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Subcellular Phototoxicity of Photofrin-II and Lutetium Texaphyrin in Cells In Vitro

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Abstract. Three cell types including bovine pulmonary artery endothelium cells (CPAE), rat kangaroo kidney cells (PTK_2), and human larynx epidermoid carcinoma cells (Hep-2) were used to study subcellular localisation and phototoxicity of Photofrin-II and lutetium texaphyrin (Lu Tex). Cells were examined for fluorescence after administration of the photosensitisers. Subcellular regions were exposed with a laser microbeam system that used an argon ion laser pumped dye laser generating a 630 nm for Photofrin-II and 730 nm for Lu Tex. Fluorescence detection suggests that the Photofrin-II is bound primarily to the mitochondria with some diffuse fluorescence in the rest of the cytoplasm. The fluorescence in Lu Tex treated cells appears to be localised to the lysosomes. The percentage of damaged cells following light exposure to the different subcellular regions after Photofrin-II or Lu Tex treatment demonstrates that the nuclear region was the most sensitive target followed by the perinuclear region and peripheral cytoplasm region.

Keywords: Endothelial cell; Fluorescence detection; Laser microirradiation; Lutetium texaphryin; Photodynamic therapy; Photofrin II; Photosensitiser; Subcellular phototoxicity

INTRODUCTION

Since the initial studies on photodynamic therapy (PDT) in 1972 [1,2], an enormous amount of basic and clinical research has suggested that PDT has the potential to be a significant cancer treatment modality [3,4]. Efficacy of PDT is related to the selective accumulation of the photosensitiser within the tumours and the subsequent induction of cytotoxic singlet oxygen following exposure to light of the appropriate wavelength. To date, most clinical PDT has used 630 nm light with the first generation sensitiser, Photofrin-II.

One aspect of the tumouricidal effectiveness of PDT is related to the depth of light penetration within the tumour mass. The effective penetration at 630 nm is 1–3 mm, whereas at 700–850 nm at least 6 mm penetration of light is observed [5]. A growing number of second-generation photosensitisers with longer wavelength absorption peaks are being synthesised. The texaphyrins are tripyrrolic pentaaza expanded porphyrins, which exhibit strong, low energy optical absorption in the 730–770 nm range [6]. One of these compounds, lutetium texaphyrin (Lu Tex) is a pure, water-soluble photosensitiser with a broad absorption band centred at 732 nm [7].

One of the suggested mechanisms of PDT destruction of tumours is that the vascular endothelium is damaged initially and tumour cells are destroyed secondarily as a result of structural damage to capillaries and functional disturbance in the microcirculation [8–10].

Within the individual cell, the subcellular sites of photodynamic damage may include the plasma membrane, lysosomes, and the mitochondria [11,12]. Biochemical analysis indicates that membrane bound mitochondrial enzymes such as cytochrome *c*, oxidase and succinate dehydrogenase are inactivated by PDT [13–15]. Damage to membranes of the endoplasmic reticulum also has been observed ultrastructurally [16]. In all of these studies, the cell damage was assayed by either microscopy or biochemical analysis after light exposure to the entire cell or cell population. Consequently, it was difficult to determine if the observed subcellular damage was due to a primary light plus drug effect in the damaged

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organelle or cell region, or due to secondary effects subsequent to absorption of light at a primary site.

In a recent study using a laser microbeam to expose subcellular sites, it was possible to demonstrate that the perinuclear cytoplasm was highly photosensitised. This observation correlated with photosensitiser fluorescence in that region. Surprisingly, it was also determined that the nucleus was a very sensitive target following laser microbeam irradiation [17]. In the present study we have expanded these early studies to examine the subcellular phototoxicity of Photofrin-II, the most frequently used photosensitiser, and Lu Tex, a second generation photosensitiser.

In the present study, we exposed specific subcellular regions of photosensitised cells to light using a laser microbeam microscope system [17]. By correlating subcellular photosensitiser fluorescence, the region of light exposure, and subsequent cell response, we are able to achieve a more complete understanding of PDT-induced cytotoxicity.

MATERIALS AND METHODS

Photosensitisers

A stock solution of Photofrin-II (Quadra Logic Technologies, Inc., Vancouver, British Columbia, Canada) was prepared according to the manufacturer's directions using sterilised 5% dextrose to give a final concentration of a 2.5 mg/ml. Lu Tex (Pharmacyclic Corp., Sunnyvale, CA, USA) was prepared in 5% mannitol and filter sterilised.

Just prior to cell exposure to the drug, culture media containing 0.15 μg/ml of Photofrin-II or 1 μg/ml of Lu Tex were prepared in the dark. The experimental cells were treated with either Photofrin-II or Lu Tex in the dark. After 4 h the medium containing photosensitiser was replaced by fresh medium free of photosensitiser.

Cells and Cell Culture

Three cell types were used in these studies.

Bovine pulmonary artery endothelium cells (CPAE) were obtained from the American Type Culture Collection (ATCC). This is an endothelial cell line derived from the main stem pulmonary artery of a young, female cow (*Box taurus*). The cells were grown in Eagle's minimal essential medium with 20% fetal bovine serum at 37°C and 5–7% CO_2 atmosphere. The cells were maintained and subcultured for 2–3 weeks before being discarded and replaced with fresh cells from the same passage.

Rat kangaroo (*Potorous tridactylis*) kidney cells (PTK_2) were originally obtained from the ATCC and grown as a monolayer in modified Eagle's minimal essential medium with 10% fetal bovine serum. The cells were subcultured once a week into T-25 culture flask and maintained in an incubator at 37°C with 5–7% CO_2 [18]. These cells have been growing in our lab for over 25 years and their flat nature facilitates selective microbeam exposure to subcellular targets.

Hep-2 cells from human larynx epidermoid carcinoma were originally obtained from the ATCC and grown as a monolayer in Eagle's minimum essential medium with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. The medium contained 10% fetal bovine serum. As with the CPAE and PTK_2 cells, these cells are relatively flat facilitating laser microbeam irradiation of selective subcellular regions.

Twenty-four hours prior to the experiments, cells were injected into standard Rose culture chambers at a density of $\sim 1 \times 10^3$ cells/ml. Prior to laser microirradiation, the cells were exposed to fresh culture medium containing 0.15 μg/ml Photofrin-II or 1 μg/ml Lu Tex for 4 h, and then exposed to fresh medium free of sensitiser. Fresh photosensitiser-free medium was applied to the cells approximately ten minutes before microirradiation. Cells exposed to the laser but not the sensitiser served as controls. In addition, cells treated with the photosensitiser and exposed to microscope illumination, but not exposed to laser irradiation, served as controls.

The chambers were covered with aluminium foil immediately after injecting the photosensitiser in order to protect the cells from ambient light. Single cells were selected for laser microirradiation that were normal in size, well attached to cover glass, and exhibiting healthy morphology (minimal number of cytoplasmic vacuoles). A Zeiss diamond marking objective was used to circle each preselected cell by etching a small circle (at 1 mm diameter) around the desired cell. Cells that did not have any neighbouring cells within the marked

circle were chosen for irradiation. The same criteria for cell selection was applied to experimental and control cells.

Fluorescence Microscopy

A Zeiss Axiovert inverted fluorescence microscope was used to visualise subcellular fluorescence of the two sensitisers. Cells were examined for fluorescence at 1 h intervals for 4 h after the initial administration of the photosensitiser. For Photofrin-II, each cell was excited at 365 nm. For Lu Tex, a wavelength of 470 nm was used. In both cases excitation filters were employed with a 75 W arc lamp as light source. An image was recorded of the fluorescence within the cell using a cooled charge-couple device (CCD) (Princeton Instruments TE/CCD-576E/UV) and stored in IP Lab format in a Macintosh IIfx computer. A phase-contrast picture of each cell was also taken for reference. Images were digitally processed improving contrast and reducing background light.

Microirradiation of Subcellular Components

A laser microbeam system was used that employed an argon ion laser (Coherent Innova 90) pumped dye laser (Coherent 599 dye) using {2-{2-{4-(dimethylamino)phenyl}ethanol}-6-methyl-4H-pyran-4-ylidene}-propanedinitrile (DCM) to generate a 630 nm beam for Photofrin-II and LDS 722 (pyridine 2) to generate a 730 nm beam for Lu Tex. Each of these wavelengths matched the absorption peak of the respective sensitisers. The beam was directed through a Zeiss Axiovert inverted fluorescence microscope and focused to a near diffraction limited spot using a Zeiss Neofluar 100 × phase-contrast objective with a numerical aperture of 1.3. For each cell, the laser beam was focused on the centre of the nucleus, the perinuclear cytoplasm or the peripheral cytoplasm. The laser spot was approximately 0.5 μm in diameter for the 630 nm microbeam irradiation and 0.6 μm for the 730 nm microbeam. A Newport Corporation model 1815 optical power meter and power detector model 818-UV (Irvine, CA) were used to measure laser power at the entrance to the objective. To determine the actual power reaching the irradiated sample, the dual objective transmittance measuring technique of Misawa et al. [19] was used. This method eliminates internal reflection errors that are encountered in a direct objective-to-power measurement in air. Using this method it was determined that each cell was exposed to 9 mW of power at a power density of 4.6×10^6 W/cm^2 for Photofrin-II and 3.2×10^6 W/cm^2 for Lu Tex. The total irradiance (energy densities) per experiment were obtained by varying exposure time. The parameters used are listed in Table 1 and Table 2.

Table 1. Power parameters used in laser microirradiation (Photofrin-II experiments)[a]

Time exposure (s)	Energy density ($\times 10^7$ J/cm^2)
300	138.0
180	82.8
60	27.6
50	23.0
40	18.4
30	13.8
20	9.2
10	4.6
5	2.3
3	1.4
1	0.5

[a]Power = 9 mW; power density = 4.6×10^6 W/cm^2.

Table 2. Power parameters used in laser microirradiation (Lu Tex experiments)[a]

Time exposure (s)	Energy density ($\times 10^7$ J/cm^2)
15	4.8
13	4.2
10	3.2
8	2.6
7	2.2
5	1.6
3	1.0

[a]Power = 9 mW; power density = 3.2×10^6 W/cm^2.

Immediately before laser irradiation, a phase-contrast image of the cell was made using the CCD camera. Each chamber was labelled and placed in the CO_2 incubator after the irradiation. Each cell was followed and recorded at 24 h and 48 h after irradiation.

RESULTS

Fluorescence Detection

The CCD camera system provided images of subcellular fluorescence in photosensitiser-

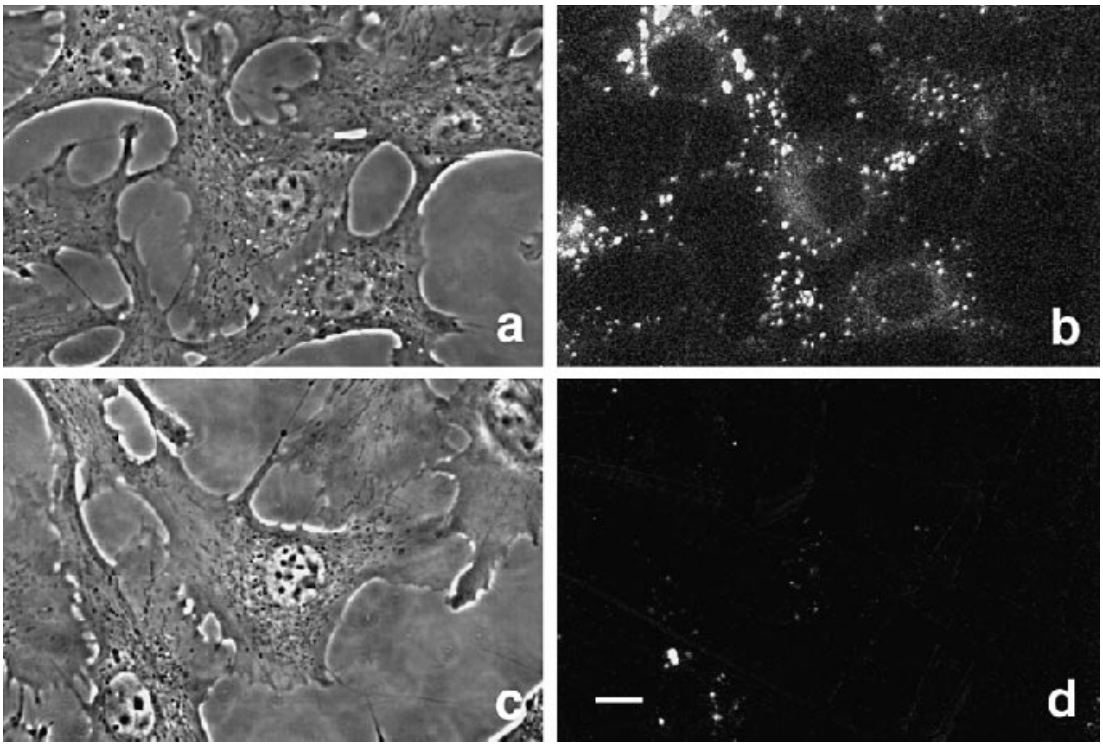

Fig. 1. Fluorescence image of CPAE cells treated by Photofrin-II. **a** Phase contrast image of cells 4 h after treatment with Photofrin-II; **b** fluorescence image of the same treated cells; **c** phase-contrast image of control cells without Photofrin-II treatment; **d** fluorescence image of the same control cells. Bar=10 μm.

treated and untreated control cells. Figure 1(a) is a phase-contrast image of CPAE cells 4 h after exposure to medium containing 0.15 μg/ml of Photofrin-II. Figure 1(b) is an image of the same cell demonstrating a large amount of fluorescence in the perinuclear area that extents towards the periphery of the cell. Figure 1(c) is the phase-contrast image of control non-Photofrin-treated CPAE cells and Fig. 1(d) is the corresponding image demonstrating a lack of fluorescence. Figure 2(a) and (b) are paired phase contrast and fluorescence images of PTK_2 cells 4 h after treatment in medium containing Photofrin-II. Figure 3(a) and (b) are paired images of Hep-2 cells. In these two cell types, fluorescence distribution is similar to the aforementioned CPAE cells, i.e. a large amount of fluorescence in the perinuclear region. Control PTK_2 cells (Fig. 2c and d) and Hep-2 cells (Fig. 3c and d) demonstrate either no detectable or weak autofluorescence.

Fluorescence detection of Lu Tex-treated CPAE and PTK_2 cells have also been studied. Figure 4(a) is a phase-contrast image of CPAE cells 4 h after exposure to medium containing 1 μg/ml Lu Tex. Figure 4(b) is the corresponding fluorescence image of these same cells. Figure 4(c) and (d) are paired images of control CPAE cells without treatment of Lu Tex. Figure 5(a) and (b) are paired Lu Tex treated PTK_2 cells. The paired control PTK_2 cells are shown in Fig. 5(c) and (d). It is evident that in both Lu Tex-treated CPAE and PTK_2 cells, fluorescence was dispersed throughout the cytoplasm and localised primarily in the lysosomes. Control CPAE and PTK_2 cells show either no detectable or weak autofluorescence.

In summary, the pattern of fluorescence of Photofrin-II is different from that of Lu Tex. The fluorescence detected in Photofrin-II treated cells suggests that the sensitiser is bound primarily to the mitochondria with some diffuse fluorescence in the rest of the cytoplasm. However, the fluorescence in Lu Tex treated cells appears to be localised to the lysosomes. In all three cell types treated with either Photofrin-II or Lu Tex, no fluorescence was detected in the nucleus.

Subcellular Phototoxicity

A laser power of 9 mW (2.3×10^6 W/cm^2) at the microscope focal point has been used in the subcellular microirradiation experiments. The total energy densities (ED) were varied by changing the duration of laser exposure (Tables 1 and 2). In the Photofrin-II experiments, a total of 242 CPAE cells were

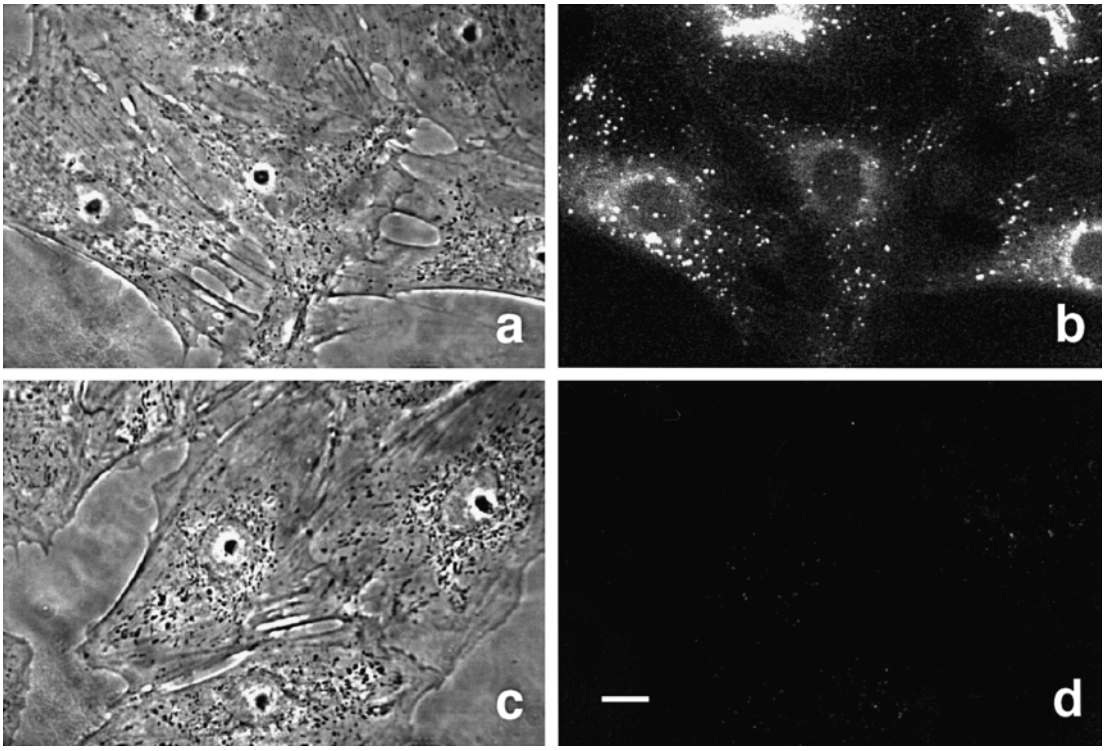

Fig. 2. Fluorescence image of PTK_2 cells treated by Photofrin-II. **a** Phase-contrast image of cells 4 h after treatment with Photofrin-II; **b** fluorescence image of the same treated cells; **c** phase-contrast image of control cells without Photofrin-II treatment; **d** fluorescence image of the control same cells. Bar=10 μm.

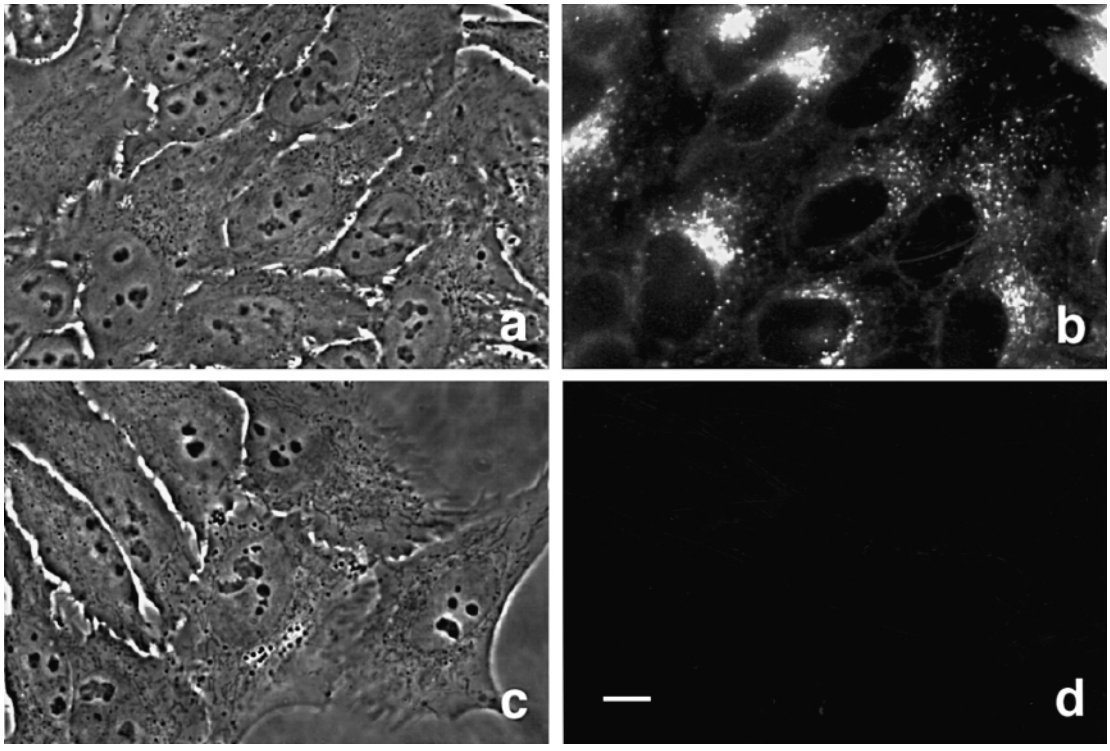

Fig. 3. Fluorescence image of Hep-2 cells treated by Photofrin-II. **a** Phase-contrast image of cells 4 h after treatment with Photofrin-II; **b** fluorescence image of the same treated cells; **c** phase-contrast image of control cells without Photofrin-II treatment; **d** fluorescence image of the same control cells. Bar=10 μm.

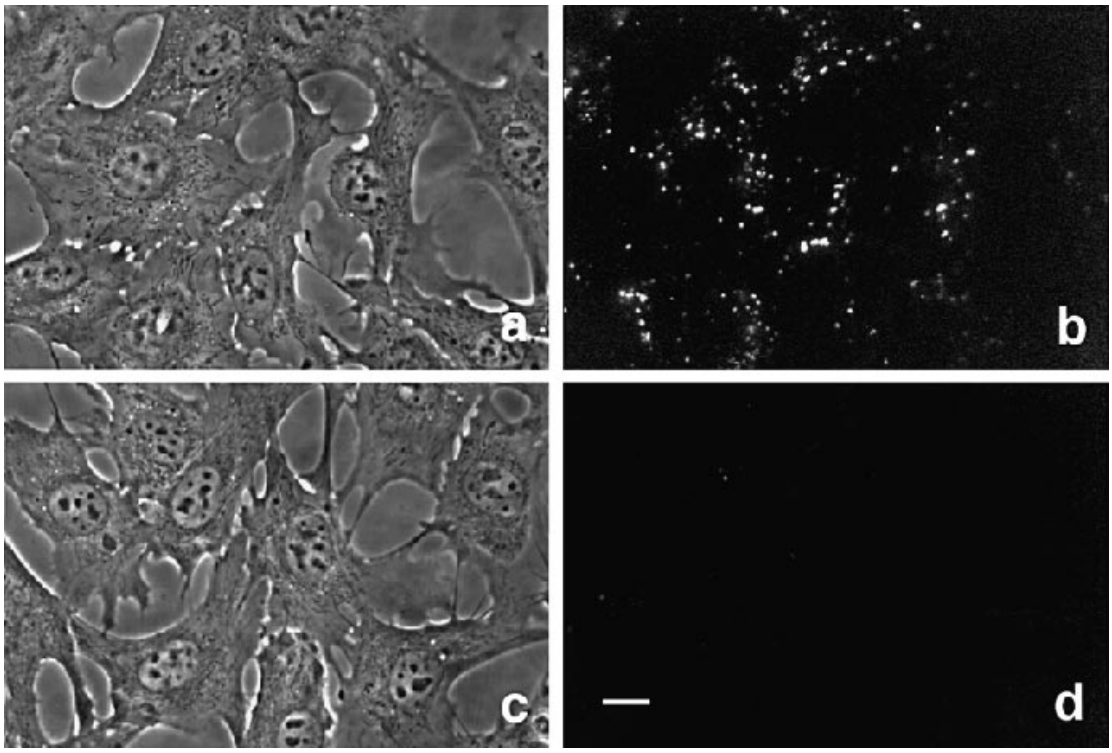

Fig. 4. Fluorescence image of CPAE cells treated by Lu Tex. **a** Phase-contrast image of cells 4 h after treatment with Lu Tex; **b** fluorescence image of the same treated cells; **c** phase-contrast image of control cells without Lu Tex treatment; **d** fluorescence image of the same control cells. Bar=10 μm.

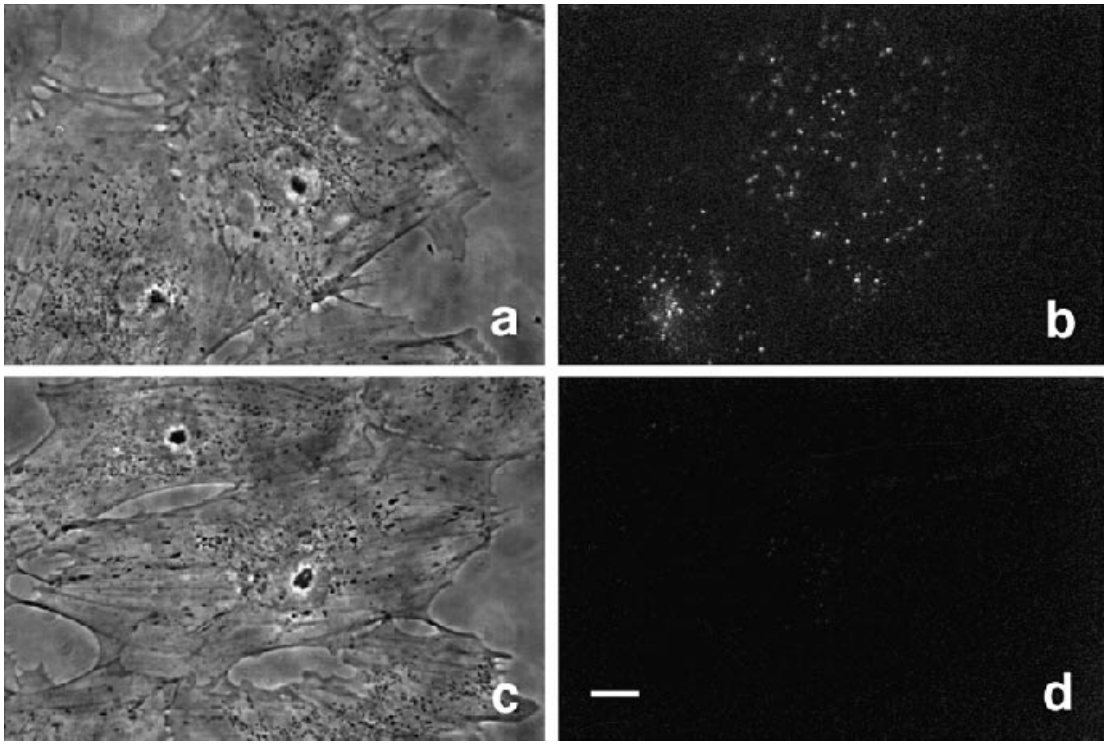

Fig. 5. Fluorescence image of PTK_2 cells treated by Lu Tex. **a** Phase-contrast image of PTK_2 cells 4 h after treatment with Lu Tex; **b** fluorescence image of the same treated cells; **c** phase-contrast image of control cells without Lu Tex treatment; **d** fluorescence image of the same control cells. Bar=10 μm.

Table 3. Cell status morphologically in CPAE after treatment of Photofrin-II plus laser microirradiation

Target of irradiation	Pretreatment of Photofrin-II	Time of exposure (s)	Total no. of cells attempted	Cell status after 24 h (healthy cells[a])		Cell status after 48 h (healthy cells[a])	
				No.	%	No.	%
Nucleus	No	300	12	12	100	12	100
	Yes	180	10	0	0	0	0
	Yes	60	10	1	10	1	10
	Yes	30	10	2	20	2	20
	Yes	20	11	7	64	7	64
	Yes	10	10	9	90	9	90
	Yes	5	10	10	100	10	100
	Yes	5	10	10	100	10	100
Perinuclear cytoplasm	No	300	13	13	100	13	100
	No	180	13	13	100	13	100
	Yes	300	10	0	0	0	0
	Yes	180	10	2	20	2	20
	Yes	60	10	5	50	3	30
	Yes	30	13	8	62	8	62
	Yes	20	20	18	90	17	85
	Yes	10	10	10	100	10	100
Peripheral cytoplasm	No	300	10	10	100	10	100
	Yes	300	10	0	0	0	0
	Yes	180	20	5	25	3	15
	Yes	60	10	8	80	8	80
	Yes	30	10	10	100	10	100
	Yes	5	10	10	100	10	100

[a]Healthy cell: cells underwent at least one additional mitosis and cells with no division but morphologically normal, alive and healthy.

irradiated in the following subcellular regions: nucleus, $n=73$; perinuclear cytoplasm, $n=99$; peripheral cytoplasm, $n=70$. For PTK_2 cells, a total of 258 cells were irradiated: nucleus, $n=84$; perinuclear cytoplasm, $n=124$; peripheral cytoplasm, $n=50$. For Hep-2 cells, a total of 262 cells were irradiated: nucleus, $n=109$; perinuclear cytoplasm, $n=113$; peripheral cytoplasm, $n=40$.

In the Lu Tex experiments, a total of 211 CPAE cells were irradiated: nucleus, $n=55$; perinuclear cytoplasm, $n=69$; peripheral cytoplasm, $n=87$. A total of 229 PTK_2 cells were irradiated: nucleus, $n=62$; perinuclear cytoplasm, $n=95$; peripheral cytoplasm, $n=72$. The representative example of the locations of the laser microspot in each cell region, and the cell status 24 h after light exposure are presented at Figs 6–10.

The raw data of cell status at 24 and 48 h after microirradiation for Photofrin-II are shown in Tables 3–5 and in Tables 6 and 7 for Lu Tex.

Individual irradiated cells at 24 and 48 h were scored with respect to: (a) cytological evidence of damage such as nuclear pycnosis, cytoplasmic vacuoles and blebbing of the cell membrane; (b) cell death, i.e. severe vacuolisation and disintegration of cytosol; (c) survival without subsequent cell division; and (d) survival with the ability to undergo a subsequent cell division. Cell morphology 24 h after laser microbeam irradiation are shown in Figs 6–8 for Photofrin-II and Figs 9–10 for Lu Tex.

Figure 11 is a graph of the combined data depicting the percentage of damaged CPAE cells after light exposure to the different subcellular regions after Photofrin-II treatment. All the cells in the three different subcellular irradiation groups were killed by the 300 s exposure, i.e. $ED=138\times10^7$ J/cm^2. At lower light doses the region that demonstrated the greatest sensitivity was the nucleus, followed by the perinuclear cytoplasm, and peripheral cytoplasm.

Figure 12 summarises the results for PTK_2 cells after Photofrin-II treatment. It was interesting that there was no significant difference between irradiation of the nuclear region and the perinuclear cytoplasm region though

Table 4. Cell status morphologically in PTK_2 after treatment of Photofrin-II plus laser microirradiation

Target of irradiation	Pretreatment of Photofrin-II	Time of exposure (s)	Total no. of cells attempted	Cell status after 24 h (healthy cells[a])		Cell status after 48 h (healthy cells[a])	
				No.	%	No.	%
Nucleus	No	60	9	9	100	9	100
	Yes	60	15	1	6	0	0
	Yes	50	10	0	0	0	0
	Yes	40	10	0	0	0	0
	Yes	30	8	0	0	0	0
	Yes	20	10	3	30	3	30
	Yes	10	12	8	67	8	67
	Yes	5	10	10	100	10	100
Perinuclear cytoplasm	No	300	10	10	100	10	100
	Yes	60	15	0	0	0	0
	Yes	30	16	3	19	2	13
	Yes	20	19	10	53	7	37
	Yes	10	22	17	77	14	64
	Yes	5	24	19	79	15	63
	Yes	3	8	7	89	6	75
	Yes	1	10	10	100	10	100
Peripheral cytoplasm	No	300	10	10	100	10	100
	Yes	300	10	0	0	0	0
	Yes	180	10	7	70	7	70
	Yes	60	10	10	100	9	90
	Yes	30	10	10	100	10	100

[a]Healthy cell: cells underwent at least one additional mitosis and cells with no division but morphologically normal, alive and healthy.

both of these regions were more sensitive than the peripheral cytoplasm. Figure 13 presents the results of Hep-2 cells. The higher sensitivity of the nucleus over the perinuclear cytoplasm was evident with an exposure of 20–30 s, i.e. ED = 9.2×10^7–13.8×10^7 J/cm^2. No differences were observed at the higher doses (up to 60 s exposure) or lower doses (down to 5–10 s exposure).

The results with Lu Tex are presented in Figs 14 and 15 for CPAE and PTK_2 cells, respectively. The results indicate that in both cell types, the nuclear region was the most sensitive target followed by the perinuclear region and the peripheral cytoplasm region.

No adverse effects to cell survival were observed in CPAE, Hep-2 and PTK_2 control cells treated by microscope illumination alone. Also, no light-alone control exposures in these three cell types resulted in cell damage even with the maximum total energy doses (Tables 3–7). In addition, it was found that the Lu Tex-treated CPAE and PTK_2 cells were more sensitive to light exposure than Photofrin-II. For example, the ED for nucleus irradiation needed to cause 100% CPAE and PTK_2 cell damage using Lu Tex were 1.6×10^7 J/cm^2 (5 s exposure) and 3.2×10^7 J/cm^2 (10 s exposure), respectively. Whereas with Photofrin-II, 82.8×10^7 J/cm^2 (180 s) and 13.8×10^7 J/cm^2 (30 s) were required to induce cell damage.

DISCUSSION

Early cell studies using haematoporphyrin derivative (HPD) demonstrated a perinuclear pattern of fluorescence due primarily to mitochondria, with little or no fluorescence in the peripheral cytoplasm or the nucleus [11]. In a more recent study on the subcellular phototoxicity of 5-aminolaevulinic acid (ALA) [17], it was found that ALA induced endogenous protoporphyrin IX production in CPAE, PTK_2 and primary culture neonatal rat myocardial cells as exhibited by fluorescence primarily confined to the mitochondria-rich perinuclear cytoplasm. A study by Uberriegler et al. [20] indicated that ALA treatment led to a bright fluorescing perinuclear region in W138 cells. The present fluorescence study with Photofrin-II together with these earlier studies confirm that this photosensitiser results in a perinuclear pattern of fluorescence. It has been reported that after

Table 5. Cell status morphologically in Hep-2 after treatment of Photofrin-II plus laser microirradiation

Target of irradiation	Pretreatment of Photofrin-II	Time of exposure (s)	Total no. of cells attempted	Cell status after 24 h (healthy cells[a])		Cell status after 48 h (healthy cells[a])	
				No.	%	No.	%
Nucleus	No	180	15	15	100	15	100
	Yes	60	10	0	0	0	0
	Yes	30	10	0	0	0	0
	Yes	20	18	6	33	5	28
	Yes	10	20	6	55	6	55
	Yes	5	20	8	75	8	75
	Yes	3	16	13	81	13	81
Perinuclear cytoplasm	No	180	10	10	100	10	100
	Yes	60	14	0	0	0	0
	Yes	30	15	5	33	4	27
	Yes	10	25	15	60	14	56
	Yes	5	35	28	80	28	80
	Yes	3	14	14	100	14	100
Peripheral cytoplasm	No	180	10	10	100	10	100
	Yes	60	10	8	80	8	80
	Yes	50	10	9	90	9	90
	Yes	30	10	10	100	10	100

[a]Healthy cell: cells underwent at least one additional mitosis and cells with no division but morphologically normal, alive and healthy.

Table 6. Cell status morphologically in CPAE after treatment of Lu Tex plus laser microirradiation

Target of irradiation	Pretreatment of Lu Tex	Time of exposure (s)	Total no. of cells attempted	Cell status after 24 h (healthy cells[a])		Cell status after 48 h (healthy cells[a])	
				No.	%	No.	%
Nucleus	No	15	10	10	100	10	100
	Yes	5	9	0	0	0	0
	Yes	4	21	10	48	8	38
	Yes	3	16	16	100	16	100
Perinuclear cytoplasm	No	15	10	10	100	10	100
	Yes	15	10	0	0	0	0
	Yes	10	12	1	8	1	8
	Yes	8	18	9	50	9	50
	Yes	5	11	10	91	9	82
	Yes	3	8	8	100	8	100
Peripheral cytoplasm	No	15	10	10	100	10	100
	Yes	15	13	0	0	0	0
	Yes	10	36	15	42	13	36
	Yes	8	18	16	89	16	89
	Yes	5	10	10	100	10	100

[a]Healthy cell: cells underwent at least one additional mitosis and cells with no division but morphologically normal, alive and healthy.

haematoporphyrin-mediated PDT, fluorescence and electron microscopy showed immediate structural changes in mitochondria, with progressive swelling and destruction of these organelles [13,14].

The fluorescence pattern in CPAE and PTK_2 cells following exposure to Lu Tex appears to be lysosomal. Other sensitisers such as chlorin-6, benzoporphyrin, and phthalocyanine have been reported to cause damage to

Table 7. Cell status morphologically in PTK_2 after treatment of Lu Tex plus laser microirradiation

Target of irradiation	Pretreatment of Lu Tex	Time of exposure (s)	Total no. of cells attempted	Cell status after 24 h (healthy cells[a])		Cell status after 48 h (healthy cells[a])	
				No.	%	No.	%
Nucleus	No	15	10	10	100	10	100
	Yes	10	10	0	0	0	0
	Yes	7	10	2	20	2	20
	Yes	5	10	4	40	4	40
	Yes	3	22	22	100	20	91
Perinuclear cytoplasm	No	15	10	10	100	10	100
	Yes	15	10	0	0	0	0
	Yes	10	10	0	0	0	0
	Yes	8	10	4	40	4	40
	Yes	5	23	17	74	14	61
	Yes	3	32	29	91	28	88
Peripheral cytoplasm	No	15	10	10	100	10	100
	Yes	13	10	0	0	0	0
	Yes	10	10	0	0	0	0
	Yes	8	20	12	60	12	60
	Yes	7	11	8	73	8	73
	Yes	5	11	11	100	11	100

[a]Healthy cell: cells underwent at least one additional mitosis and cells with no division but morphologically normal, alive and healthy.

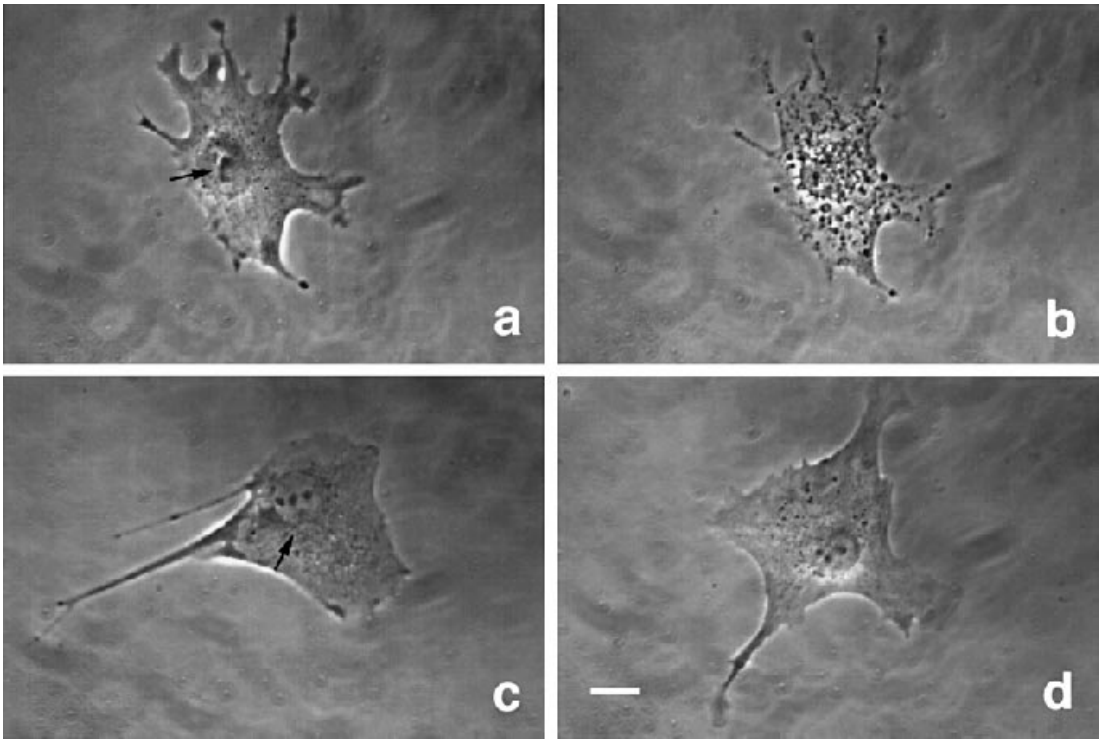

Fig. 6. Cellular damage in single CPAE cells pretreated by Photofrin-II after laser microirradiation. **a** A single cell with Photofrin-II treatment just before laser microirradiation (*arrow* denotes the nuclear region ready to trigger); **b** the same cell 24 h after 60 s laser microirradiation. **c** A single cell with Photofrin-II treatment just before laser microirradiation (*arrow* denotes the perinuclear region ready to trigger); **d** the same cell 24 h after 20 s laser microirradiation. Bar=10 μm.

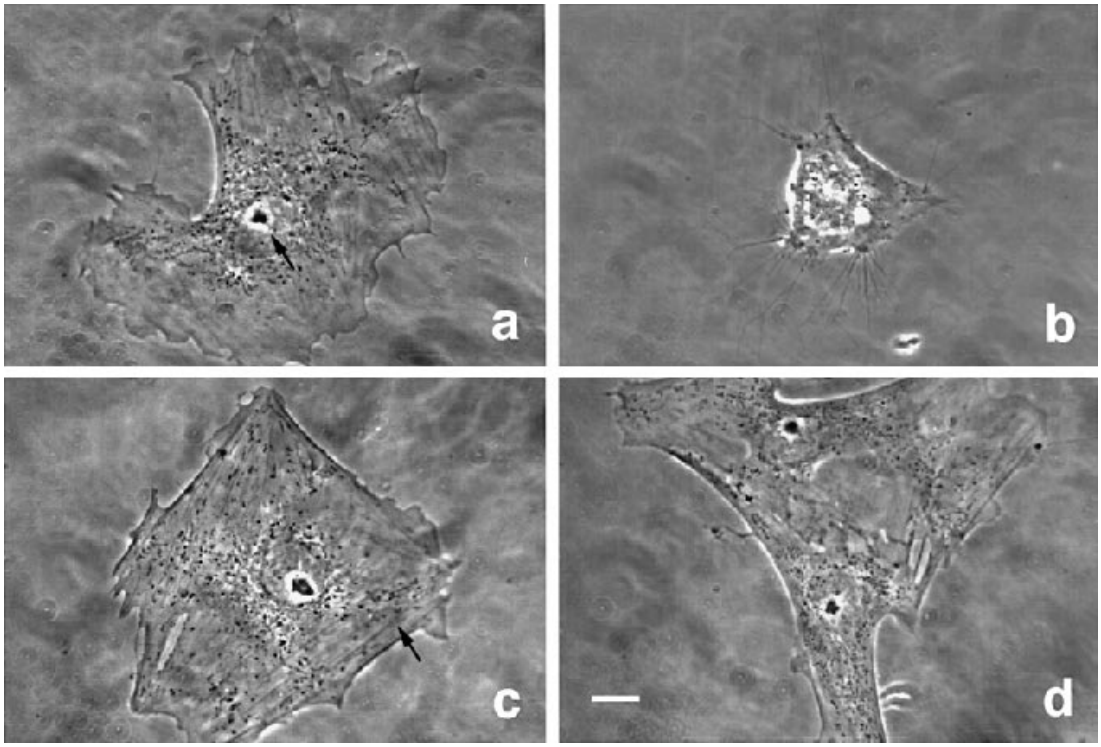

Fig. 7. Cellular damage in single PTK_2 cells pretreated by Photofrin-II after laser microirradiation. **a** A single cell with Photofrin-II treatment just before laser microirradiation (*arrow* denotes the nuclear region ready to trigger); **b** the same cell 24 h after 10 s laser microirradiation. **c** A single cell with Photofrin-II treatment just before laser microirradiation (*arrow* denotes the peripheral cytoplasm region ready to trigger); **d** the same cell divided to two cells 24 h after laser microirradiation. Bar=10 μm.

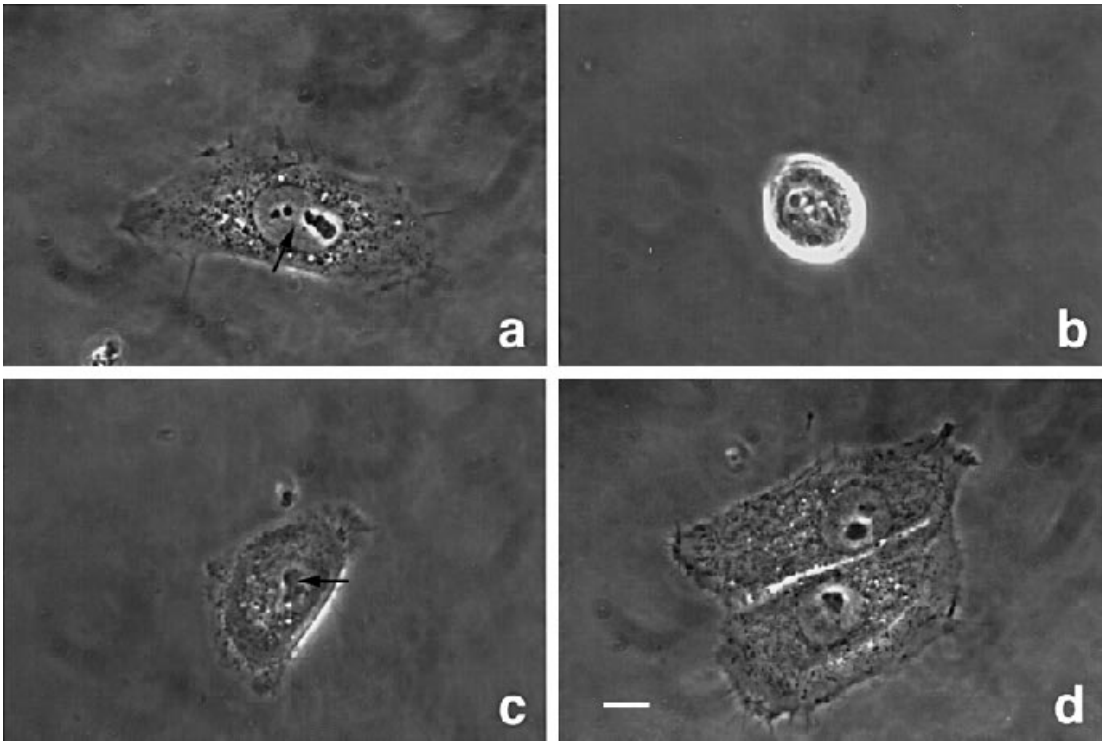

Fig. 8. Cellular damage in single Hep-2 cells pretreated by Photofrin-II after laser microirradiation. **a** A single cell with Photofrin-II treatment just before laser microirradiation (*arrow* denotes the nuclear region ready to trigger); **b** the same cell 24 h after 30 s of laser microirradiation. **c** A single cell with Photofrin-II treatment just before laser microirradiation (*arrow* denotes the nuclear region ready to trigger); **d** the same cell divided to two cells 24 h after 10 s of laser microirradiation. Bar=10 μm.

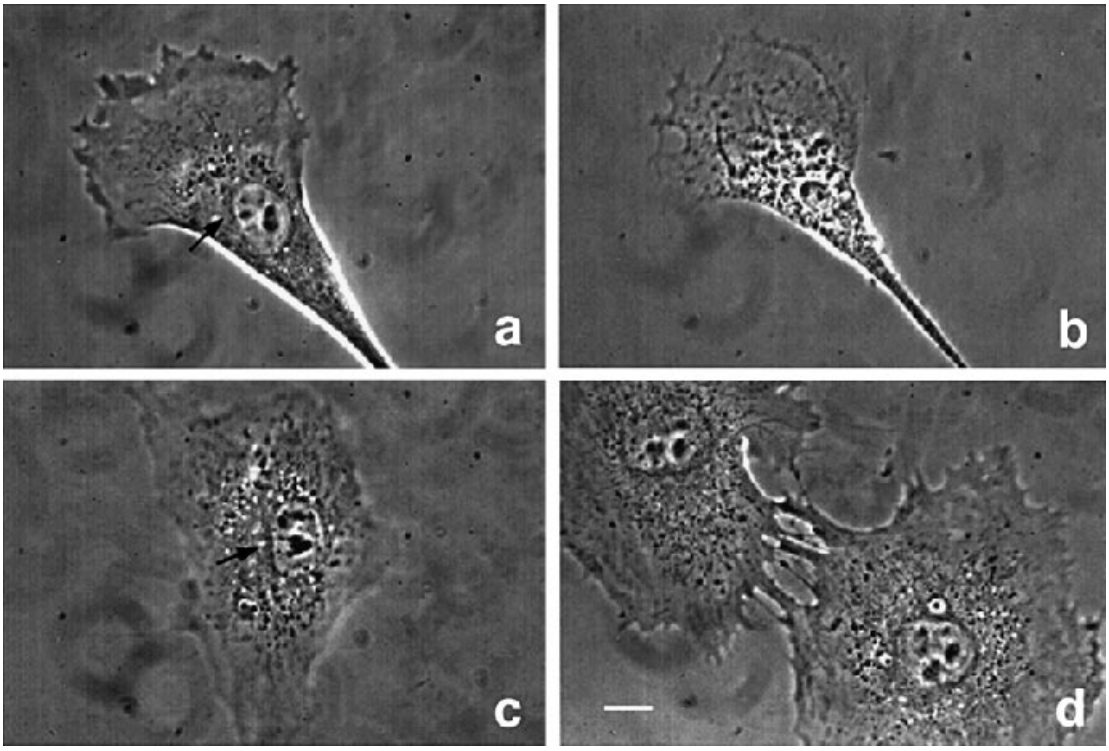

Fig. 9. Cellular damage in single CPAE cells pretreated by Lu Tex after laser microirradiation. **a** A single cell with Lu Tex treatment just before laser microirradiation (*arrow* denotes the perinuclear region ready to trigger); **b** the same cell 24 h after 10 s of laser microirradiation. **c** A single cell with Lu Tex treatment just before laser microirradiation (*arrow* denotes the perinuclear region ready to trigger); **d** the same cell divided to two cells 24 h after 5 s of laser microirradiation. Bar=10 μm.

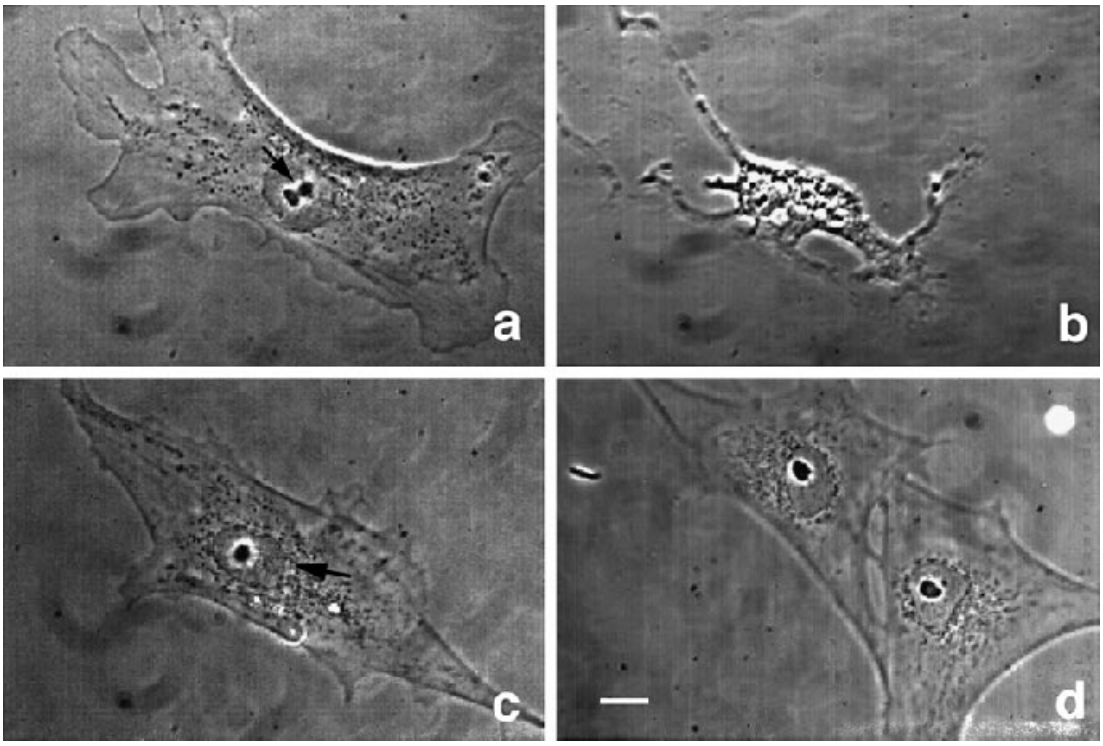

Fig. 10. Cellular damage in single PTK_2 cells pretreated by Lu Tex after laser microirradiation. **a** A single cell with Lu Tex treatment just before laser microirradiation (*arrow* denotes the nuclear region ready to trigger); **b** the same cell 24 h after 5 s of laser microirradiation. **c** A single cell with Lu Tex treatment just before laser microirradiation (*arrow* denotes the perinuclear region ready to trigger); **d** the same cell 24 h after 5 s of laser microirradiation. Bar=10 μm.

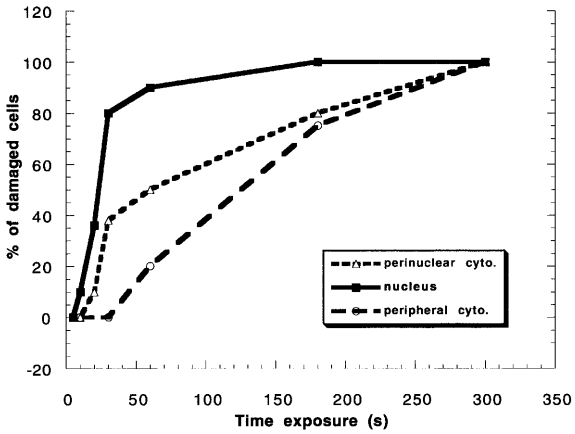

Fig. 11. Cellular toxicity at 24 h after subcellular laser microirradiation in CPAE cells treated with Photofrin-II.

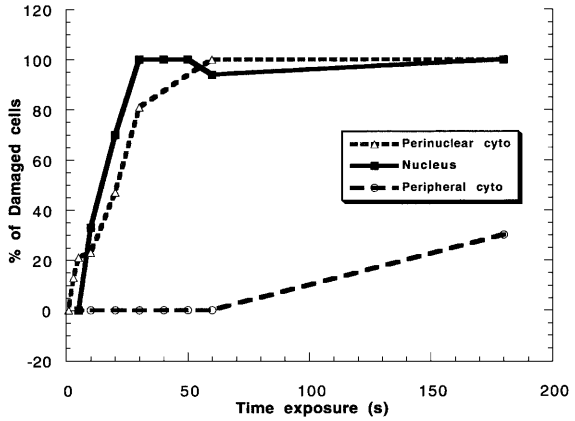

Fig. 12. Cellular toxicity at 24 h after subcellular laser microirradiation in PTK_2 cells treated with Photofrin-II.

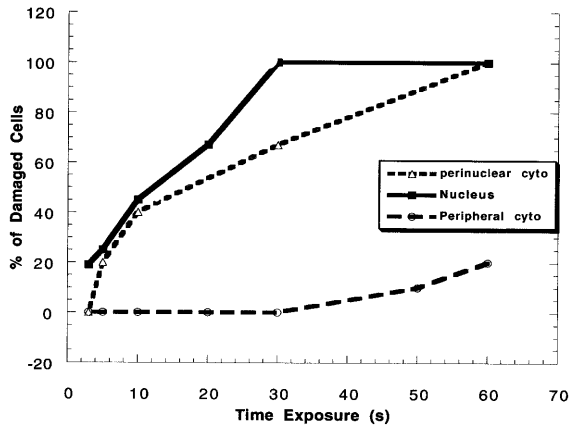

Fig. 13. Cellular toxicity at 24 h after subcellular laser microirradiation in Hep-2 cells treated with Photofrin-II.

lysosomes which resulted in hydrolytic enzyme leakage leading to cell death [21].

Our experimental results indicate that the light dose needed to kill the cells is less for Lu Tex than for Photofrin-II. Cells treated with

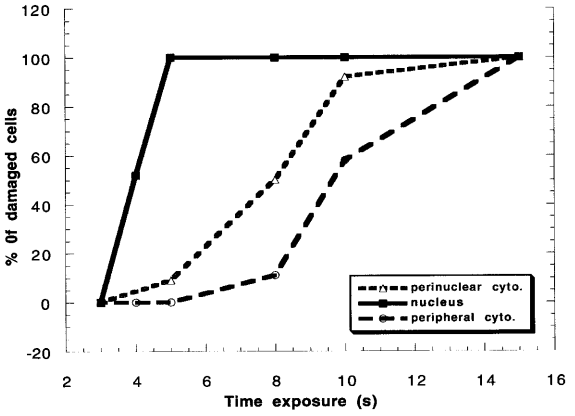

Fig. 14. Cellular toxicity at 24 h after subcellular laser microirradiation in CPAE cells treated with Lu Tex.

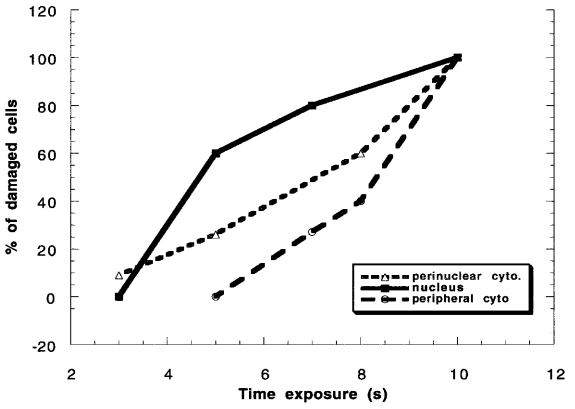

Fig. 15. Cellular toxicity at 24 h after subcellular laser microirradiation in PTK_2 cells treated with Lu Tex.

either of these compounds are far more sensitive to light than cells exposed to ALA. Photofrin-II and Lu Tex are incorporated directly into cells and rapidly bind to mitochondria, lysosomes and other cytoplasmic components. ALA is converted intracellularly into monomeric protoporphyrin IX which is the photoactive species. Differences in cell phototoxicity between the three compounds may also be due to differences in cellular concentration of the compounds as well as the quantum efficiency of singlet oxygen generation.

The present study confirms previous work on the subcellular phototoxicity of ALA [17], in which it was possible to demonstrate a correlation between perinuclear sensitiser fluorescence and subcellular site of phototoxicity. However, of greater interest is the finding that the nucleus is a very sensitive site despite the fact that most of the literature and the fluorescence localisation data suggest that the nucleus is not a primary target site of PDT.

This study validates the hypothesis that subcellular fluorescence can be correlated with cellular target site, i.e. perinuclear cytoplasm versus peripheral cytoplasm. However, the results demonstrating selective nuclear sensitivity raises basic questions with respect to localisation of photosensitiser in the nucleus and the role of the nucleus as a primary subcellular target. It is possible that the photosensitiser may be present at a concentration below the detection of the CCD camera system. Under this condition, it would be possible that the genetic material in the nucleus may be sensitive to even small amounts of generated singlet oxygen.

CONCLUSIONS

The fluorescence detection in CPAE, PTK_2, and He-2 cells after administration of photosensitisers demonstrated that the Photofrin-II is bound primarily to the mitochondria with some diffuse fluorescence in the rest of the cytoplasm. However, the Lu Tex appears to be localised to the lysosomes. The percentages of damaged cells following light exposure to the different subcellular regions after Photofrin-II or Lu Tex treatment indicated that the nuclear region was the most sensitive target followed by the perinuclear and peripheral cytoplasmic region.

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