) and variable data (MTT assay; Fig. 7, lower panel). Dose-response curves were assessed by evaluating cell viability with the trypan blue or MTT assay at fixed cisplatin concentration ($8 \mu\text{M}$) followed by irradiation with fluences between 0 and 60 J cm^{-2} ($20 \mu\text{M}$ ICG).

Based on MTT data, the fluence rate required to obtain 75% lethality (ED_{75}) was lower than that indicated by trypan blue assay data. In fact, as shown in Fig. 7, MTT was better than trypan blue in detecting cell viability. The first isobologram (trypan blue data) shows that PDT plus cisplatin had an additive effect, whereas the second

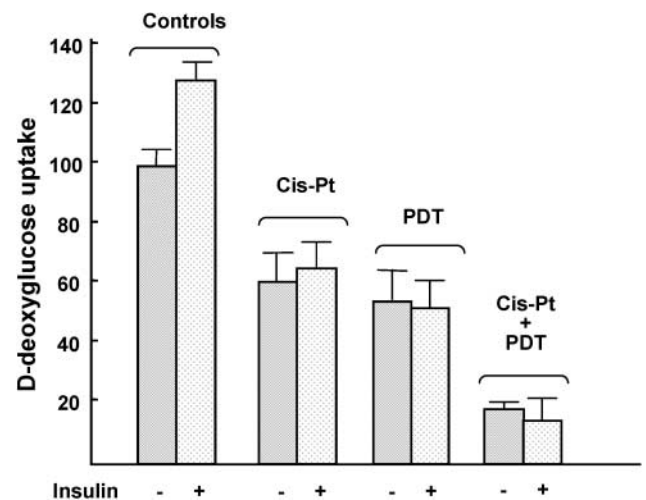


Figure 6. Basal versus insulin-stimulated [^{14}C]deoxyglucose incorporation into MCF-7 cells after single ($8 \mu\text{M}$ cisplatin or $20 \mu\text{M}$ ICG/ 25 J cm^{-2}) and combined ($8 \mu\text{M}$ cisplatin and $20 \mu\text{M}$ ICG/ 25 J cm^{-2}) treatments. Columns, average of three determinations; bars, SD.

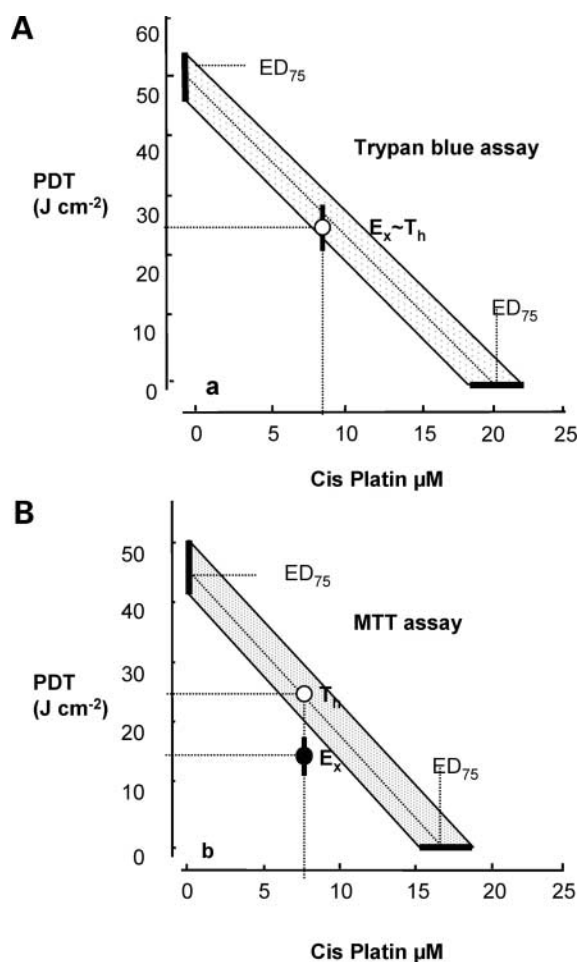


Figure 7. Isobolographic analysis of data obtained with combined treatments. **A**, isobologram constructed with E₇₅ data measured by the trypan blue assay. The theoretical light fluence (T_h) that must be used with 8 μM cisplatin to obtain an E₇₅ coincides with the experimental value (E_x). The effect is additive. **B**, isobologram constructed with E₇₅ data measured by MTT assay. The theoretical light fluence (T_h) that must be used with 8 μM cisplatin to obtain an E₇₅ is about 10 J cm⁻² higher than the experimental value (E_x). The effect is synergistic.

isobologram (MTT data) shows that it had a synergistic effect. This observation is in line with the possibility that metabolic impairment of cells (measured by MTT), which occurs well before cells are irreversibly damaged (detected by trypan blue), is a more sensitive indicator of cell fitness.

Although the response to the various treatments, as assessed by trypan blue and MTT data, was similar, the magnitude of change differed between the two techniques. In fact, the response was additive based on trypan blue data and quasi-synergistic based on MTT data. In both cases, combined treatment resulted in mutual reinforcement of destructive efficacy. This observation is supported by the cell cycle analysis of cells exposed to PDT, cells incubated with low-dose cisplatin, and cells exposed to PDT after incubation with low-dose cisplatin. In fact, cisplatin killed mostly S-phase cells, whereas ICG/PDT killed mostly G₁-phase cells. Only a small fraction of cells

survived combined treatment, although cisplatin and ICG/PDT administered singly would kill only a limited number of cells. Obviously, preincubation of MCF-7 cells with the usual cisplatin dose (8 μM) followed by their exposure to higher light fluences (e.g., 40 J cm⁻²) results in the rapid death of all cells. Therefore, in principle, a combination consisting of a low dose of cisplatin and appropriately adjusted ICG/PDT dosage (i.e., higher light fluence and/or photosensitizer concentration) would be effective. This strategy was among those listed by Dolmans *et al.* (35) in their review of PDT for cancer treatment. Our study supports the validity of this strategy and provides a clinical perspective. In fact, it appears from our data that the adverse effects of chemotherapy can be partially abated without reducing the efficacy of treatment. Obviously, this hypothesis should be tested in *in vivo* models before being applied in human therapy.

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