

Photodynamic therapy of malignant glioma with hypericin: Comprehensive *in vitro* study in human glioblastoma cell lines

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Abstract. The poor prognosis of patients suffering from malignant glioma requires further efforts. Photodynamic therapy (PDT) might be a therapeutic option to increase surgical radicality. Hypericin (HY) exhibit high phototoxicity to malignant cells and accumulates to a higher extent in glioblastoma cells as compared to neurons. Therefore, the impact of various experimental parameters on cytotoxicity, intracellular accumulation and phototoxicity of HY was quantitatively assessed in the three human glioblastoma cell lines U373 MG, LN229 and T98G. Additionally, intracellular location of HY was studied with fluorescence microscopic techniques. For all three cell lines, no cytotoxicity was found for incubation concentrations up to 5 μ M. For short-time incubation (2 h), maximum HY fluorescence was achieved at an incubation concentration of about 5 μ M. However, uptake kinetics of HY was dependent on its incubation concentration. Moreover, increase in HY fluorescence was negligible at 4°C, which strongly indicates that the compound is taken up by an energy-dependent process. HY exhibited high phototoxicity (at 595 nm) in all three cell lines with ID₅₀-values ranging from 0.15 J/cm² to 0.22 J/cm², but sensitivity decreased in the order U373 MG > LN229 > T98G. However, assessment of phototoxicity at different wavelengths revealed that highest cell inactivation was achieved at 600 nm. Fluorescence microscopy showed that HY fluorescence arose predominantly from the perinuclear region and the nuclear membrane. Fluorescence pattern of HY was significantly different from those observed for organelle markers staining lysosomes or mitochondria. Location of HY in the plasma membrane was proven by total internal reflection fluorescence microscopy. Thus, the present study demonstrates that glioblastoma cells can be effectively

inactivated by HY-PDT after short-time incubation and exposure to low light doses. These results obtained in cell culture are encouraging and justify further evaluation HY-PDT for the treatment of malignant glioma in animal experiments.

Introduction

Photodynamic therapy (PDT) is a relatively new modality of cancer treatment, which rely on the administration of a photosensitizer, which preferentially accumulates in tumour tissue. Upon illumination at the appropriate wavelength the photosensitizer molecules are excited from their ground state S₀ to an electronically excited singlet state S_x. The excited state energy can be dissipated via several competing relaxation pathways. Transition of the photosensitizer molecules from the lowest excited singlet state S₁ to the lowest triplet excited state T₁ (intersystem crossing) is required for the generation of cytotoxic reactive oxygen species (ROS). Superoxide radicals result from several electron transfer reactions (type I mechanism), whereas singlet oxygen is generated by energy transfer from triplet state molecules to molecular oxygen (type II mechanism). ROS react with various biomolecules (e.g. proteins) causing tumour cell death (1).

Hypericin (HY), a phenanthroperylene quinone, is a naturally occurring compound found in plants of the genus *Hypericum*, of which *Hypericum perforatum* (St. John's wort) is most common. Besides pharmacological activity in the dark, e.g. antiviral (2), antitumoral (3) and antiangiogenic activity (4), HY exhibit excellent photosensitizing properties [reviewed by Agostinis *et al* (5)], mainly due to its high triplet quantum yield and its efficient generation of ROS (6,7). Consequently, phototoxicity of HY was diminished when specific quenchers of ROS were applied (8) or under hypoxic conditions (9). HY-mediated photodynamic inactivation of malignant cell lines of different origin (9-15), including glioblastoma cells (16,17), has been demonstrated during the last decade.

Patients suffering from malignant glioma have a very poor prognosis. Although median survival of patients treated with surgery, radiation and chemotherapy has been prolonged during the last few years (18,19), many patients die within the first year after diagnosis. Recurrence of malignant glioma occurs most frequently at the margins of resection (20).

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Surgical radicality was successfully improved by fluorescence-guided resection of the tumours after administration of 5-aminolaevulinic acid (5-ALA) (21,22). This intermediate of porphyrin biosynthesis is converted to protoporphyrin IX (PP IX) in most cell types. Quite selective accumulation of 5-ALA-induced PP IX has been demonstrated for malignant glioma cells *in vitro* (23,24) and *in vivo* (25,26) as well as for many other tumour cells. In addition, due to the photosensitizing properties of PP IX, photodynamic treatment of malignant glioma might be considered as a promising additional therapeutic option, which is currently investigated *in vitro* (27,28) and *in vivo* (29-32).

In particular, the observation that HY accumulates to a higher extent in glioblastoma cells as compared to neurons (33) recommends a comprehensive evaluation of the sensitivity of glioblastoma cells to HY-PDT. Thus, the objective of the present study was to investigate the impact of various experimental parameters, e.g. photosensitizer concentration or incubation time, on cytotoxicity, intracellular accumulation and phototoxicity of HY in three human glioblastoma cell lines. In addition, intracellular location of HY was studied with different fluorescence microscopic techniques.

Materials and methods

Chemicals. Hypericin (HY) was obtained from Phytochem (Ichenhausen, Germany) with a purity higher than 99%. Stock solutions of HY were made up in DMSO at a concentration of 2 mM and stored in the dark at -20°C. For all experiments, incubation media were freshly prepared in cell culture medium (at a final FCS content of 10%; see below) under sterile conditions.

Cell cultivation. Human glioblastoma cell lines were obtained from either the European Collection of Cell Cultures, Wiltshire, UK (U373 MG cells, ECACC No. 890811403) or from the American Type Culture Collection, Manassas, VA, USA (LN229, ATCC No. CRL-2611; T89G, ATCC No. CRL-1690). All three cell lines were routinely grown in RPMI-1640 medium (Gibco-BRL, Paisley, UK; supplemented with GlutaMAX™) supplemented with 10% FCS (Biochrom, Berlin, Germany) and antibiotics [penicillin (100 IU/ml) and streptomycin (100 µg/ml); Gibco-BRL] maintained at 37°C and 5% carbon dioxide in an incubator (Cellstar; Nunc, Wiesbaden, Germany). Cells were detached by incubation with Accutase™ (PAA Laboratories, Pasching, Austria) at 37°C for 5-10 min after rinsing with phosphate-buffered saline (PBS, Gibco-BRL). For all experiments, cells were seeded at a defined density of 150 cells/mm² and grown at same conditions for 24 h prior to incubation.

Cytotoxicity. To determine cytotoxicity of HY, cells were grown in 24-well plates (Nunc; seeding of 3x10⁴ cells/well) and incubated (37°C) with HY at concentrations of 0.5, 1.0, 2.5, 5 and 10 µM for 2 h or 24 h. After rinsing with PBS cells were reincubated with (photosensitizer-free) culture medium and grown for 48 h (37°C/5% CO₂). Cell survival was assessed with the neutral red (NR) assay (10,34). Cultures were thus incubated (37°C) with an aqueous NR solution [Biochrom; diluted with culture medium 1:25

(V/V) resulting in final NR concentration of 0.012% and adjusted to pH 7.4] for 2-3 h (by addition of 500 µl NR solution to each well). Supernatants were discarded and cells were rinsed twice with isotone saline. Neutral red was extracted from the cells by addition of 150 µl of a mixture of water/ethanol/acetic acid (1/1/0.02, V/V/V). After gentle agitation for 10 min, a 100 µl aliquot of each sample was transferred into a microtiter plate (Greiner, Frickenhausen, Germany). Absorbance was measured at 570 nm (with a reference wavelength of 690 nm) on a plate reader (Lucy 1, anthos Mikrosysteme, Krefeld, Germany) and analysed with the anthos WinRead Software (Version 2.3). Cell survivals were calculated as percentages of the non-incubated controls. For each cell line, at least three independent experiments were performed in quadruplicate.

Phototoxicity. To determine phototoxicity of HY, cells were cultured in 4-well plates (Nunc; seeding of 3x10⁴ cells/well) and incubated (37°C) with HY at a non-cytotoxic concentration of 2.5 µM for 2 h. After rinsing with PBS and reincubation with culture medium (without photosensitizer) cells were illuminated using a dye laser (model 375, Spectra Physics, Mountain View, USA) pumped by an argon ion laser (model 2030, Spectra Physics). All three cell lines were illuminated with energy densities (light doses) of 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4 and 0.5 J/cm² at 595 nm (12) and a power density (fluence rate) of 5 mW/cm² by increasing exposure times from 10 sec to 100 sec. Photodynamic inactivation of T98G and U373 MG cells was also determined for various incubation concentrations of HY (0.1-2.5 µM/2 h/37°C). Cells were illuminated with energy densities of up to 1.5 J/cm² at 595 nm (5-10 mW/cm²) as described above. In addition, LN229 cells were illuminated at different wavelengths ranging from 575 nm to 615 nm (in 5 nm steps). For each wavelength, the light was delivered at a power density of 10 mW/cm² and cells were exposed for 30 sec (corresponding to an energy density of 0.3 J/cm²).

All illumination experiments were monitored with a power meter (model TPM-310, Gentec, Quebec, Canada). In each case, cell survival was assessed photometrically using the NR assay (as described above) after an additional growth period of 48 h (37°C, 5% CO₂). Cell survivals were calculated as percentages of the incubated and non-illuminated controls. As a quantitative measure of phototoxicity, ID₅₀-values [inactivation doses (light doses) required to reduce cell survival to 50% of the controls] were deduced from dose response curves. In each case, at least three independent experiments were performed in quadruplicate.

Intracellular accumulation. To determine the kinetics of intracellular accumulation of HY, U373 MG cells were cultivated in 24-well plates (seeding of 3x10⁴ cells/well) and incubated (37°C) with either different HY concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 5, 10, 15 and 20 µM) for 2 h or different incubation times (5, 10, 15, 30 min and 1.5, 2, 3, 4, 6 and 24 h) at HY concentrations of 2.5, 5 and 20 µM. After rinsing with PBS (500 µl) cells were detached with Accutase™ (300 µl as described above). Cells of 4 identically treated wells were pooled and filtered using a metal mesh (100-112 µm pore size; Haver & Boecker, Oelde, Germany).

After addition of propidium iodide (Sigma-Aldrich, Steinheim, Germany) dissolved in PBS (2 mg/ml; 10 μ l per 1 ml cell suspension) fluorescence of HY-treated cells was measured by flow cytometry (FACSSORT, Becton-Dickinson, San Jose CA, USA; excitation wavelength: 488 nm). Measurements were done considering the parameters forward scatter (FSc) and side scatter (SSc) to identify the cell population (dot plot FSc vs. SSc), fluorescence channel 2 (FL2, emission wavelength: 585 \pm 21 nm) to determine cellular HY uptake and fluorescence channel 3 (FL3, emission wavelength: >670 nm) to assess cell viability and analysed with the CellQuest™ software (version 4.0.2, Becton-Dickinson). In each case, at least 4 independent experiments were performed in duplicate and each data point represents the mean fluorescence intensity of 25.000 cells. To study the uptake mechanism of HY, U373 MG cells were cultivated in 24-well plates. After pre-cooling of cells and incubation medium at 4°C for 1 h, cells were incubated with 2.5 μ M HY at 4°C for 2 h and subsequently at 37°C for another 2 h. Cells concomitantly incubated with HY (2.5 μ M/ without pre-cooling of the medium) at 37°C for 4 h served as controls. After rinsing the cultures with PBS and detachment of the cells with Accutase™ intracellular accumulation of HY was determined by flow cytometry as described above.

Intracellular location. To determine the intracellular location of HY, cells were cultivated on object slides (Marienfeld, Lauda-Königshofen, Germany; seeding of 2.85 \times 10⁵ cells/slide) and incubated (37°C) with HY at concentrations of 0.5, 1.5 and 2.5 μ M for 2 h. In addition, cells were incubated with either the mitochondrial marker rhodamine 123 (R 123, Fluka, Buchs, Switzerland; 1 μ M/1 h) or the lysosomal marker acridine orange (AO, Molecular Probes, Eugene, USA; 1 μ M/1 h). In all cases, cells were rinsed with Earl's buffered salt solution (EBSS, Sigma-Aldrich) prior to fluorescence microscopy (Axioplan 1, Carl Zeiss Jena, Jena, Germany). Two different fluorescence microscopic techniques were applied: a) Conventional fluorescence microscopy {light source: high pressure mercury lamp (HBO 50, Carl Zeiss) in combination with appropriate filters [band-pass filters at 510-560 nm (HY/AO) and 450-490 nm (HY/R 123)] as well as dichroic mirrors [580 nm (HY/AO) and 510 nm (R 123)]} was used to investigate the distribution of HY and the organelle markers within whole cells (epi-illumination). b) Total internal reflection fluorescence microscopy [TIRFM, light source: pulsed laser diode (LDH C470 with driver PDL 800-B, Picoquant, Berlin, Germany; wavelength: 470 nm, repetition rate: 40 MHz, average power: 1.2 mW) adapted to the fluorescence microscope using a single mode fiber (Point Source, Southampton, UK)] was applied to demonstrate HY location within the plasma membrane of glioblastoma cells (TIR illumination). In the second case, fluorescence is excited by an evanescent electromagnetic field (arising upon total internal reflection of the excitation laser light at the cell substrate interface) with a penetration depth $d(\Theta, \lambda)$ of about 100 nm (range between 70 nm and 250 nm depending on the angle of incidence Θ and the wavelength of the excitation light λ) enabling an almost selective examination of the plasma membrane (with quite little contribution of adjacent cellular sites). The variable angle TIRFM device used in this

study has been described in detail previously (35). In all cases, fluorescence was detected using an EMCCD camera (with Peltier cooling, sensitivity below 10⁻¹⁷ W/Pixel; DV887DC-BV, ANDOR Technology, Belfast, UK) in combination with appropriate long-pass filters [>590 nm (HY/AO) and >520 nm (R 123)].

Statistical analysis. Values of the quantitative data are given as median (central tendency) \pm median absolute deviation (MAD, variability) except otherwise noted.

The relation between incubation concentration of HY c_i and cellular fluorescence $I_F(c_i)$ was fitted by a non-linear least square estimation with 4 parameters according to:

$$I_F(c_i) = I_0 + \frac{I_{\max} - I_0}{1 + \left(\frac{K_{50}}{c_i}\right)^a} \text{ for } c_i > 0 \quad \text{i)}$$

with minimum intensity I_0 (a.u.) (i.e. cellular autofluorescence), maximum intensity I_{\max} (a.u.), K_{50} -value (μ M) corresponding to c_i (μ M) resulting in half-maximal fluorescence as well as the fitting parameter $a = 1.5$.

For an incubation concentration of 2.5 μ M, the relation between incubation time of HY t and cellular fluorescence $I_F(t)$ was fitted according to:

$$I_F(t) = \frac{\nu}{\alpha} (1 - e^{-\alpha t}) \quad \text{ii)}$$

with the two fitting parameters ν and α describing increase and decline of HY fluorescence, respectively.

For incubation concentrations of 10 μ M and 20 μ M, the relation between incubation time of HY t and cellular fluorescence $I_F(t)$ was fitted by a compartment model with 4 parameters according to:

$$I_F(t) = I_{\max} (1 - e^{-\gamma t}), \text{ for } t < t_{\max} \quad \text{iii) and}$$

$$I_F(t) = I_{\infty} + (I_{\max} (1 - e^{-\gamma t_{\max}}) - I_{\max}) e^{-\alpha (t - t_{\max})} \text{ for } t \geq t_{\max} \quad \text{iv)}$$

with maximum intensity I_{\max} (a.u.) achieved at the incubation time t_{\max} (h) and I_{∞} (a.u.) corresponding to the intensity at infinite incubation time t_{∞} of HY. Parameters γ and α are characterizing uptake of HY from the incubation medium into the cells (at short incubation times) and cellular release of HY into the medium (at longer incubation times), respectively.

Phototoxicity, i.e. the relation between cell survival and light doses, was fitted according to:

$$SF = \frac{100}{1 + \left(\frac{E}{ID_{50}}\right)^a} \quad \text{v)}$$

with surviving fraction SF (% of control), energy density E (J/cm²), inactivation dose ID_{50} (J/cm²) and a fitting parameter a .

For post-hoc pair-wise comparison the Tukey-Kramer HSD test was used. The global significance level was 5%. All statistical calculations and curve fittings were carried out with the software package JMP version 5.1 (www.JMP.com).

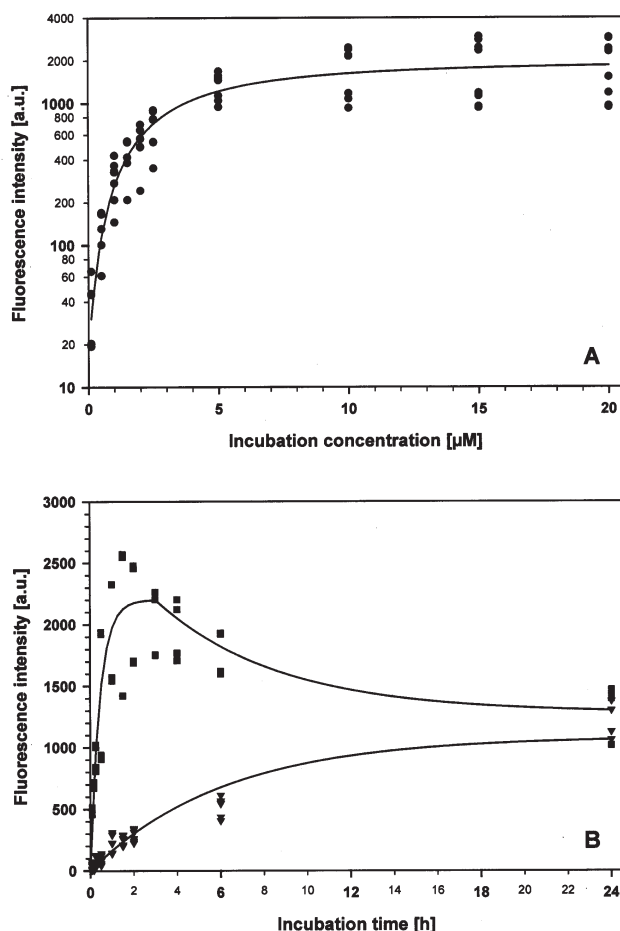


Figure 1. Cellular accumulation of hypericin in U373 MG glioblastoma cells dependent on (A) incubation concentration (incubation time of 2 h) and (B) incubation time [incubation concentrations of 2.5 μM (\blacktriangledown) and 20 μM (\blacksquare)] measured by flow cytometry.

Results

Cytotoxicity. Cytotoxicity of HY was determined in the three human glioblastoma cell lines U373 MG, LN229 and T98G for two different incubation times. After short-time incubation of 2 h the photosensitizer exhibited no cytotoxicity (defined as $>95\%$ viable cells as compared to non-incubated controls) up to an incubation concentration of 5 μM . Extremely weak cytotoxicity (cell survival $>90\%$ of the controls) was found after increasing either the incubation concentration to 10 μM or the incubation time to 24 h. No significant differences between the three different cell lines were observed for any of the incubation conditions investigated.

Intracellular accumulation. U373 MG glioblastoma cells were selected as an example to study intracellular accumulation of HY in a time- and concentration-dependent manner by flow cytometry, i.e. cellular fluorescence served as a measure for intracellular HY accumulation. Cells were either incubated at HY concentrations up to 20 μM for 2 h or at HY concentrations of 2.5, 10 or 20 μM up to 24 h. In each case, the fraction of viable cells within the samples was $>90\%$ as determined by propidium iodide staining, although the highest incubation concentration of HY (20 μM) was not considered in the cell-based assay for cytotoxicity (see above).

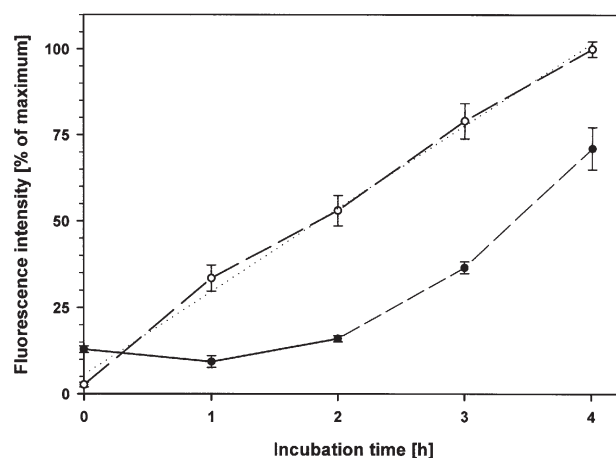


Figure 2. Cellular accumulation of hypericin in U373 MG glioblastoma cells (incubation concentration 2.5 μM) dependent on incubation temperature [4°C for 2 h and subsequently 37°C for further 2 h (\bullet) vs. 37°C for 4 h (\circ)] measured by flow cytometry.

For short-time incubation of 2 h, cellular fluorescence increased up to incubation concentrations of about 5 μM , whereas no further increase in HY accumulation was found at higher incubation concentrations. Measured values as well as the fitted curve (according to equation i) are shown in Fig. 1A.

After incubation of U373 MG cells with HY at a concentration of 2.5 μM a continuous increase in cellular fluorescence was observed up to 6 h. Extension of the incubation period up to 24 h resulted in further intracellular HY accumulation with a doubling of fluorescence intensity. In contrast, kinetics of cellular HY accumulation was significantly different at higher incubation concentrations of 10 and 20 μM . In both cases, the photosensitizer was rapidly taken up and highest intracellular accumulation was found after about 2 h incubation. In agreement with the results described above, maximum fluorescence intensities were quite similar for both HY concentrations. Increase in incubation time up to 6 h resulted in a lowering of cellular fluorescence by about 20–30% as compared to maximum fluorescence. After 24 h incubation cellular fluorescence was further reduced and intensity was in the same order of magnitude as attained for an incubation concentration of 2.5 μM . Measured values as well as the fitted curves [according to equation ii (2.5 μM incubation) and equations iii and iv (20 μM incubation)] are shown in Fig. 1B.

Cellular uptake mechanism. To study the mechanism of cellular HY uptake in more detail, U373 MG glioblastoma cells were incubated with HY (2.5 μM) at different temperatures. Cellular HY accumulation was negligible in the first half of the incubation period at 4°C, whereas cellular HY fluorescence increased rapidly when cells were subsequently incubated at 37°C for another 2 h. At the end of the incubation period fluorescence intensity was about two-thirds of that found for control cells incubated at 37°C for 4 h. These data are depicted in Fig. 2.

Phototoxicity. In first set of experiments, phototoxicity of HY was determined for all three glioblastoma cell lines

Table I. Phototoxicity of HY in human glioblastoma cell lines (incubation: 2.5 μ M for 2 h) after illumination at 595 nm (5 mW/cm²).

Cell line	ID ₅₀ -value (J/cm ²)
U373 MG	0.15
LN229	0.19
T98G	0.22

Table II. Phototoxicity of HY in human T98G glioblastoma cells incubated at different concentrations (2 h) after illumination at 595 nm (10 mW/cm²).

HY concentration (μ mol)	ID ₅₀ -value (J/cm ²)
0.5	0.88
1.5	0.34
2.5	0.20

U373 MG, LN229 and T89G at a non-cytotoxic incubation concentration of 2.5 μ M (incubation time of 2 h each) and an illumination wavelength of 595 nm. Illumination of HY treated cells resulted in considerable reduction in cell viability and ID₅₀-values of 0.15 J/cm² (U373 MG), 0.19 J/cm² (LN229) and 0.22 J/cm² (T98G) were calculated. The ID₅₀-value for U373 MG cells was significantly different from that for T98G cells using one-way analysis (Tukey-Kramer test, $p < 0.05$). These data are summarized in Table I. Exposure of the cell to 0.4 J/cm² resulted in a decrease in cell viability of about one order of magnitude and cell survival varied between 9 % (U373 MG) and 12-13% (LN229 and T98G).

Subsequently, the relation between incubation concentration of HY and photodynamic inactivation of glioblastoma cells was studied in some more detail. Therefore, phototoxicity of HY was also determined for different non-cytotoxic concentrations in T98G and U373 MG cells at an illumination wavelength of 595 nm. In case of T98G cells, complete dose-response curves were determined for incubation concentrations of 0.5, 1.5 and 2.5 μ M at incubation times of 2 h each. ID₅₀-values increased with decreasing HY concentration from 0.2 J/cm² (2.5 μ M) over 0.34 J/cm² (1.5 μ M) to 0.88 J/cm² (0.5 μ M). These data are summarized in Table II and dose-response curves are shown in Fig. 3A. In case of U373 MG cells, cultures were exposed to HY concentrations between 0.1 μ M and 2.5 μ M (incubation times of 2 h each) and equal light doses of 0.3 J/cm². Photodynamic treatment at incubation concentrations of up to 1 μ M resulted in weak to moderate cell inactivation (>75% cell survival). In contrast, pronounced cell damage with survival rates of about 25% was observed at HY concentrations ≥ 1.5 μ M (Fig. 3B). These results indicate that no simple relation exists between photodynamic efficacy of HY and its incubation concentration.

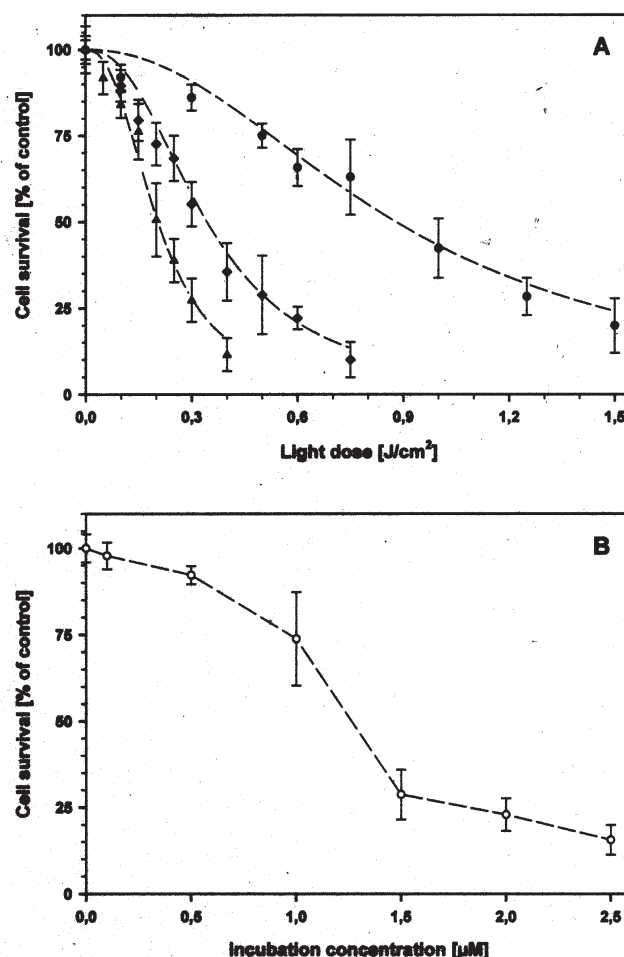


Figure 3. Phototoxicity of HYP in (A) T98G [0.5 μ M (●), 1.5 μ M (◆) and 2.5 μ M (▲)] and (B) U373 MG human glioblastoma cells dependent on incubation concentration (incubation time of 2 h). Illumination was performed at 595 nm and light was delivered at 5-10 mW/cm². Cell viability was determined using the NR-assay 48 h after illumination. Cell survivals were calculated as percentages of the incubated and non-illuminated controls. Each value represents median \pm MAD of at least three independent experiments performed in quadruplicate.

To determine the optimal wavelength for HY-PDT within cellular environment more precisely, LN229 glioblastoma cells were illuminated at different wavelengths between 575 nm and 615 nm in steps of 5 nm; a light dose of 0.3 J/cm² was applied at each wavelength after incubation with HY (2.5 μ M/2 h). Weak to moderate cell inactivation with survival rates decreasing from 90% to 60% was observed for wavelengths between 575 nm and 590 nm. Illumination at 595 nm and 600 nm resulted in considerable photodynamic damage of the cells with survival rates of about 20%, respectively. At higher wavelength cell survival increased rapidly and was negligible at wavelengths >610 nm. These results, i.e. the action spectrum of HY within cellular environment, are shown in Fig. 4.

Intracellular location. Intracellular location of HY was investigated in the three human glioblastoma cell lines U373 MG, LN229 and T98G for non-cytotoxic incubation concentrations of 0.5, 1.5 and 2.5 μ M (incubation time of 2 h each) with two different fluorescence microscopic techniques. For all three cell lines, fluorescence of HY upon

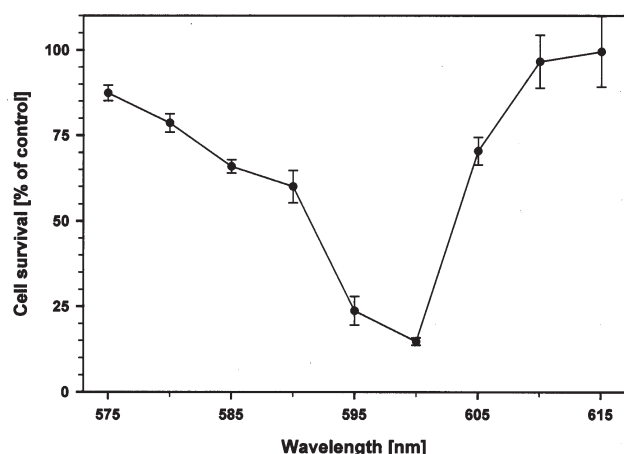


Figure 4. Phototoxicity of HY in human LN229 glioblastoma cells (incubation: $2.5 \mu\text{M}$ for 2 h) after illumination at different wavelengths ($0.3 \text{ J}/\text{cm}^2$ delivered at $5 \text{ mW}/\text{cm}^2$ each). Cell viability was determined using the NR-assay 48 h after illumination. Cell survivals were calculated as percentages of the incubated and non-illuminated controls. Each value represents median \pm MAD of at least three independent experiments performed in quadruplicate.

epi-illumination (i.e. conventional fluorescence microscopy) arose predominantly from the perinuclear region (typically on one side of the nucleus with some small granules) and the nuclear membrane. In addition, faint fluorescence was detected within the whole cytoplasm. As depicted in Fig. 5 [A1 (U373 MG), B1 (T98G) and C1 (LN229)], no obvious differences in intracellular location were observed for the

three glioblastoma cell lines incubated at a HY concentration of $2.5 \mu\text{M}$. Moreover, intracellular distribution of HY remained basically unchanged when the incubation concentration of HY was reduced to $1.5 \mu\text{M}$ and $0.5 \mu\text{M}$ as exemplarily shown for T98G cells [Fig. 5, B1 ($2.5 \mu\text{M}$), B2 ($1.5 \mu\text{M}$) and B3 ($0.5 \mu\text{M}$)], although fluorescence intensity decreased with decreasing HY supply. In all cases, cell borders have been visible suggesting that HY was also located in the plasma membrane of the glioblastoma cells.

Therefore, total internal reflection fluorescence microscopy (TIRFM) was applied. This microscopic technique enables a quite selective excitation of fluorophores in (or in close vicinity to) the plasma membrane, since the penetration depth $d(\Theta)$ of the excitation light is limited (see above). For all three cell lines, HY fluorescence could be easily detected upon TIR illumination as exemplarily shown for LN229 cells [Fig. 5, C2 ($d(\Theta) \approx 150 \text{ nm}$ at $\Theta = 66^\circ$) and C3 ($d(\Theta) \approx 80 \text{ nm}$ at $\Theta = 70^\circ$)]. In all cases, fluorescence images upon TIR illumination showed some structured pattern with highest intensity in those sites where the plasma membrane is in immediate vicinity to the glass substrate.

Staining of the three glioblastoma cell lines with the mitochondrial marker rhodamine 123 (R123) and the lysosomal marker acridine orange (AO) resulted in fluorescence patterns which have been distinctly different from those observed after HY incubation as shown for U373 MG cells [Fig. 5, A1 (HY), A2 (R123) and A3 (AO)]. Therefore, mitochondria and lysosomes seem to be not the predominant location sites of HY within these glioblastoma cells.

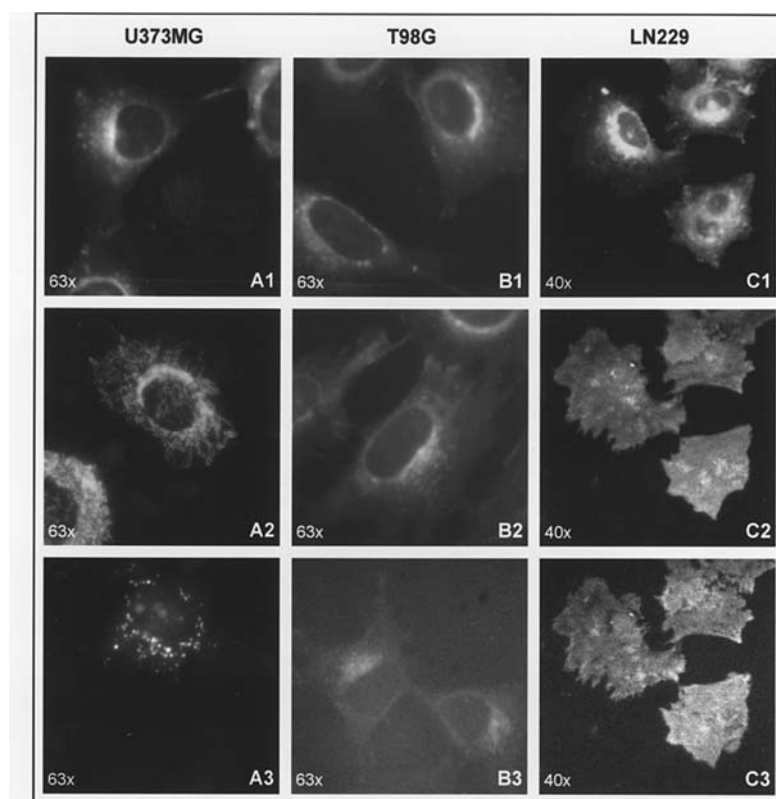


Figure 5. Fluorescence microscopic images of U373 MG, T98G and LN229 glioblastoma cells; U373 MG cells incubated with HY ($2.5 \mu\text{M}/2 \text{ h}$, A1), rhodamine 123 ($1 \mu\text{M}/1 \text{ h}$, A2) and acridine orange ($1 \mu\text{M}/1 \text{ h}$, A3); T98G cells incubated with HY at concentrations of $2.5 \mu\text{M}$ (B1), $1.5 \mu\text{M}$ (B2) and $0.5 \mu\text{M}$ (B3) for 2 h each; LN229 cells incubated with HY ($2.5 \mu\text{M}/2 \text{ h}$) upon epi-illumination (C1) and TIR illumination (C2/ $\Theta = 66^\circ$ and C3/ $\Theta = 70^\circ$ with Θ corresponding to the angle of incidence). Fluorescence was measured at $\lambda_{\text{em}} = >590 \text{ nm}$ (HY/acridine orange) or $\lambda_{\text{em}} = >520 \text{ nm}$ (rhodamine 123).

Discussion

For all three glioblastoma cell lines, HY exhibited high phototoxicity combined with weak to negligible cytotoxicity of the compound itself (dark toxicity). Illumination of HY-treated glioblastoma cells (2 h/2.5 μ M) at 595 nm with light doses as low as 0.15-0.20 J/cm² resulted in a decrease in cell survival to 50% (ID₅₀-values); after exposure to 0.4 J/cm² cell survival was reduced to about 10% as compared to non-illuminated controls. These results are basically in line with results obtained for other cell lines (11,13,16). In the case of AY-27 cells (transitional cell carcinoma), incubated with HY (0.05-10 μ M/2 h) and illuminated (595 nm) of under quite similar experimental conditions as used in the present study, an incubation concentration of about 2.5 μ M was required to reduced cell survival to 50% (IC₅₀-value) when cells were illuminated with 0.45 J/cm² (12). Another study, which compared the sensitivity of four different cell lines to HY-PDT, cell survival could be decreased to about 50% with incubation concentrations of 1.25 μ M and 2.5 μ M (incubated for 5 h) and light exposure to 0.4 J/cm² (11). Finally, sensitivity of U373 MG cells to HY-PDT seem to be somewhat higher as compared to that of the two other cell lines. This might be caused by an increased uptake of HY in U373 MG or (and) an increased sensitivity of this cell line to oxidative stress. As demonstrated previously, photodynamic efficacy of HY varied between different cell lines, and was directly related to the intracellular accumulation of the photosensitizer (10).

As exemplified for LN229 glioblastoma cells (Fig. 4), efficient photodynamic cell inactivation could also be achieved at wavelength slightly >595 nm; considering the experimental protocol of the present study, i.e. illumination at wavelength from 575 nm to 615 nm in steps of 5 nm, an optimal wavelength of 600 nm for HY-PDT was determined. This is basically in agreement with previous results obtained with RT-112 (human transitional cell carcinoma), where illuminations at 592, 595 and 598 nm resulted in almost identical cell survival (36). However, it should be mentioned that the surviving fractions of RT-112 cells were very small (far below 10%), which usually compromises the reliability of data on cell survival obtained by an optical test. In addition, it has been demonstrated *in vivo* (C26 colon carcinoma cells inoculated subcutaneously) that HY-PDT was more effective at 590 nm as compared to that at 550 nm. This was deduced from an increase in the depth of tumour necrosis, which was not solely due to the increased penetration depth of yellow light as compared to green light (13).

Cellular accumulation of HY in U373 MG glioblastoma cells was determined in a time- and concentration-dependent manner by flow cytometry. Maximum intracellular HY fluorescence was achieved after an incubation time of about 2 h and incubation concentrations greater than 5 μ M (Fig. 1). These findings are concordant with a fluorescence microscopic study reported by us recently (33), where 20 different glioma cells (8 permanent cell lines and 12 primary cultures) exhibited maximum fluorescence at 1-2 h after incubation with HY at a concentration of 20 μ M. Although similar fluorescence intensities were found after 24-h incubation with HY at concentrations of 2.5 and 20 μ M, accumulation kinetics of HY in U373 MG cells seem to differ considerably.

However, it should be mentioned that reliability of fluorescence measurements will be compromised when the fluorescence quantum yield of the chromophore changes, e.g. in cellular environment. For PAM-212 cells (murine keratinocytes) incubated with HY at concentrations as high as 50 μ M, the intracellular formation of weakly fluorescent aggregates has been supposed (37). Incubation of U373 MG glioblastoma cells at a concentration of 2.5 μ M resulted in an almost linear increase in intracellular HY fluorescence up to an incubation time of 4 h (Fig. 2), which increased further with increasing incubation time (Fig. 1). Again, these data are basically in line with previous results obtained for other cell lines (10,11).

Since contradictory results concerning the cellular uptake mechanism of HY have been published (16,38), this issue was addressed in some more detail. No increase in cellular HY accumulation was observed in U373 MG glioblastoma cells after 2-h incubation at 4°C (Fig. 2). Since cellular metabolism is severely impaired at low temperatures, these data strongly indicate that HY is taken up into this cell line by an energy-dependent process, e.g. by receptor-mediated endocytosis or pinocytosis, rather than by (passive) diffusion. In contrast, no noticeable difference in the HY staining pattern in WiDr cells (human colon carcinoma cell line) was found when comparing fluorescence images after 1 h incubation at 4°C or at room temperature by Uzdensky *et al* (16). Consequently, they concluded that HY penetrates cells mostly by diffusion. Although the uptake mechanism of HY in cells of different origin might vary, it should be stressed that is virtually essential to inhibit energy metabolism early enough prior to cell incubation, e.g. reversibly by pre-cooling of cells and media as described above. However, an active uptake of HY in cells is basically in agreement with data obtained with Caco-2 cell monolayers, a model system to investigate intestinal absorption. Transcellular transport of HY at 4°C was significantly lower than at 37°C. Concomitantly, binding studies of HY to Caco-2 cells revealed a relatively low affinity constant (about 0.1 mM). Taken together, these data obtained for Caco-2 cells under serum free conditions suggest that HY is actively taken up after non-specific binding to membrane lipids (38). In addition, alteration of individual steps of endocytosis and membrane cycling changed the intracellular HY location (39), which also indicate the involvement of energy-dependent processes in uptake and distribution of HY in living cells.

Comparison of the fluorescence pattern observed for HY with that found for the two organelle markers, revealed that neither mitochondria nor lysosomes are the primary location sites of the photosensitizer. For all three glioblastoma cell lines, intracellular distribution of HY was quite similar and not affected by its incubation concentration. HY fluorescence was detected predominantly in the perinuclear region and the nuclear membrane. Thus, in agreement with previous localisation studies (16,40,41), it is supposed that HY localises preferentially within the membranes of the endoplasmic reticulum and the Golgi complex. However, it should be mentioned that location of HY in mitochondria and lysosomes has been also supposed for other cell lines (11,42).

Concerning the location of HY within the plasma membrane, also contradictory results obtained by confocal

fluorescence microscopy have been published (16,38). In the present study, total internal reflection fluorescence microscopy (TIRFM) was used to address this issue. Excitation of fluorophors is carried out by an evanescent field with a penetration depth of about 100 nm depending preferentially on the angle of incidence. Thus, this microscopic technique is virtually unique with respect to its axial resolution in close vicinity to the cell-substrate interface. HY fluorescence could be detected with TIRFM even at penetration depths below 100 nm, where the contribution of intracellular fluorophors is almost negligible. Thus, HY location in (and/or immediate vicinity to) the plasma membrane can be considered as proven for all three glioblastoma cell lines.

Due to the hydrophobicity of HY, serum proteins are necessary to deliver the photosensitizer to the cells; alternatively, solubilizers, e.g. cyclodextrins or liposomes (38), were essential under serum free conditions. Since cellular accumulation of HY seem to be crucially affected by the composition of the cell culture medium (15,38,42), comparability of the results of the individual studies is hampered.

HY is preferentially bound to low-density lipoproteins (LDL) and smaller fractions are associated with high-density lipoproteins or very low-density lipoproteins as shown with density-gradient ultracentrifugation (41) and spectroscopic methods (41,43). However, interaction with albumin has also been shown (41,44). Binding of HY to LDL might suggest that one reasonable active uptake mechanism of this photosensitizer in tumour cells is LDL-receptor-mediated endocytosis, as supposed previously also for porphyrin-based photosensitizers (1). This hypothesis may in particular hold for glioblastoma cells, which have very high numbers (several hundreds of thousands per cell) of LDL receptors (45). Cellular uptake via LDL receptor-mediated endocytosis is directed to the lysosomal compartment for further processing (46). However, fluorescence microscopy revealed that lysosomes seem not to be the primary location site of HY in glioblastoma cells after 2 h incubation (Fig. 5).

Due to a very short lifetime, diffusion length of singlet-oxygen is limited to a few tens of nanometers in cellular environment (47). Therefore, close proximity between location and damage site of the photosensitizer is required. Although accumulation of HY in mitochondria was not found in any of the three glioblastoma cells studied (see above), there is sufficient evidence in literature that mitochondria are primary cellular damage sites of HY-PDT (11,48-50). Thus, an ultra-weak amount of HY seem to be sufficient for light-induced mitochondrial damage initiating apoptotic cell death. This has been demonstrated for numerous cell lines, including glioblastoma cells, as reviewed by Agostinis *et al.* (5). Location of HY in the plasma membrane, as demonstrated in the present study with TIRFM, might induce damage of membrane constituents (51,52) and subsequently necrotic cell death. Finally, it has been shown that the ratio between apoptotic and necrotic cell death induced by HY-PDT depends on drug and light dose, and probably also on the genetic background of the cell line (5).

In conclusion, the present study demonstrates that glioblastoma cells can be effectively inactivated by HY-PDT after short-time incubation and exposure to low light doses. Due to its lipophilicity, HY seems to be preferentially located

in cellular membranes, including the plasma membrane. In addition to its low cytotoxicity (in the dark), HY has biological properties, such as inhibition of cell proliferation and signal transduction (53,54), which might be of additional advantage for the treatment of malignant glioma. These results obtained in cell culture are encouraging and justify further evaluation of HY-PDT for the treatment of malignant glioma in animal experiments.

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