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ORIGINAL ARTICLE

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Effects of chlorin-mediated photodynamic therapy combined with fluoropyrimidines in vitro and in a patient

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Abstract Although photodynamic therapy (PDT) is becoming an additional cancer therapy, only little is known about its interactions with other drugs and treatment modalities in vitro and in vivo. We investigated the combination of 5-fluoro-2′-deoxyuridine (5FdUr), a chemotherapeutic drug, with 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (mTHPC), a potent photosensitizer. Two cell lines, MCF-7 and LNCaP, were either simultaneously or consecutively incubated with both drugs and irradiated with laser light to activate mTHPC, and cell survival was determined. The combination of the two treatments was additive or antagonistic in LNCaP cells but additive or synergistic in MCF-7 cells depending on the protocol and concentration of 5FdUr. In one patient with multiple basal cell carcinoma, the effect of the combination of 5-fluorouracil administration followed by PDT resulted in significantly stronger effects than expected, leading to severe oedema, redness and ulceration. The healing process was delayed by 2 months compared to PDT alone. It is therefore important to find optimal conditions under which PDT and chemotherapy combinations do not fall outside the therapeutic window.

Keywords Photodynamic therapy · Tetra(*m*-hydroxyphenyl)chlorin · 5-Fluoro-2′-deoxyuridine · Chemotherapy · Basal cell carcinoma

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Introduction

Photodynamic therapy (PDT) is based on light activation of a photosensitizer resulting in the formation of radicals and reactive oxygen species (for review see reference 8). Photosensitizers are nontoxic substances that accumulate preferentially in the target tissue, e.g. tumour, upon systemic administration or topical application. They absorb light in the visible (preferably above 600 nm) or infrared range of the spectrum. Upon activation, a photosensitizer can emit light (fluorescence) or undergoes type I (electron or hydrogen transfer) or type II (formation of singlet oxygen) photochemical reactions. This leads to oxidative damage to proteins, lipids and DNA, resulting in apoptotic or necrotic cell death, depending on the photosensitizer, cell type [10] and treatment protocol [5].

5,10,15,-Tetra(*m*-hydroxyphenyl)chlorin (mTHPC, Foscan) is a neutral lipophilic second-generation photosensitizer. It inhomogeneously localizes in the cytoplasm around the nucleus of MCF-7 and V79 cells [11] and photosensitizes isolated mitochondria [15]. It has been tested in vivo in mouse models [13, 19]. Clinical trials with mTHPC-mediated PDT are ongoing [6, 21].

PDT is becoming a treatment modality for, for example, age-related macular degeneration [22], skin diseases [14] and gynaecological tumours [7, 26]. PDT can be used as an alternative treatment, or supplement surgery or chemotherapy. It has been shown in one study that mTHPC-mediated PDT does not induce resistance to chemotherapy, radiotherapy or PDT in MCF-7 cells [12], indicating that PDT can be combined with other treatment modalities. Still, interaction or interference of PDT with classical cancer treatments has been poorly investigated but is important to be understood in order to improve combined treatments.

Fluoropyrimidines are used in the chemotherapeutic treatment of breast, gastrointestinal tract and head and neck cancer, and might be good candidates to enhance PDT because of their different mode of action within

cells. In this study we investigated the combined effects of mTHPC-mediated PDT and 5-fluoro-2′-deoxyuridine (5FdUr) or 5-fluorouracil (5-FU) on survival of two cell lines, MCF-7 and LNCaP, and in one patient with multiple basal cell carcinoma (BCC), respectively. These cell lines, a human breast cancer and a human prostate carcinoma cell line, were chosen mainly because of their relevance to PDT administration and our knowledge of mTHPC-mediated effects on MCF-7 cells.

Materials and methods

Cell lines

LNCaP (human prostate carcinoma) cells were cultured in RPMI medium (Gibco, Basel, Switzerland) enriched with 10% fetal calf serum, 25 IU/ml penicillin and 25 mg/ml streptomycin. MCF-7 (human breast cancer) cells were grown in OptiMEM medium (Gibco, Basel, Switzerland) supplemented with 10% fetal calf serum, 25 IU/ml penicillin and 25 mg/ml streptomycin. Cells were kept at 37°C in an atmosphere containing 5% CO_2 at 100% humidity.

Chemicals

mTHPC (Foscan) was from Biolitec Pharma (Edinburgh, UK) and was dissolved in ethanol. 5FdUr was purchased from Fluka (Buchs, Switzerland), and M30 CytoDEATH antibody from Roche Diagnostics (Schweiz, Rotkreuz, Switzerland).

Incubation protocols

LNCaP and MCF-7 cells (10,000 and 300 respectively) were seeded in Petri dishes (4 cm diameter) 24 h before addition of drugs (two dishes for each condition). Depending on the protocol, mTHPC and 5FdUr were added simultaneously or consecutively (Fig. 1). In preliminary experiments various concentrations of the drugs were tested. For the experiments presented here concentrations of 0, 3 and 10 μ*M* for 5FdUr and 0 and 0.1 μ*M* for mTHPC were chosen.

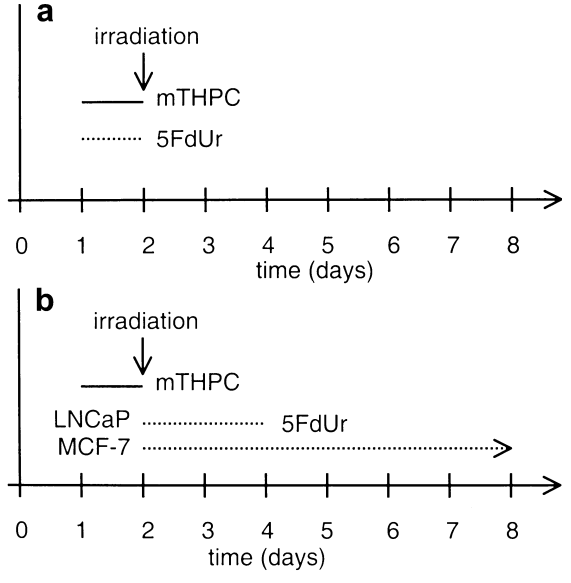

Fig. 1a, b Incubation protocols. **a** Simultaneous incubation with mTHPC and 5FdUr, incubation times with both drugs were the same (24 h) for both cell lines. **b** Consecutive incubation with mTHPC and 5FdUr, incubation with 5FdUr lasted until the end of the experiments (day 5 for LNCaP cells, day 10 for MCF-7 cells)

Irradiation

For irradiation a diode laser (Applied Optronics Corporation, South Plainfield, N.J.) emitting at 652 nm was used. The output power was set to 25 mW, and the irradiation times were 0, 30 and 60 s, resulting in total doses of 0, 0.26 and 0.52 J/cm^2, respectively. The Petri dishes were placed into the laser beam so that the entire dishes were irradiated and not only their central areas. The medium was changed prior to irradiation without further washing of the cells.

Survival assays

LNCaP cells were counted on the 5th day of each experiment, MCF-7 cells were fixed with methanol/acetic acid (3:1) on the 10th day, stained with Giemsa, and colonies (more than 50 cells) were counted. The plating efficiency for MCF-7 cells was 35% on average.

Detection of apoptosis

Apoptosis was detected with M30 CytoDEATH antibody, which binds to a cytokeratin 18 neo-epitope exposed upon cleavage by caspases [17]. Cells were trypsinized 2 h after irradiation with 0.52 J/cm^2, washed with phosphate-buffered saline, fixed with cold methanol for 30 min at –20°C and washed again with phosphate-buffered saline containing 0.1% Tween 20. For 1 h the cells were incubated with M30 CytoDEATH antibody (dilution 1:250). Then they were washed and mounted on a coverslip. Fluorescence was detected with a confocal laser scanning microscope (Leica TCS 4D, Glattbrugg, Switzerland). Excitation was at 488 nm, and emission was detected with an FITC bandpass filter.

Statistics and calculation of synergism/antagonism

Each experiment was repeated five times, and the figures show the mean of all five experiments plus standard error. For statistical analysis, a paired *t*-test was used, and $P < 0.05$ was considered significant.

A combined treatment was considered synergistic (antagonistic), when the surviving fraction of cells treated with PDT plus 5FdUr was lower (higher) than the survival fractions of cells treated with PDT alone multiplied by the survival fraction of cells treated with 5FdUr alone: survival[PDT + 5FdUr] less than survival[PDT]×survival[5FdUr]. The expected effect for the combined treatment was calculated for every individual experiment and compared to the actual combined treatment, and the means of all five experiments are given in Tables 1, 2, 3 and 4.

Clinical treatment

One patient was treated with mTHPC-mediated PDT after the application of 5-FU cream (Efudix) for 3 weeks to one side of the chest and back. The patient was admitted to the hospital and injected intravenously with a dose of 0.15 mg mTHPC/kg within 5 min. The patient had to remain in subdued light during the first 7 days after injection. The application of 5-FU cream was stopped the day before illumination. On the 3rd and 4th day after injection, various lesions with a size between 3 and 10 mm were treated with red light (645–655 nm) from an LED source (Diomed LED, Cambridge, UK) with a fluence rate of 100 mW/cm^2. Lesions on the 5-FU-treated side were irradiated to a total dose of 10 or 15 J/cm^2 with illumination times of 100 or 150 s for each lesion. Lesions on the nonpretreated side were illuminated with 15 J/cm^2.

Table 1 Effects on LNCaP cells of simultaneous incubation with mTHPC and 5FdUr followed by irradiation

5FdUr concentration (μ*M*)	Total dose (J/cm^2)	Survival (%)		Effect	*P* value
		Measured	Calculated		
–	0.26	36.4			
3	–	76.2			
3	0.26	28.4	31.7	Additive	0.5585
3	0.52	15.5	21.1	Additive	0.4409
–	0.52	19.0			
10	–	61.0			
10	0.26	28.2	20.2	Additive, slightly antagonistic	0.0620
10	0.52	19.2	11.6	Antagonistic	0.0475

Table 2 Effects on LNCaP cells of consecutive incubation with mTHPC (followed by irradiation) and 5FdUr

5FdUr concentration (μ*M*)	Total dose (J/cm^2)	Survival (%)		Effect	*P* value
		Measured	Calculated		
–	0.26	39.1			
3	–	71.1			
3	0.26	30.7	29.9	Additive	0.9021
3	0.52	8.3	6.8	Additive	0.3985
–	0.52	8.9			
10	–	70.0			
10	0.26	52.8	28.2	Antagonistic	0.0157
10	0.52	13.3	6.5	Additive, slightly antagonistic	0.0704

Table 3 Effects on MCF-7 cells of simultaneous incubation with mTHPC and 5FdUr followed by irradiation

5FdUr concentration (μ*M*)	Total dose (J/cm^2)	Survival (%)		Effect	*P* value
		Measured	Calculated		
–	0.26	22.6			
3	–	98.8			
3	0.26	21.4	22.2	Additive	0.8314
3	0.52	6.4	6.1	Additive	0.8670
–	0.52	6.1			
10	–	94.3			
10	0.26	17.6	21.0	Synergistic	0.0422
10	0.52	11.3	5.7	Additive	0.2903

Table 4 Effects on MCF-7 cells of consecutive incubation with mTHPC (followed by irradiation) and 5FdUr

5FdUr concentration (μ*M*)	Total dose (J/cm^2)	Survival (%)		Effect	*P* value
		Measured	Calculated		
–	0.26	25.0			
3	–	75.1			
3	0.26	10.8	17.9	Additive, slightly synergistic	0.0930
3	0.52	0.4	1.3	Synergistic	0.0240
–	0.52	1.9			
10	–	57.8			
10	0.26	9.8	13.7	Additive	0.3194
10	0.52	0.4	0.8	Additive	0.2278

Results

Incubation with 5FdUr

Incubation of MCF-7 cells with 3 and 10 μM 5FdUr for 7 days induced up to 25% and 42% reduction in colony formation, respectively, but had hardly any effect when incubation was for 24 h only (Fig. 2). When LNCaP cells were incubated with 5FdUr for 24 h, their survival was reduced by about 24% (3 μ*M*) or 40% (10 μ*M*), and prolongation of the incubation time to 48 h did not induce significantly higher cell death (not shown). These

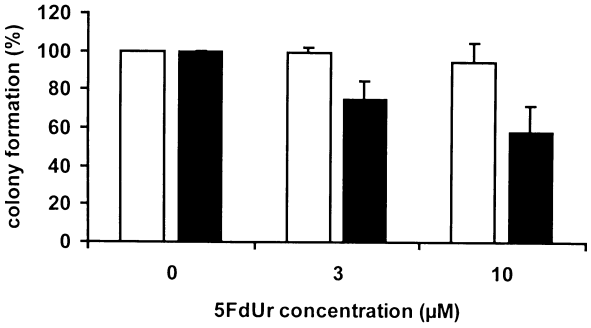

Fig. 2 Survival (colony formation) of MCF-7 cells after incubation with 5FdUr for 24 h (*white bars*) or 7 days (*black bars*)

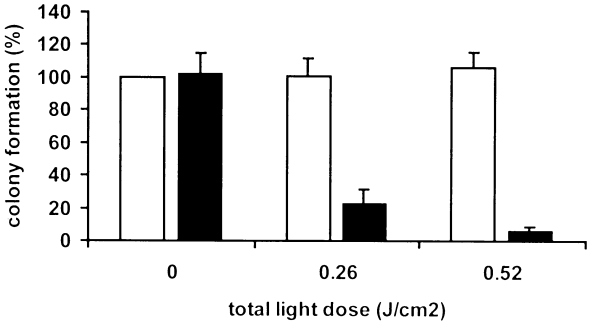

Fig. 3 Survival (colony formation) of MCF-7 cells after in vitro PDT. Cells were irradiated after incubation for 24 h in the absence (*white bars*) or presence (*black bars*) of 0.1 μ*M* mTHPC

conditions were tested in preliminary experiments and were chosen because they resulted in an intermediate level of cell death.

Incubation with mTHPC and irradiation

Figure 3 shows MCF-7 cells that were incubated for 24 h in the absence or presence of 0.1 μ*M* mTHPC and subsequently irradiated with laser light of 652 nm. Neither mTHPC nor irradiation alone resulted in a significantly changed number of colonies growing, but their combination killed 77% (0.26 J/cm^2) and 94% (0.52 J/cm^2) of MCF-7 cells. Comparable results were obtained for LNCaP cells (not shown).

Combination of 5FdUr, mTHPC and irradiation

Suitable conditions being found, incubation with 5FdUr and mTHPC (followed by irradiation) were now combined. The results for MCF-7 cells are shown in Fig. 4 (simultaneous incubation) and Fig. 5 (consecutive incubation); results for LNCaP cells are not shown. Cell survival measured after combined treatments was compared to the survival calculated from the numbers of single treatments. The results are shown in Tables 1 and 2 for LNCaP cells, and in Tables 3 and 4 for MCF-7 cells. Interestingly, combination of the higher concentration of 5FdUr (10 μ*M*) with in vitro PDT resulted in

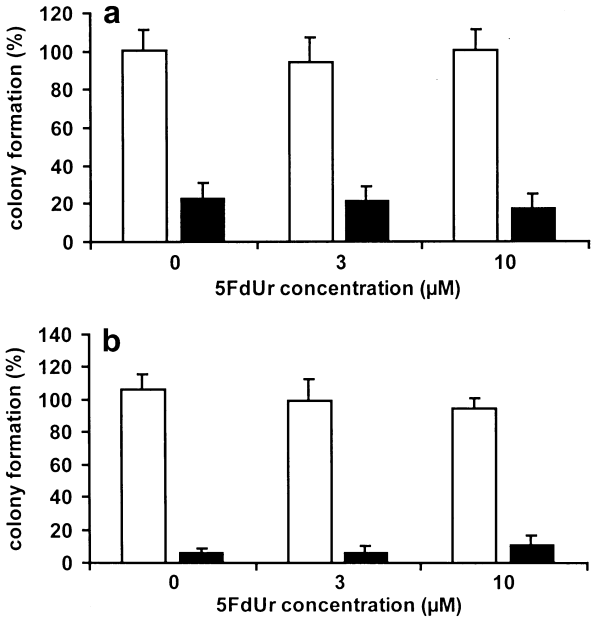

Fig. 4a, b Survival (colony formation) of MCF-7 cells after in vitro PDT and incubation with 5FdUr. The cells were simultaneously incubated with mTHPC and 5FdUr. The total doses of irradiation were (**a**) 0.26 J/cm^2 and (**b**) 0.52 J/cm^2 (*white bars* cells incubated in the absence of mTHPC, *black bars* cells incubated in the presence of 0.1 μ*M* mTHPC)

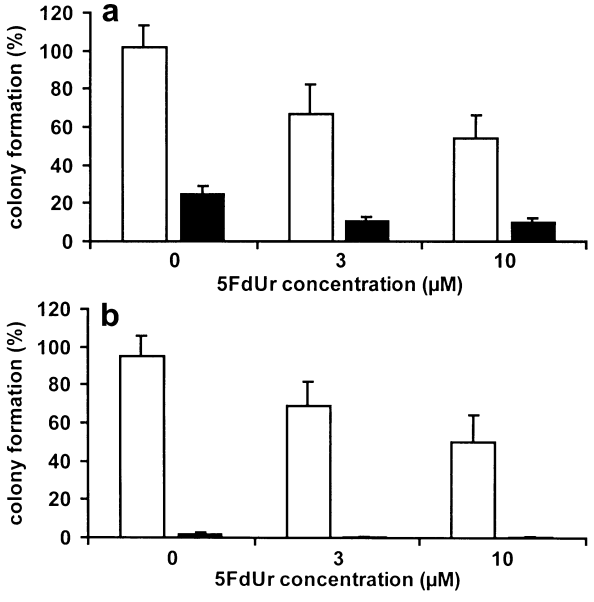

Fig. 5a, b Survival (colony formation) of MCF-7 cells after in vitro PDT and incubation with 5FdUr. The cells were incubated first with mTHPC and after irradiation with 5FdUr. The total doses of irradiation were (**a**) 0.26 J/cm^2 and (**b**) 0.52 J/cm^2 (*white bars* cells incubated in the absence of mTHPC, *black bars* cells incubated in the presence of 0.1 μ*M* mTHPC)

antagonistic effects in LNCaP cells independent of the protocol (simultaneous vs consecutive), whereas the toxicity of the lower concentration of 5FdUr (3 μ*M*) combined with in vitro PDT was additive. A number of

effects were observed in MCF-7 cells. In particular, the protocol with consecutive incubation of cells with the two drugs showed synergistic effects, i.e. cell death induced by the combined treatment was higher than expected from the survival rates from the single treatments.

Induction of apoptosis

Since MCF-7 cells are known to have a mutation in caspase-3 [20], the following experiment was only done with LNCaP cells. Cells were incubated with 0.1 μ*M* mTHPC and/or 10 μ*M* 5FdUr for 24 h, irradiated and stained with M30 CytoDEATH antibody for apoptosis. Incubation with mTHPC and irradiation alone or in combination with 5FdUr induced an apoptotic response in LNCaP cells, whereas control cells or cells incubated only with 5FdUr showed no staining (Fig. 6).

Clinical treatment

On the 3rd day after administration of the photosensitizer a total of 20 lesions (< 11 mm diameter) were treated on the 5-FU pretreated side: 8 received 10 J/cm^2 and 12 received 15 J/cm^2. On the "control" side, 10 lesions (< 11 mm diameter) were treated with 15 J/cm^2 [2]. On the 4th day the light dose was reduced to 10 J/cm^2 because of intense swelling and redness of the lesions treated on the previous day. A total of five lesions were treated on both the 5-FU-treated side and the "control" side.

In Fig. 7a the appearance of the patient before treatment is shown. Figure 7b shows the effect of PDT 1 day after illumination (day 4) for the combined treatment and Fig. 7c the effect of mTHPC-mediated PDT alone during a previous treatment session. Figure 7b shows that both the 5-FU pretreated side and the untreated side had nearly similar intense reactions. The lesions were painful, requiring treatment with morphine and the healing process was impaired. After 4 to 6 months the treated lesions had healed, but in half of the lesions scar formation had occurred. With a follow-up of 2 years the lesions had all been treated successfully.

Discussion

Our in vitro study showed that a combination of two treatments, mTHPC-mediated PDT and the chemother-

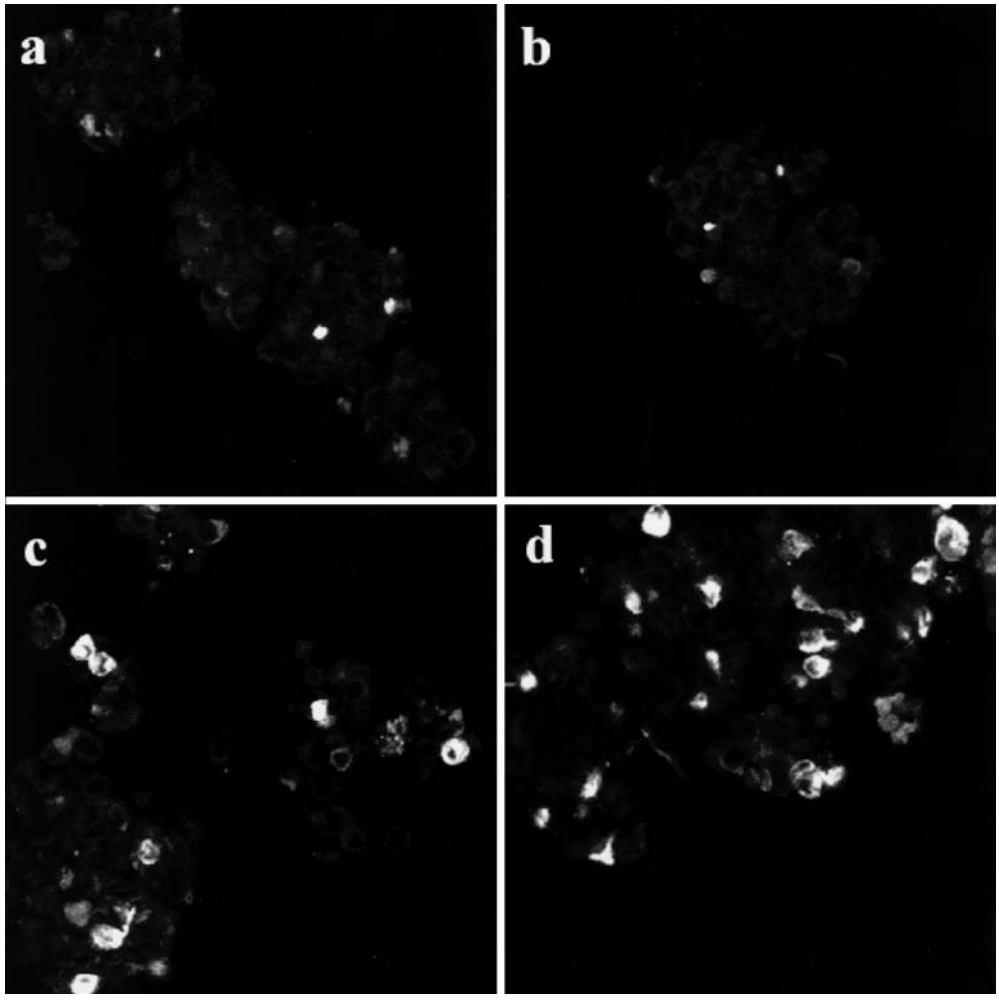

Fig. 6a–d Detection of apoptosis in LNCaP cells after incubation with M30 CytoDEATH antibody by confocal laser scanning microscopy (**a** control cells, **b** cells incubated with 10 μM 5FdUr, **c** cells incubated with 0.1 μM mTHPC and irradiated with 0.52 J/cm^2, **d** combination of **b** and **c**). Typical pictures from one experiment out of four are shown (picture size 250×250 μm)

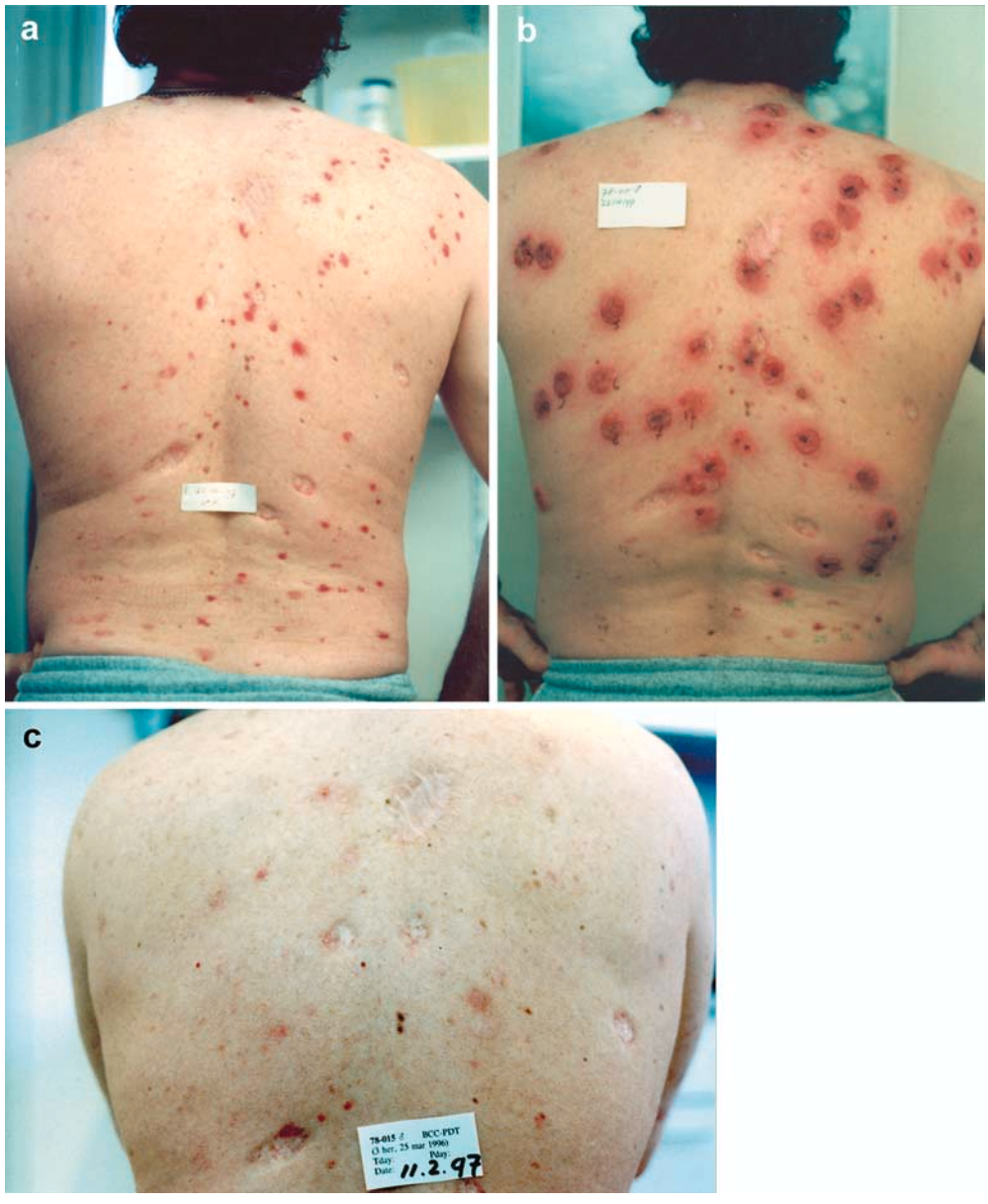

Fig. 7 **a** Photograph of the patient with multiple basal cell carcinomas injected with 0.15 mg/kg of mTHPC before PDT. The right part of his back was treated with 5-FU cream for 3 weeks. The lesions on the pretreated right side are red and slightly swollen. **b** Photograph of the patient 1 day after illumination with 10 J/cm^2 on the right side and 10–15 J/cm^2 on the left side. The overall reaction is severe with intense swelling, reddening and pain. There was no clear difference between the pretreated and untreated side. **c** For comparison, a detailed photograph of the left part of the chest of the same patient treated with the same dose of mTHPC and 15 J/cm^2 alone 1 year previously. The photograph was taken 1 day after PDT. There is some redness and some swelling

apeutic drug 5FdUr, resulted in a lower cell survival than single-mode treatment. Mostly, additive effects were observed. However, the results were strongly dependent on experimental conditions and/or cell lines. LNCaP cells showed antagonistic effects when a higher (10 μM) concentration of 5FdUr was used (Tables 1 and 2). MCF-7 cells, on the contrary, showed synergistic effects regarding colony formation. Since MCF-7 cells grew in colonies, but LNCaP cells did not, different assays have to be chosen to determine survival of these cell lines, and the results may therefore be difficult to compare.

Contradictory results have also been reported by other groups. K562 human leukaemic cells have been shown to respond synergistically to a combination of etoposide and aluminium tetrasulphophthalocyanine-mediated PDT [9], whereas in a study with C6 rat glioma cells, methotrexate alone was under some conditions more effective than in combination with Photofrin-mediated PDT [18]. Streckyte et al. [24] found in an animal model no enhancement by combining Adriamycin and photohem-mediated PDT. Spectroscopically they showed an interaction between photohem and Adriamycin and therefore proposed a consecutive protocol for drug administration. However, Canti et al. [3] showed that the combination of Adriamycin or cisplatinum with photoactivated aluminium disulphonated phthalocyanine had an additive antitumour effect allowing them to use lower doses of chemotherapeutic drugs which reduced side effects. Promising results in mice have also been obtained by repeating chemotherapy and PDT [23]. A similar conclusion was drawn by Baas et al. [1], who treated patients with mammary skin metastasis. By combining photofrin-mediated PDT and mitomycin C, lower light doses could be used compared

to PDT alone. Even combinations of two photosensitizers seem to be possible, as accidentally seen in a patient who had taken aminolaevulinic acid and a hypericin-containing antidepressant and showed severe phototoxic reactions compared to other patients taking only aminolaevulinic acid. The synergistic effects of these two compounds have also been shown in vitro in HaCaT cells [16].

mTHPC and 5FdUr were chosen because of their different modes of action and expected synergism in cells. mTHPC is a lipophilic compound, accumulating mainly in membranes but not in the nucleus, whereas 5FdUr acts in the cytosol by inhibiting thymidylate synthase and is incorporated into RNA and DNA [25]. Therefore, inhibition via direct interaction of the two drugs seems to be unlikely. Spectroscopic analysis of the compounds after uptake into cells might clarify this issue. However, uptake of 5FdUr into the cells might be inhibited, especially in the consecutive incubation protocol where membranes and membrane-bound enzymes are damaged as a consequence of mTHPC-mediated PDT. Drug uptake studies are needed to investigate this situation.

One hint that the rather nonspecific actions of PDT are less likely to be influenced by the defined actions of 5FdUr than vice versa was given by our last experiment. Apoptosis elicited by PDT, as assessed qualitatively in LNCaP cells, was not influenced by simultaneous incubation with 5FdUr (Fig. 6). Incubation of LNCaP cells with 5FdUr alone did not result in apoptosis, most likely because a very short incubation time (24 h) was chosen in this experiment [4].

The results of the patient treatment were unexpected for this combination. The reason for testing this combination was based on the fact that during a previous PDT treatment the patient had decided himself to administer 5-FU cream to several lesions and reported afterwards that these lesions had responded very well. Both the patient and the dermatologist suggested that a therapeutic trial using the combination could be useful. In order to limit possible side effects the PDT treatment was given on days 3 and 4 using moderate light doses.

From the combination treatment of the patient with multiple BCCs, it has become clear that there is a strong interaction between 5-FU (and/or its metabolized products) and PDT with mTHPC. We can also conclude that the skin readily absorbs the 5-FU which then acts as a systemic drug. We had expected minimal swelling, less redness and better healing based on our previous experiences in patients with mTHPC-mediated PDT. The scars we observed in our patient were comparable or even more intense than those seen after surgery in previously treated lesions. Care must be taken in the use of PDT in combination with other drugs that may have photodynamic properties or increase PDT-triggered effects.

Based on the in vitro results and on this patient's history, it is clear that more studies are needed to determine how chemotherapeutic drugs and photosensitizers interact and how these two can be combined in order to result in increased cell killing with reduced side effects.

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