Abstract. Compared to current treatments including surgery, radiation therapy, and chemotherapy, PDT offers the advantage of an effective and selective method of destroying diseased tissues without damaging surrounding healthy tissues. One of the aspects of antitumour effectiveness of PDT is related to the distribution of photosensitizing drugs. The localization of photosensitizers in cytoplasmic organelles during PDT plays a major role in the cell destruction; therefore, intracellular localization of Ph in malignant and normal cells was investigated.

The cell lines used throughout the study were: human malignant A549, MCF-7, Me45 and normal endothelial cell line HUV-EC-C. After incubation with Ph cells were examined using fluorescence and confocal microscopy to visualize the photosensitizer accumulation. For cytoplasm and mitochondria identification, cells were stained with CellTracker Green and MitoTracker Green, respectively. Distribution of Ph was different in malignant and normal cells and dependent on the incubation time. The maximal concentration of Ph in two malignant cell lines (A549 and MCF-7) was observed after 4 hours of incubation, and the most intensive signal was observed around the nuclear envelope. Intracellular distribution of Ph in the Me45 cell line showed that the fluorescence emitted by Ph overlaid that from MitoTracker. This indicates preferential accumulation of the sensitizer in mitochondria.

Our results based on the mitochondrial localization support the idea that PDT can contribute to elimination of malignant cells by inducing apoptosis, which is of physiological significance.

Introduction

Photodynamic therapy (PDT) is an approved, minimally invasive therapeutic approach to the management of a variety of tumours and certain benign diseases. It is based on the specific photosensitizer accumulation in the tumour tissue, followed by irradiation with visible light, which induces cell death and tumour ablation. The hydrophobic photosensitizer tends to localize in the plasma and subcellular membranes, making these structures especially sensitive to the photooxidative damage (Nowis et al., 2005). Subsequently, the tissue is photo-illuminated at the wavelength that is adequate for maximal absorption of the photosensitizer (Brown et al., 2004). The tissue destruction by PDT requires molecular oxygen and involves the photochemical generation of reactive oxygen species (ROS) that can directly kill the target cells or destroy the vascular endothelial cells, leading to tumour ischaemia, depending on the distribution of the drug at the moment of irradiation (Nowis et al., 2005). Photosensitizers can be categorized by direct chemical structure and come from several broad families. The first discovered family is based on haematoporphyrin and its derivatives. After the process of purification and manipulation, haematoporphyrin derivatives (HpD) are transformed into commercial products of various names such as Photofrin® (Ph), Photosan, Photocan, etc. Fluorescence in the wavelength range 600-700 nm has so far been reported for haematoporphyrin and protoporphyrin with the quantum yields of 0.04–0.09 (Allison et al., 2004). The photosensitizers should have a high triplet quantum yield to convert triplet-oxygen \( ^3O_2 \) to singlet-oxygen \( ^1O_2 \). However, the detection of these photosensitizing agents in cells and tissues is often limited by a strong photobleaching effect at power densities above 30–50 mW/cm². Determination of the maximum drug concentration in tumour and healthy tissue allows optimizing the time interval between drug administration and tumour irradiation (Allison et al., 2004).
Intracellular accumulation of the photosensitizers is one of the most important factors to determine the efficacy of the PDT (Lam et al., 2001; Ogura et al., 2003). Depending on their physicochemical properties and their uptake mechanism, sensitizers can reach different intracellular concentrations and localize in different subcellular compartments. Additionally, the preferential localization of a sensitizer in target organelles verifies the cell death mechanism after PDT (Kumala et al., 2003; Ouédraogo and Redmond, 2003).

Photofrin® was the first photosensitizer used in PDT of cancer and has been successfully employed in the treatment of many types of tumours (Almeida et al., 2004). Currently, it is one of the most relevant sensitizers; however, its intracellular localization still remains unclear and contradictory.

In the present study we investigated the intracellular distribution of Ph in A549, MCF-7 and HUV-EC-C cultured cell lines and the localization of Ph in Me45 cells marked with CellTracker Green CMFDA specific to the cytoplasm and with MitoTracker Green FM specific to mitochondria.

Material and Methods

Cells and cell culture

The cell lines used throughout the study were: human malignant A549, MCF-7, Me45, and one normal endothelial cell line HUV-EC-C.

Human lung carcinoma cell line (A549) was initiated in 1972 by D. J. Giard et al. during the explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male (Giard et al., 1973). Human breast adenocarcinoma cell line (MCF-7) was derived from the pleural effusion taken from a 69-year-old Caucasian female with metastatic breast cancer. Human malignant melanoma (Me45) cell line (derived from a lymph node metastasis of skin melanoma in a 35-year-old male) was established in 1997 at the Radiobiology Department of the Centre of Oncology in Gliwice, Poland. The HUV-EC-C endothelial normal cells originated from human umbilical vein (ATCC).

The tumour cells were grown in culture medium MEM (Sigma-Aldrich, St. Louis, MO) with 3% glutamine, 10% foetal calf serum and antibiotics: gentamycin (100 µg/ml) and penicillin (100 U/ml). The HUV-EC-C cells were grown in complete growth medium: Ham's F12K with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and supplemented with 0.1 mg/ml heparin and 0.03–0.05 mg/ml endothelial cell growth supplement (ECGS), and 10% foetal bovine serum. The cell lines were incubated at 37°C with 5% CO₂.

Fluorescence microscopy

Microcultures, derived from the culture dishes, were conducted on 8-well glass slides. Subsequently, the cells were incubated with photosensitizer – Photofrin® (Ph) (QLT Phototherapeutics, Inc., Vancouver, Canada) at concentrations of 30 µg/ml for various times (1 h, 2 h, 3 h, 4 h). After incubation cells were fixed in 4% buffered formalin, washed in PBS and examined under a fluorescence microscope using a UV excitation filter (300–400 nm). Emission was recorded in the red region of the spectrum (400– 800 nm).

Confocal microscopy

To visualize the sensitizer accumulation within the cells 1.5×10⁴ Me45 cells were plated in 600 µl growth medium into 8-well chambered coverglass (Nunc, Rochester, NY) and incubated for 18 h. Then, the cells were treated with Ph (30 µg/ml). Intracellular localization was monitored using an LSM 510 confocal laser scanning microscope (Carl Zeiss GmbH, Jena, Germany) after 4 hours of incubation of the cells with the photosensitizer. Prior to microscopic analysis cells were washed three times in phosphate-buffered saline (PBS, pH = 7.2) and incubated with fluorescent probes to visualize the subcellular organelles targeted by the sensitizer. For cytoplasm identification, cells were incubated with 5 µM CellTracker Green CMFDA and for mitochondria with 90 nM MitoTracker Green (Molecular Probes, Eugene, OR). At the end of the double staining, the labelling solution was removed by gentle rinsing with PBS.

CellTracker Green CMFDA, MitoTracker Green were excited using argon-ion laser 488 nm line and photosensitizer by helium-neon laser 543 nm line. The fluorescence was filtered with BP505–530 nm band-pass and 560 nm long-pass emission filters. To reduce interchannel cross-talk, the multitracking technique was used. Image acquisition was performed using a 40x oil immersion objective lens, optical slice thickness: 1 µm. Images were analyzed using KS400 software (Carl Zeiss GmbH).

Results

Fluorescence microscopy

Distribution of Ph was different in malignant and normal cells and dependent on the incubation time. After 1 hour of incubation the dye was localized next to the inner side of the plasma membrane in the MCF-7 and A549 cells (Fig. 1A, 1D). In the MCF-7 cell line after 3 hours of incubation the photosensitizing drug penetrated into the cytoplasm (Fig. 1B). In A549 cells the photosensitizer diffused throughout the cytoplasm after 3 hours (Fig. 1E). The maximal concentration of Ph in both malignant cell lines was observed after 4 hours and the most intensive signal was observed around the nuclear envelope (Fig. 1C, 1F). The localization of the photosensitizer was similar in both malignant and normal cell lines. The results confirm time-dependent accumulation of fluorescent dyes.
There was no difference between various times of incubation in normal endothelial cell line HUV-EC-C. In this line the photosensitizer diffused throughout the cytoplasm and the most intensive signal was observed around the nuclear envelope after 1, 2, 3 and 4 hours after incubation (Fig. 2A, 2B, 2C, 2D).

**Confocal microscopy**

Intracellular distribution of Ph in the Me45 cell line was monitored after 4 hours of incubation with porphyrins. Cell-Tracker Green and Mito-Tracker were used as molecule markers. Ph was mainly localized in the intracellular compartments of Me45 cells (Fig. 3A and 3B). However, in several cases this photosensitizer penetrated mitochondrial membranes (Fig. 3C and 3D). The fluorescence emitted by Ph overlaid that from Mito Tracer, indicating its preferential accumulation in mitochondria.

*Fig. 1. Intracellular fluorescence of Photofrin® in MCF-7 cells: A - after 1 h of incubation with Ph; B - after 3 h of incubation with Ph; C - after 4 h of incubation with Ph; and in A549 cells: D - after 1 h cells of incubation with Ph; E - after 3 h of incubation with Ph; F - after 4 h of incubation with Ph.*
Discussion

The anti-cancer effects of PDT are thought to occur at two different levels: direct lethal effects on tumour cells leading either to apoptosis and/or necrosis; and indirect effects that cause the recruitment of inflammatory cells and the non-specific activation of the immune system. The balance between these responses is monitored by numerous parameters such as the intracellular localization of the drug, the physical and chemical properties of the photosensitizer and the cell type.

Wide varieties of photosensitizers, including Ph, have been involved in several possible mechanisms in PDT, cytotoxicity and apoptosis (Konan et al., 2002). The fluorescing properties of the photosensitizers can be used to evaluate their intracellular localization and treatment effects (Oleinick et al., 2002). Fluorescence microscopy studies of two malignant (A549, MCF-7) and one normal (HUV-EC-C) cell lines were investigated after different time of incubation with Ph. Additionally, the intracellular localization of Ph in a cultured malignant (Me45) cell line was compared with the distribution of fluorescent probes specific to the cytoplasm (CellTracker Green CMFDA) and to the mitochondria (MitoTracker Green) by confocal microscopy. However, the intracellular localization of this photosensitizer remains unclear and contradictory.

Our in vitro studies showed that there was a time-dependent increase of accumulation of the fluorescent dye in the malignant cell lines compared to the normal endothelial cells. We observed time-independent accumulation of Ph in HUV-EC-C. Initially, in this cell line the photosensitizer diffused throughout the cytoplasm. The most intensive signal was observed around the nuclear envelope. In the investigated malignant cell lines fluorescence of Ph was localized in the inner side of the plasma membrane, the cytoplasm, and around the nuclear envelope. The redistribution of this sensitizer from the parts of the plasma membrane to the nuclear envelope and adjacent intracellular sites was observed with increasing time of incubation. After the longer incubation time with Ph (4 h) the intracellular fluorescence was most pronounced in the nuclear envelope and distinct spots around. Liang at al. demonstrated a perinuclear localization of Ph, particularly in mitochondria, with little fluorescence in the peripheral cytoplasm or the nucleus in two normal and one malignant cell lines (Liang et al., 2000). Moreover, they investigated subcellular localization and phototoxicity of lutetium texaphyrin (Lu-Tex). The fluorescence pattern in
bovine pulmonary artery endothelial cells (CPAE) and kangaroo kidney cells (PTK2) following exposure to Lu Tex appeared to be lysosomal (Liang et al., 2000). This has also been reported by other groups (Kubler et al., 2001; Abels, 2004).

It has also been presented that after haematoporphyrin-mediated PDT, fluorescence and electron microscopy showed immediate structural changes in the mitochondria, with progressive swelling and destruction of these organelles (Kessel et al., 1997). Ricchelli et al. reported that Ph was localized in the mitochondria and not in the plasma membrane in vitro (Ricchelli et al., 1990). Marchetti et al. indicated that Ph is a ligand for the mitochondrial peripheral benzodiazepine receptor, which is responsible for triggering pore transition (Marchetti et al., 1996). We observed that Ph was mainly localized in intracellular compartments, but in several cases also in mitochondrial membranes. Other study showed that about 15% of haematoporphyrin derivative (HpD) was localized in the nucleus of two cell lines: human glioma (BMG-1) and squamous carcinoma (4451) cell lines. Differences in the distribution of HpD in the perinuclear region were not apparent in these cell lines. Additionally, the oral squamous carcinoma cells accumulated significantly higher amounts of HpD than glioma cells, but were less sensitive to the photodynamic treatment (Gupta et al., 2003). It was established that the anionic meso-tetra(4-sulphonatophenyl) porphyrin (TPPS₄) and the cationic meso-tetra(4-N-methylpyridyl) porphyrin (TMPyP) initially localize in the lysosomes, while irradiation induces relocalization of both photosensitizers to the cytoplasm and to the nucleus (Olivo et al., 2003). Olivo et al. showed that hypericin localizes in both mitochondria and lysosomes in bladder cell lines (RT112, SD) (Ali and Olivo, 2002). However, they found that double labelling with DAPI, a nuclear stain, did not show localization of hypericin in the nucleus (Ali and Olivo, 2002). These results suggest no possible genotoxic effects of hypericin.

Our studies showed a diffuse distribution of the photosensitizing drugs in the cells. As can be expected in

![Fig. 3. Intracellular distribution of Photofrin® in Me45 cells monitored 4 hours after incubation with the photosensitizer: A - Me45 cells with Ph; B - Me45 with Ph and cytoplasm staining; C and D – Me45 with Ph and mitochondria staining.](image)
other cell lines, this work confirms specific and time-dependent accumulation of fluorescent dyes in the human malignant cell lines in comparison to the normal endothelial cell line.

It should be stressed that localization of photosensitizers in cytoplasmic organelles during PDT plays a major role in cell damage. Mitochondria play an important role in the early events of apoptosis (Lam et al., 2001; Patito et al., 2001). Recent reports indicate that membrane photodamage can yield a variety of effects. The membrane contains diverse receptors, transport receptors, transport systems and signal transduction proteins (Kessel et al., 2000). The cellular uptake, intracellular distribution and retention of Ph in cells depend on micro-environmental factors and cell characteristics including cell volume, state of proliferation, the capacity and affinity of the intracellular target sites to bind Ph, and cell type.

References


