

Photo-oxidative killing of human colonic cancer cells using indocyanine green and infrared light

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Summary Despite of the approval of Photofrin® in various countries, chemically defined sensitizers for photodynamic therapy (PDT) are still needed for the absorption of light in the infrared spectrum, which provides a maximal penetration of light into tissue. Therefore, both the efficacy and the mechanism of action of the clinically approved dye indocyanine green (ICG) and laser irradiation were investigated in vitro. For the investigation of phototoxic effects, HT-29 cells were incubated 24 h prior to irradiation by using different concentrations of ICG (10–500 µM). In each experiment, cells were irradiated using a continuous wave (cw)-diode laser ($\lambda_{\text{ex}} = 805 \text{ nm}$, 30 J cm^{-2} , 40 mW cm^{-2}). After laser irradiation, cell viability of dark control and of cells incubated with 500 µM ICG was 1.27 ± 0.11 or 0.28 ± 0.05 respectively. Using 100 µM ICG and D₂O, cell viability was further decreased from 0.46 ± 0.03 (H₂O) to 0.11 ± 0.01 (D₂O). Using D₂O and 100 µM ICG, the concentration of malondialdehyde, a marker of lipid peroxidation, increased from $0.89 \pm 0.10 \text{ nmol } 10^{-6} \text{ cells}$ to $11.14 \pm 0.11 \text{ nmol } 10^{-6} \text{ cells}$. Using 100 µM ICG and laser irradiation sodium azide or histidine (50 mM), quenchers of singlet oxygen reduced the cell killing significantly. In contrast, when using mannitol, a quencher of superoxide anion and hydroxyl radical, cell killing was not inhibited. According to the present results, photoactivated ICG seems to kill colonic cancer cells due to the generation of singlet oxygen and the subsequent formation of lipid peroxides. Therefore, ICG might present a promising photosensitizer for PDT; first clinical results confirm these findings.

Keywords: HT-29 cells; ICG; singlet oxygen; lipid peroxidation

Indocyanine green (ICG), which was approved by the United States Food and Drug Administration in 1956, is widely applied in medical diagnosis (Fox et al, 1956; Flower and Hochheimer, 1976; Moneta et al, 1987).

ICG shows a low incidence of adverse reactions (Hope-Ross et al, 1994) and has been intensively studied regarding its pharmacokinetics (Paumgartner et al, 1970). The absorption spectrum of this watersoluble, anionic tricyanobenzene dye exhibits a strong absorption band around 800 nm coinciding with the emission wavelength of a diode-laser (805 nm, see Figure 1).

For the evaluation of ICG as a possible photosensitizer, various photophysical parameters of ICG were determined by us in cooperation with the Department of Physics (University of Regensburg, Germany). The yields of triplet formation $\phi_{\text{S}_1\text{-T}_1}$, by S₁-T₁ intersystem-crossing are 14% (in water, H₂O), 16% (in methanol), 17% (in dimethyl sulphoxide, DMSO) and 11% (in aqueous albumin solution) (Reindl et al, 1997). In comparison to HPD (in methanol, 82%) and aluminium phthalocyanine (in water, 38%) (Grossweiner, 1994) the triplet yields of ICG appear low, but the values of ICG might be high enough to act as a photosensitizer for photodynamic therapy (PDT).

First, in vitro experiments using ICG and normal keratinocytes (Fickweiler et al, 1997) showed phototoxic effects. However, the mechanism of action – photo-oxidative versus photothermal cell killing – remained unclear and was investigated again using carcinoma cells (colon cancer, HT-29).

MATERIALS AND METHODS

Cell culture and dye preparation

The immortalized human colon carcinoma cell line HT-29 (Fogh and Trempe, 1975) was maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemie, Deisenhofen, Germany) and supplemented with 10% fetal calf serum (FCS; Sigma Chemie), 1% L-glutamine and 1% streptomycin–penicillin (Gibco, Eggenstein, Germany) in a humidified atmosphere containing 8% carbon dioxide at 37°C. Cells were washed with phosphate-buffered saline (PBS; Biochrom, Berlin, Germany) and harvested by a 10 min treatment with 0.05% trypsin/0.02% EDTA (Gibco) in PBS. For in vitro assays, ICG (molar mass of the ICG sodium iodide: 924.9 g mol^{-1} ; PULSION Medizintechnik, München, Germany) was dissolved in growth medium at concentrations of 10, 50, 100 and 500 µM.

Treatment protocol and phototoxicity assay

HT-29 cells were seeded at equal concentrations (15×10^3 cells in 100 µl medium per well) into 96-well microtitre plates (Costar, Tecnomara, Fernwald, Germany). After overnight cell attachment, medium was replaced by 100 µl ICG solution at various concentrations (10, 50, 100 or 500 µM). Following an incubation time of 24 h at 37°C, supernatants were removed. In order to eliminate remaining dye, cells were carefully washed with medium and covered with 100 µl of drug-free medium prior to irradiation. The cells for control experiments were processed in the same way.

Irradiation of cells incubated with ICG was performed using a continuous wave (cw)-diode-laser emitting light at 805 nm (Opto Power Corp., CA, USA) with a maximum optical output power of 15 W. The laser light was coupled into a monocoire fibre with 1.5 mm

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diameter and distributed to a flat homogeneous area (150 cm², Ø 14 cm) sufficient to cover a 96-well microtitre plate by a biconvex lens with a fluence of 40 mW cm⁻². The fluence used was 30 J cm⁻². Measuring the temperature of supernatants by a thermocouple immediately after irradiation ensured that no hyperthermic conditions were imposed by this irradiation arrangement.

Following irradiation, cells were maintained under normal culture conditions for a further 24 h. Viability of cells after ICG and/or light treatment was assessed by means of the 3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) assay (for details see McHale and McHale, 1988). The ratio of the optical densities of treated cells to untreated cells, which served as control, was referred to as cell viability (CV).

Effect of sodium azide, histidine and mannitol on cell killing

Sodium azide, histidine or mannitol effective physical quencher of reactive oxygen species (Moan et al, 1979; Agarwal et al, 1991; Hampton et al, 1993; Zaidi et al, 1993) were added to the cell culture 60 min prior to irradiation. HT-29 cells incubated with 100 µM ICG for 24 h were irradiated (30 J cm⁻²) as described above but in presence of sodium azide, histidine or mannitol (Merck) at a concentration of 50 mM in PBS. CV was assessed as described above.

Effect of D₂O on cell killing

Cell killing effects are enhanced using D₂O in vitro (Lin et al, 1991). D₂O was added 60 min prior to irradiation. HT-29 cells incubated with 100 µM ICG for 24 h were irradiated (30 J cm⁻²) as described above but in presence of D₂O. CV was assessed as described above.

Lipid peroxidation assay

Unsaturated lipids are important targets of membrane photo-oxidation. Malondialdehyde (MDA), an ultimate marker of lipid peroxidation, can be measured by the fluorometric thiobarbituric acid (TBA) assay (Perret et al, 1994). After irradiation the cells were homogenized using ultrasound and centrifuged for 15 min. The suspension was mixed with phosphotungstic acid (10% in H₂O), sodium dodecyl sulphate (7% in H₂O), hydrochloric acid (0.1 M), thiobarbituric acid (0.5% in H₂O) and shaken for 60 min at a temperature of 95°C. After cooling down, the MDA-TBA complex was extracted by *n*-butanol. The quantification of the MDA-TBA complex was performed by measuring its fluorescence at 553 nm; a MDA standard solution (0–3 nmol ml⁻¹) served as control.

Data analysis and statistics

Each individual experiment was carried out at least in triplicate. For the MTT assay, at least ten individual wells were plated with cells treated in an identical manner with their mean optical density used for data analysis. The effects of the different treatment modalities were expressed as CV of treated cells compared with untreated controls. Differences were tested for statistical significance using the two-sided *t*-test. All primary data are presented as mean and standard deviations of the mean.

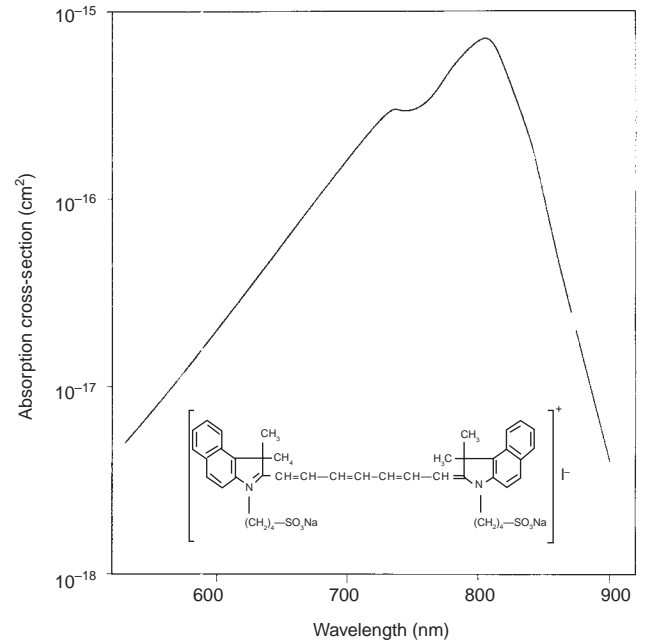


Figure 1 Chemical structure and absorption spectrum of ICG sodium iodide

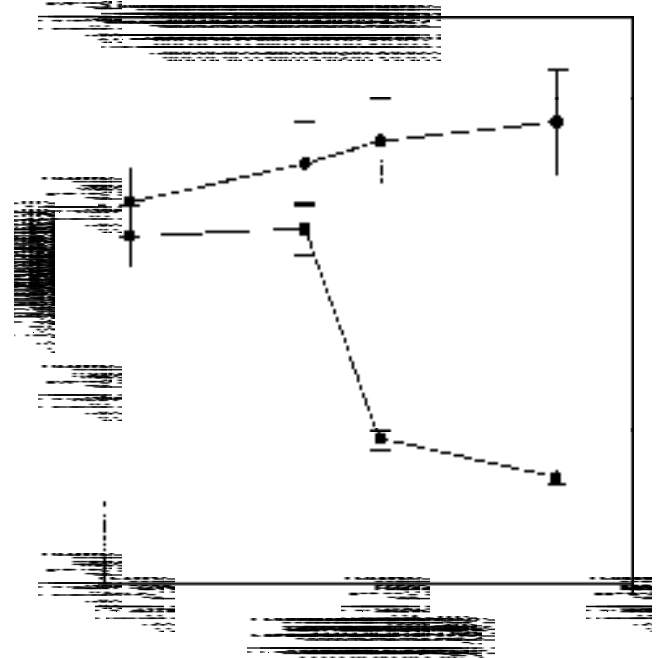


Figure 2 Concentration finding studies with (■) and without (●) irradiation by a cw-diode laser (805 nm, 40 mW cm⁻², 30 J cm⁻²). Cell viability of colonic cancer cells HT-29 following incubation for 24 h using different concentrations of ICG. Each point is the mean of at least three determinations ± standard deviation

RESULTS

ICG phototoxicity in vitro

The experiments revealed no dark toxicity in any ICG concentration used. Irradiation used on its own did not result in a significant decrease of CV. Viability assays 24 h after irradiation exhibited a

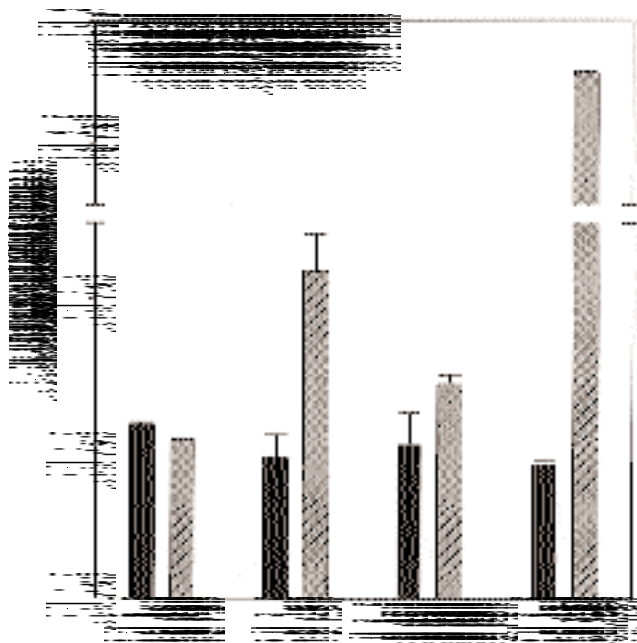


Figure 3 Effects of histidine (50 mM) and D₂O on ICG-incubated HT-29 cells (100 μM ICG) with and without light irradiation (diode-laser, 805 nm, 40 mW cm⁻²). The black bars represent MDA concentration using ICG alone and ICG in combination with histidine or D₂O, the grey bars represent the MDA concentration using ICG alone and ICG in combination with histidine or D₂O following irradiation. Each bar is the mean of 3 determinations ± standard deviation

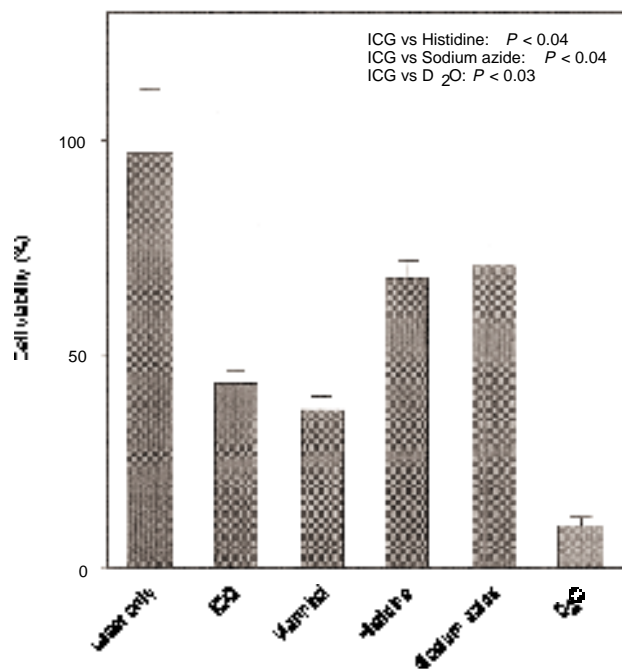


Figure 4 Effect of sodium azide (50 mM), histidine (50 mM) mannitol (50 mM) and D₂O on ICG (100 μM) mediated cell killing determined by the MTT assay (incubation time 24 h, total light dose 30 J cm⁻²). Each bar is the mean of three determinations ± standard deviation

behaviour inversely proportional to the used ICG concentrations as illustrated in Figure 2. Starting with a concentration of 10 μM ICG, the CV decreased according to an increasing ICG concentration by using a light dose of 30 J cm⁻². CV of dark control and cells

incubated with 500 μM ICG and irradiated with 30 J cm⁻² was 1.27 ± 0.14 and 0.28 ± 0.02 respectively.

Lipid peroxidation

MDA, a marker for lipid peroxidation, increased from 0.93 ± 0.16 nmol 10⁻⁶ cells to 2.17 ± 0.26 nmol 10⁻⁶ cells after light irradiation (30 J cm⁻²) of HT-29 cells incubated with 100 μM ICG for 24 h (Figure 3). Using histidine, the amount of MDA was 1.42 ± 0.05 nmol 10⁻⁶ cells with light and 1.01 ± 0.24 nmol 10⁻⁶ cells without light. Adding D₂O, however, dramatically increased MDA from 0.89 ± 0.10 nmol 10⁻⁶ cells to 11.14 ± 0.11 nmol 10⁻⁶ cells.

Effects of different quenchers and D₂O on cell killing

Figure 4 shows the effect of incubation with ICG (100 μM) and quenchers (50 mM) or D₂O prior to light irradiation (30 J cm⁻²). Using ICG and laser irradiation only, CV was 0.46 ± 0.03. The cell killing of ICG was reduced in the presence of sodium azide indicated by an increase of the CV from 0.46 ± 0.03 to 0.65 ± 0.04. In the presence of histidine, CV increased from 0.46 ± 0.03 to 0.64 ± 0.05. By adding 50 mM mannitol to the cells, CV did not change significantly, 0.46 ± 0.03 and 0.40 ± 0.04 respectively, whereas the addition of D₂O revealed a dramatic decrease of CV from 0.46 ± 0.03 to 0.11 ± 0.06.

DISCUSSION

The present results show that human colon carcinoma cells can be effectively destroyed by photoactivated ICG. Irradiation with a low energy density (30 J cm⁻²) significantly decreased the cell viability. Other experiments in vitro using different sensitizers and a different setup, yielded a similar cell toxicity (Ma et al, 1994; Jori, 1996; Rossi et al, 1996; Fickweiler et al, 1997). However, ICG concentrations up to 500 μM did not exhibit a dark toxicity. The photosensitizers used, e.g. haematoporphyrin (Bachor et al, 1995), methylene blue (Wainwright et al, 1997) or porphycenes (Bäumler et al, 1995), usually resulted in an increasing dark toxicity with increasing sensitizer concentrations in vitro.

Evaluating the mechanism of action regarding the cell killing by a photosensitizer, specific experiments should be performed since the absorbed energy of the laser light by the molecule of the used photosensitizer can reach the ground state by either radiative (fluorescence) or non-radiative decay. In the non-radiative decay the absorbed energy of the molecule is converted either to heat (internal conversion) and transferred to other molecules (photo-oxidation, type I), or transferred to molecular oxygen (photooxidation, type II) via triplet-state.

Photothermal effects due to internal conversion damage cells by an increase of the intracellular temperature, shown by the use of ICG for photocoagulation or tissue welding (Decoste et al, 1992; Reichel et al, 1994). If photothermal effects are responsible for cell killing by ICG, quenchers like sodium azide or histidine should fail to reduce ICG-induced phototoxicity.

In order to investigate whether photo-oxidation of type I or type II are the predominant mechanisms of action mannitol, a quencher of superoxide anion, hydroxyle radical (type I) and histidine or sodium azide, quenchers of singlet oxygen (type II) were used (Agarwal et al, 1991; Zaidi et al, 1993; Wlaschek et al, 1995). The results seen in Figure 4 show that mannitol did not protect HT-29 cells against photoactivated ICG, whereas the quenchers sodium

azide or histidine significantly reduced the cell killing. Thus, in the present *in vitro* experiments, the killing of HT-29 cells by ICG and laser irradiation seem to be mediated mainly by singlet oxygen. Moreover, adding D₂O to the culture medium, photo-oxidative cell killing was further enhanced due to a prolonged lifetime of singlet oxygen in the presence of D₂O, about 40 μs versus 3 μs in H₂O (Matheson et al, 1978). In addition, the increase of lipid peroxides *in vitro* as measured by means of the fluorometric TBA assay (Perret et al, 1994), confirms that photo-oxidation of HT-29 cell structures is responsible, to some extent, for the cell killing in the chosen experimental setup (Figure 3). The increase of MDA adding D₂O to the cell culture medium may be a hint for oxidative destruction of membranes due to the presence of singlet oxygen and its prolonged lifetime in D₂O.

In contrast to many other sensitizers, e.g. porphyrine derivatives, which are photoactivated by red light ranging from 600 to 700 nm, ICG exhibits a high absorption cross-section in the infrared region at 805 nm. Infrared light penetrates deeper into tissue than red light (Anderson et al, 1981), presenting an outstanding advantage, if ICG acts as a photosensitizer *in vivo*.

Due to the clinical approval of ICG, the successful treatment of Kaposi's sarcomas was already shown in a first pilot study, using a different experimental setup (Abels et al, 1998). Further investigations regarding the exact mechanism of action *in vivo* – photo-dynamic versus photothermal – will be performed.

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