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THE STUDY OF CYTOTOXICITY AND PHOTOTOXICITY OF INDOCYANINE GREEN IN VITRO

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Abstract

The present study investigates the photochemical properties of a potential photosensitiser, indocyanine green (ICG), in vitro. A marked cytotoxicity of ICG on HeLa cells after 24 hours from ICG application into the cells was proved beginning from a concentration of 206 μM ICG ($P<0.001$). We further followed the HeLa proliferation curve under a combined influence of ICG at concentrations of 24 and 94 μM and laser irradiation at an energy density of 0, 24, 60, and 99 J/cm^2. Phototoxicity was evaluated at 1, 24, and 48 hours after irradiation. No phototoxic effect was detected in any of the experiments at the time of 1 hour after irradiation. At a concentration of 24 μM a phototoxic effect can only be found at the highest applied irradiation density of 99 J/cm^2 at the time of 24 hours ($P_{99}=0.004$) and 48 hours ($P_{99}=0.002$) after irradiation. For the time being, an ICG concentration of 94 μM appears to be optimal; a phototoxic effect occurred with all the applied irradiation densities (24, 60, and 99 J/cm^2) at the time of 24 hours ($P_{24}=0.028$, $P_{60} < 0.001$, $P_{99} < 0.001$). At the time of 48 hours after irradiation, a phototoxic effect was determined for the applied irradiation densities of 60 and 99 J/cm^2($P_{60} = 0.045$, $P_{99} = 0.007$).

Key words

Indocyanine green, Photosensitiser, HeLa cells

INTRODUCTION

Photodynamic therapy (PDT) is a method of choice of destruction of certain types of tumours, the destruction being based on photochemical formation of oxygen radicals. In comparison with radiotherapy and chemotherapy, PDT is a patient-friendly method. It may be only indicated in certain types of tumours; accessibility to light and size of tumour play a role here. A key factor of PDT usage is the application of a photosensitiser, i.e., a chemical compound which is activated by light of a suitable wavelength. The present study investigates in vitro properties of indocyanine green as a potential photosensitising agent.

Indocyanine green (ICG) is a tricarbon type of dye with an infrared type of spectrum and an absorbance maximum of around 800 nm, which enables a maximum penetration of light into the tissues (*1*) and thus a more efficient procedure of PDT application; in the visible region ICG absorbs little or nothing at all. ICG (*Fig. 1*) has been used in medical diagnostics since 1956 for determination of cardiac output, blood plasma volume, in the diagnostics of hepatic function, capillary microscopy,

and for localisation of objects in tissues. ICG is also usually employed in vivo in cases of dye-elevated photocoagulation and ablation of tissues (*2*). The application of ICG also appears as promising in photochemotherapy, which is currently considered to be an efficient palliative therapeutic modality in the treatment of Kaposi sarcoma (*3*). The photooxidation mechanism seems to be the principal mechanism of the phototoxic effect of ICG on tumour cells (*4*). Our study investigates the cytotoxic and phototoxic effects of ICG on HeLa tumour cells and is aimed at determining the maximum phototoxic effect of ICG on HeLa cells.

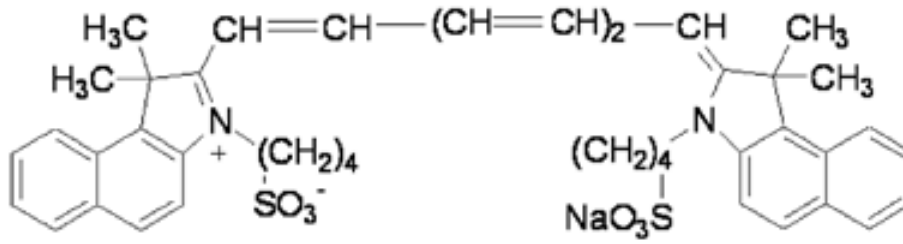

Fig. 1.
Chemical structure of ICG

MATERIAL AND METHODS

Cell culture and preparation of the compound

The HeLa tumour cell line was kept in Dulbecco's modified Eagle's medium (Sigma Aldrich) supplemented with 5 % fetal serum (Sigma Aldrich) and 1 % L-glutamine (Sigma Aldrich) in a humidified atmosphere containing 8 % carbon dioxide at 37 ^{0}C. The cells were washed in a PBS phosphate buffer solution (Sigma Aldrich) and lysed for 10 min with 0.1 % trypsin – 0.04 % EDTA in PBS. ICG (M = *947.87* g/l: Sigma Aldrich) was dissolved in PBS.

ICG cytotoxicity tests

For pilot cytotoxicity tests, HeLa cells were seeded at a concentration of 10^4 cells/ml in 200 µl of the DMEM culture medium per well into 96 microtitre plates. An ICG solution in a total volume of 20 µl was added to the individual wells, so that ICG concentration per well was 2 µM–200 µM.

Cytotoxicity was evaluated after 24 hours by means of MTT assay. This method utilises the capacity of living cells to reduce the tetrazolium salt – MTT (3,4,5–dimethylthiazol–2-yl)–2,5-diphenyltetrazolium bromide (Sigma Aldrich) by means of mitochondrial dehydrogenases. The amount of the blue formasan dye originating is proportional to the number of living cells in the culture. Altogether 10 wells were analysed; 20 µl of MTT from the stock solution at a concentration of 5 µg/ml in PBS was added to them and the cells were incubated for 4 hours at 37 ^{0}C. After sucking off the supernatant, formasan was dissolved in DMSO (Sigma Aldrich) and absorbance was measured by a microplate reader (Spectra Shell) at 570 nm. The absorbances obtained in individual experiments were subjected to statistical analysis.

ICG phototoxicity tests

For phototoxicity tests, HeLa cells were seeded at concentrations of 10^4 cells/ml in 200 µl of the DMEM culture medium per well into 96 microtitre plates. An ICG solution at a volume of 20 µl at concentrations of 2 µM–200 µM was added after 24 hours from cell seeding. The cells were then

incubated for 24 hours under standard conditions (37 ^{0}C, 5 % CO_2, 95 % air humidity) and the DMEM medium was exchanged. The HeLa cells were then irradiated with a Beautyline BTL-10 laser (Beautyline, Ltd., Prague) at a wavelength of 830 nm, energy densities of 0, 24, 60, and 99 J/cm^2, and an output of 360 mW. The cells were repeatedly exposed to standard conditions and cell viability (MTT assay) was determined after 1, 24, and 48 hours following laser irradiation.

Data analysis and statistics

Each cytotoxicity and phototoxicity test was performed three times. To determine cell viability by means of MTT assay, the absorbances from individual wells were statistically evaluated in 10 repeats; a non-parametric Mann-Whitney t-test was used for the evaluation of the data measured, and data dispersion was recorded using a 25% and 75% quartile.

RESULTS AND DISCUSSION

ICG cytotoxicity of HeLa cells in vitro

We studied the cytotoxic effect of ICG on HeLa cells, when ICG was added to the cells immediately after the seeding. Cytotoxicity was evaluated after 24 hours following addition of the substance to the cells by means of MTT assay. A statistically significant cytotoxic effect in comparison with the control was found beginning with the concentration of 206 μM ($P < 0.001$), see *Fig. 2*. From our first experiments we know that if ICG is added to the cells only 24 hours after the seeding, cytotoxicity appears at higher concentrations of ICG. With regard to the character of the experimental cells we chose stricter testing criteria because of the subsequent choice of ICG concentrations for the phototoxicity tests.

The study (*5*) reports that a combination of exposure to 0.5 % ICG and the excitation source of radiation may cause damage to Muller cells. Despite the fact that the usually employed ICG preparations in clinical practice do not cause any significant damage (e.g., application of ICG in liver function diagnostics), relatively small changes in ICG concentrations in the above study (*5*) did cause damage.

In view of the above-mentioned information ICG cytotoxicity for cell systems has to be tested.

ICG phototoxicity of HeLa cells in vitro

In connection with the above results of cytotoxicity tests we chose lower concentrations of ICG for the testing of ICG phototoxicity. We observed the proliferation curve of HeLa cells under a combined effect of ICG at concentrations of 24 and 94 μM and laser irradiation at densities of 0, 24, 60, and 99 J/cm^2 at the time of 1, 24, and 48 hours after irradiation. No phototoxic effect was detected in any of the experiments at the time of 1 hour after irradiation. For the reason of a more perfect attachment of adherent HeLa cells, ICG was added 24 hours after the seeding.

In all graphs indirectly describing the proliferation curves at the time of 24 and 48 hours after irradiation, we always compare the individual experimental variants

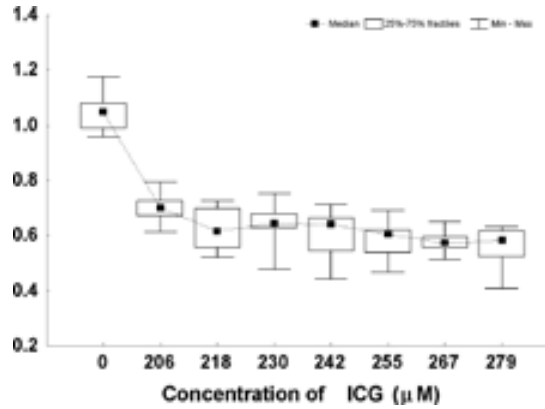

Fig. 2.
Cytotoxicity of ICG with HeLa cells after 24 h incubation
Comparison of the individual variants (absorbances measured in MTT assay in relation to concentrations of μM ICG) with control 0 μM ICG. All the differences were significant (all have the same $P < 0.001$)

(absorbances measured in MTT assay) with the control, and determine significant difference among them by means of a statistical test. For these experiments we standardised a variant as the control denoted as C, where ICG at a chosen concentration was applied to HeLa cells. The designation C-0 denotes the variant of cells without any experimental intervention. In the figures we can always see 2 graphs next to each other with a common axis X. The first type of graph describes variants with ICG application and laser-irradiated, the other type of graph describes variants with no ICG applied (i.e., only laser-irradiated, at energy densities of 0, 24, 60, and 99 J/cm^2).

At a concentration of 24 μM a phototoxic effect can only be found at the highest applied irradiation density of 99 J/cm^2 at the time of 24 hours (*Fig. 3*, P_{99}=0.004) and 48 hours (*Fig. 4*, P_{99}=0.002) after irradiation. For the time being, an ICG concentration of 94 μM appears to be optimal; a phototoxic effect occurred with all the applied irradiation densities (24, 60, and 99 J/cm^2) at the time of 24 hours (*Fig. 5*, P_{24}=0.028, $P_{60} < 0.001$, $P_{99} < 0.001$). At the time of 48 hours after irradiation, a phototoxic effect was determined for the applied irradiation densities of 60 and 99 J/cm^2 (*Fig. 6*, $P_{60} = 0.045$, $P_{99} = 0.007$). A maximum phototoxic effect of ICG on HeLa cells was thus detected for an ICG concentration of 94 μM, a laser output of 360 mW, and an energy density of 99 J/cm^2 at the time of 24 hours after irradiation ($P_{99} < 0{,}001$).

The article (*6*) finds a phototoxic effect of the ICG on the SKMEL 188 line and the S91 mouse cell line. The study (*7*) registers a phototoxic effect of ICG in vitro on pancreatic cells MIA PaCa-2, PANC-1, and BxPC-3, where an

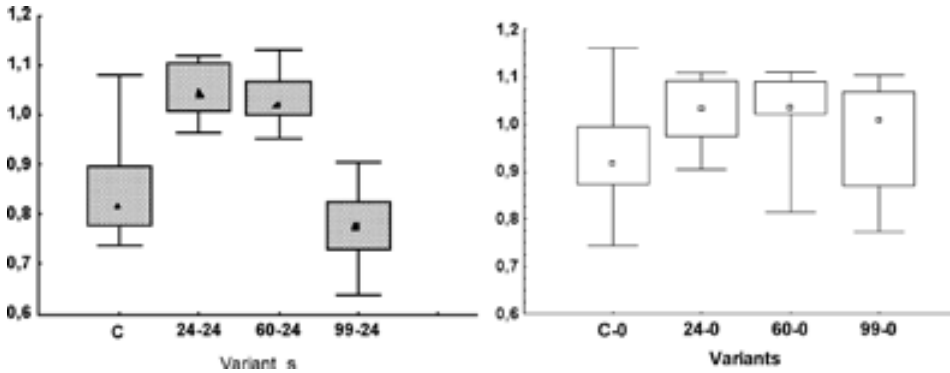

Fig. 3.
Phototoxicity of ICG (24 μM) for HeLa cells 24 h after irradiation
Comparison of the individual variants (absorbances measured in MTT assay) with the control C = 0–24 (0 J/cm^2 – 24 μM ICG). The following differences were significant:
P = 0.034 Variant 24–0 (24 J/cm^2 – 0 μM ICG)
P = 0.010 Variant 24–24 (24 J/cm^2 – 24 μM ICG)
P = 0.023 Variant 60–24 (60 J/cm^2 – 24 μM ICG)
P = 0.004 Variant 99–24 (99 J/cm^2 – 24 μM ICG)

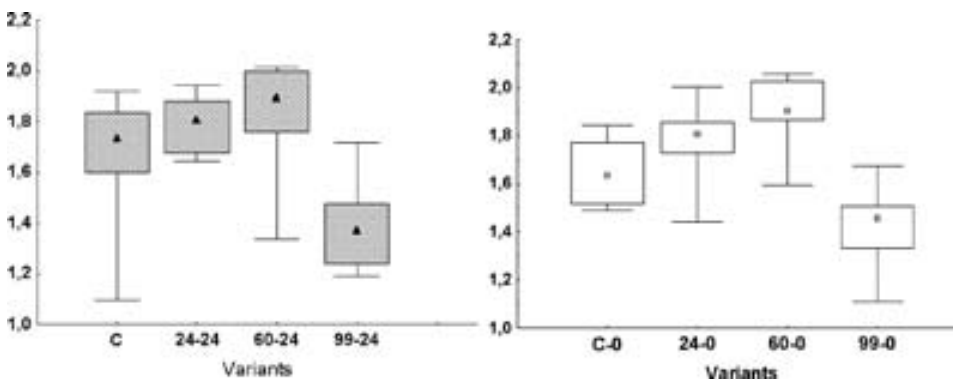

Fig. 4.
Phototoxicity of ICG (24 μM) for HeLa cells 48 h after irradiation
Comparison of the individual variants (absorbances measured in MTT assay) with the control C = 0–24 (0 J/cm^2 – 24 μM ICG). All the differences were significant:
P = 0.034 Variant 24–0 (24 J/cm^2 – 0 μM ICG)
P = 0.023 Variant 24–24 (24 J/cm^2 – 24 μM ICG)
P = 0.002 Variant 60–0 (60 J/cm^2 – 0 μM ICG)
P = 0.023 Variant 60–24 (60 J/cm^2 – 24 μM ICG)
P = 0.005 Variant 99–0 (99 J/cm^2 – 0 μM ICG)
P = 0.002 Variant 99–24 (99 J/cm^2 – 24 μM ICG)

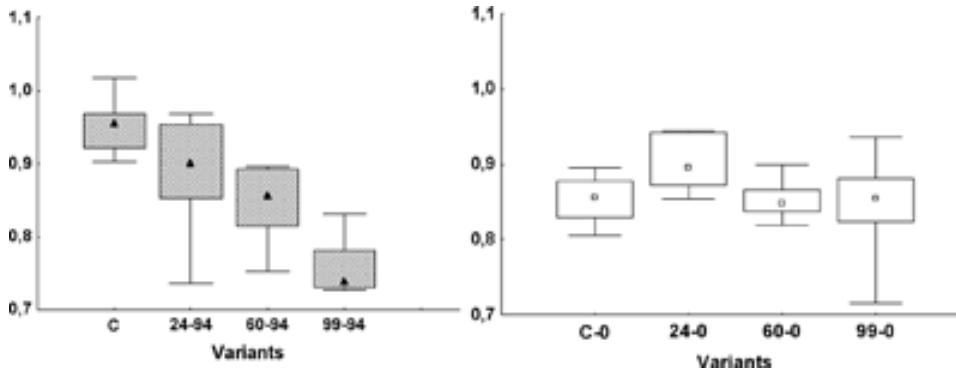

Fig. 5.
Phototoxicity of ICG (94 μM) for HeLa cells 24 h after irradiation
Comparison of the individual variants (absorbances measured in MTT assay) with the control C = 0–94 (0 J/cm^2 – 94 μM ICG). All the differences were significant:

P < 0.001 Variant 0–0 (0 J/cm^2 – 0 μM ICG)
P = 0.009 Variant 24–0 (24 J/cm^2 – 0 μM ICG)
P = 0.028 Variant 24–94 (24 J/cm^2 – 94 μM ICG)
P < 0.001 Variant 60–0 (60 J/cm^2 – 0 μM ICG)
P < 0.001 Variant 60–94 (60 J/cm^2 – 94 μM ICG)
P < 0.001 Variant 99–0 (99 J/cm^2 – 0 μM ICG)
P < 0.001 Variant 99–94 (99 J/cm^2 – 94 μM ICG)

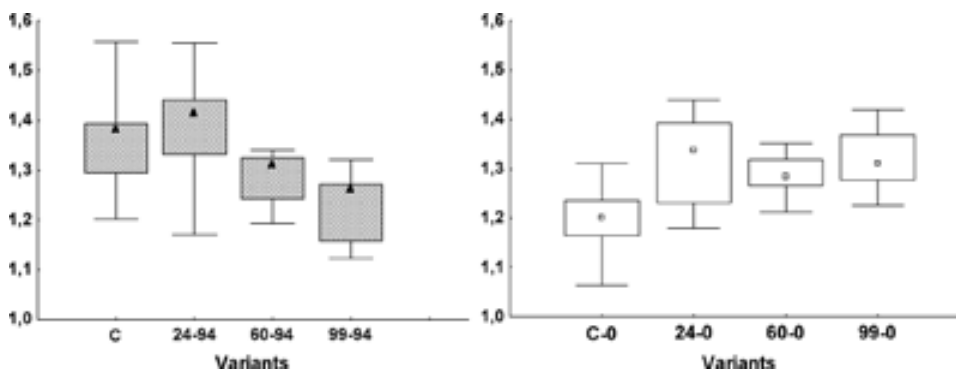

Fig. 6.
Phototoxicity of ICG (94 μM) for HeLa cells 48 h after irradiation
Comparison of the individual variants (absorbances measured in MTT assay) with the control C = 0–94 (0 J/cm^2 – 94 μM ICG). The following differences were significant:

P = 0.002 Variant 0–0 (0 J/cm^2 – 0 μM ICG)
P = 0.045 Variant 60–94 (60 J/cm^2 – 94 μM ICG)
P = 0.007 Variant 99–94 (99 J/cm^2 – 94 μM ICG)

ICG concentration of 20 μg/ml combined with laser irradiation had caused destructionof cells in all the three lines. In our experiments with ICG we had chosen a tumour model of HeLa cells for reasons of a possible later application of PDT on uterine cervix carcinoma, because the focus would be accessible for an exciting radiation probe.

Further experiments are necessary to research the phototherapeutic potential of ICG in vivo on model organisms, because so far the phototherapeutic potential of ICG has mostly been tested in vitro (*8*).

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STUDIUM CYTOTOXICITY A FOTOTOXICITY INDOCYNINOVÉ ZELENĚ IN VITRO

Souhrn

Práce se zabývá fotochemickými vlastnostmi potenciálního fotosensitizéru indocyninové zeleně (ICG) in vitro. U HeLa buněk byla prokázána význačná cytotoxicita ICG 24 hod. po aplikaci, a to při koncentracích vyšších než 206 μM. Dále byla sledována proliferační křivka HeLa buněk po kombinovaném účinku ICG při koncentracích 24 a 94 μM a laserového záření (830 nm) při hustotách energie 0, 24, 60, a 99 J/cm^2. Fototoxicita byla hodnocena 1, 24, a 48 hodin po ozáření. jednu hodinu po ozáření nebyl pozorován žádný fototoxický účinek v žádném z experimentů. Fototoxický účinek u koncentrace ICG 24 μM byl zjištěn po 24 a 48 hod. pouze u nejvyšší hustoty energie 99 J/cm^2. Prozatím se zdá být optimální koncentrace ICG 94 μM, u které se fototoxický účinek objevil při všech aplikovaných hustotách energie (24, 60, and 99 J/cm^2) již po 24 hodinách. 48 hodin po ozáření byl fototoxický účinek patrný pro aplikované hustoty energie 60 a 99 J/cm .

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