Doctoral Thesis

Subcellular events in photodynamic therapy
role of mitochondria

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Subcellular events in photodynamic therapy: role of mitochondria

A dissertation submitted to the
Swiss Federal Institute of Technology (ETH) Zürich
for the degree of Doctor of Natural Sciences

presented by
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2000
Für meine Eltern in Liebe und Dankbarkeit
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1. **Summary**

In photodynamic therapy (PDT), a tumour-localised photosensitiser (PS) is excited by visible light, resulting in the formation of reactive oxygen species and destruction of cells. The nature of the PS and the experimental protocol determine its subcellular localisation, and hence the primary target site and following events. Reactive oxygen species formed upon irradiation are short-lived and react with biomolecules in their immediate vicinity.

In MCF-7 cells the second-generation PS 5,10,15,20-tetra(m-hydroxyphenyl)-chlorin (mTHPC) localises in the nuclear membrane, plasma membrane and organelles, some of which are identified as mitochondria by co-localisation with rhodamine 123. When isolated rat liver mitochondria are incubated with mTHPC and subsequently irradiated with light of 652 nm, their lipids and proteins become oxidatively modified. In consequence, mitochondrial functions including Ca\(^{2+}\) uptake, respiration and ATP synthesis are impaired, and the transmembrane potential (ΔΨ) is decreased. Incubation of MCF-7 cells with mTHPC and irradiation results in decreased viability, oxygen consumption and ΔΨ. One to two hours after irradiation intracellular Ca\(^{2+}\) levels rise. This is accompanied by a biphasic formation of nitrite and nitrate. The use of scavengers of reactive oxygen species demonstrates that mTHPC acts via type I (electron or hydrogen transfer) and type II (energy transfer to molecular oxygen) photochemical reactions.

Cells with increased ΔΨ, e.g., carcinoma cells or L929 cells overexpressing Bcl-2, are protected against apoptosis and take up higher concentrations of lipophilic cationic compounds. Therefore, positively charged PS should preferentially accumulate in these cells and permit selective destruction. The sensitivity of control (BV3) and Bcl-2 overexpressing (B22) L929 cells to three, charged and uncharged, PS alone or in combination with light was investigated. In both cell lines, victoria blue BO (VB-BO; positively charged) localises in mitochondria, mTHPC (uncharged) in membranous structures and meso-tetra(4-sulfonatophenyl)porphine (TPPS\(_4\); negatively charged) in lysosomes. Uptake of mTHPC and TPPS\(_4\) into both cell lines is equal, whereas B22 cells take up more VB-BO than BV3 cells. They, consequently, display higher sensitivity to this PS both in the dark and after light exposure. Dark toxicity of TPPS\(_4\)
and mTHPC, and light-induced toxicity of mTHPC are equal in both cell lines. Incubation with TPPS₄ and illumination with white light is more toxic to BV3 cells. After incubation with these PS and illumination cells show signs of apoptosis including the release of cytochrome c from mitochondria, membrane blebbing, enhanced annexin V binding and rounding off. Overexpression of Bcl-2, which protects against apoptosis-inducing stimuli, renders B22 cells more sensitive than control cells to the toxicity of VB-DO, a lipophilic cationic PS. This provides a potential strategy for cancer cell targeting in vivo.

A major side effect of PDT is skin photosensitivity caused by accumulation of PS in the skin. Protection of patients cannot be achieved by sunscreens, but only by creams that do not allow penetration of visible light. Cover creams containing pigments, e.g., TiO₂ and Fe₂O₃, show in vitro efficacy to reduce light-induced cell killing of human keratinocytes incubated with mTHPC. A Hypericum extract, used in the treatment of depression, also contains compounds that accumulate in the skin of patients and display characteristics of a PS. Synergistic effects of Hypericum perforatum extract with δ-aminolaevulinic acid, a PS precursor, are observed in vitro and in vivo. Incubation of human keratinocytes with both compounds in the dark or followed by light exposure results in higher cell killing than expected for additive toxic effects.
2. ZUSAMMENFASSUNG


Zellen mit erhöhtem \(\Delta \Psi\), z. Bsp. Karzinomzellen oder Bel-2 überexprimierende L929 Zellen, sind gegen Apoptose geschützt und nehmen höhere Konzentrationen lipophiler kationischer Substanzen auf. Deshalb sollten positiv geladene PS vorzugsweise in diesen Zellen akkumulieren und eine selektive Zerstörung ermöglichen. Die Sensitivität von Kontrollzellen (BV3) und Bel-2 überexprimierenden (B22) L929 Zellen auf drei, geladene und ungeladene, PS allein oder in Kombination mit Licht wurde untersucht. In beiden Zelllinien ist Victoria Blau BO (VB-BO; positiv geladen) in Mitochondrien, mTHPC (ungeladen) in Membranstrukturen und Meso-tetra(4-

### 3. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>antimycin A</td>
</tr>
<tr>
<td>ALA</td>
<td>δ-aminolaevulinic acid</td>
</tr>
<tr>
<td>arsenazo III</td>
<td>3,6-bis[2-arsenophenylazo]-4,5-dihydroxy-2,7-naphthalene-disulfonic acid</td>
</tr>
<tr>
<td>Asc/TMPD</td>
<td>ascorbate/tetramethylphenylenediamine</td>
</tr>
<tr>
<td>AV</td>
<td>annexin V</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxytoluene</td>
</tr>
<tr>
<td>C2 ceramide</td>
<td>N-acetylsphingosine</td>
</tr>
<tr>
<td>C6 ceramide</td>
<td>N-hexanoylsphingosine</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonylcyanide-3-chlorophenylhydrazone</td>
</tr>
<tr>
<td>CF</td>
<td>colony formation</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
</tr>
<tr>
<td>DHC</td>
<td>C2 dihydroceramide</td>
</tr>
<tr>
<td>DHR</td>
<td>dihydrorhodamine 123</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylfuran</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>ΔΨ</td>
<td>mitochondrial transmembrane potential</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenedinitilotetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>FCCP</td>
<td>carbonylcyanide-4-trifluoromethoxyphenylhydrazone</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Heps</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HpD</td>
<td>haemoporphyrin derivative</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LH</td>
<td>unsaturated lipid</td>
</tr>
<tr>
<td>LOOH</td>
<td>lipid hydroperoxide</td>
</tr>
<tr>
<td>mTHPC</td>
<td>5,10,15,20-tetra(m-hydroxyphenyl)chlorin</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide; thiazolyl blue</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>PDT</td>
<td>photodynamic therapy</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PPIX</td>
<td>protoporphyrin IX</td>
</tr>
<tr>
<td>PS</td>
<td>photosensitiser(s)</td>
</tr>
<tr>
<td>Rh123</td>
<td>rhodamine 123</td>
</tr>
<tr>
<td>SDS</td>
<td>sodiumdodecylsulfate</td>
</tr>
<tr>
<td>TBARS</td>
<td>thiobarbituric acid-reactive substances</td>
</tr>
<tr>
<td>TPPS₄</td>
<td>meso-tetra(4-sulfonatophenyl)porphine</td>
</tr>
<tr>
<td>VB-BO</td>
<td>victoria blue BO</td>
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4. INTRODUCTION

4.1. Photodynamic therapy

Photodynamic therapy (PDT) is based on light activation of a photosensitiser (PS) that has accumulated at a target site, e. g., tumour tissue, resulting in the formation of reactive oxygen species and, finally, cell death (for reviews, see Fisher et al., 1995; Henderson and Dougherty, 1992). The basic steps of PDT are summarised in Table 1 and will be discussed in the following paragraphs.

Table 1. Basic steps in PDT (modified from Penning and Dubbelman, 1994).

1. Systemic or topical application of the PS
2. Accumulation/retention of the PS in the target tissue (e. g. tumour)
3. Photoexcitation: penetration and absorption of light
4. Intersystem crossover to triplet state of the PS
5. Generation of reactive intermediates (radicals, reactive oxygen species)
6. Photooxidation of cellular constituents (proteins, lipids, nucleic acids)
7. Functional and structural alterations (enzyme activities, membrane fluidity, mutations)
8. Cell damage/death: necrosis or apoptosis (primary cell death)
9. Vascular occlusion, inflammation (secondary cell death)

PDT is used to treat a variety of dysplasias and superficial cancers, i. e., gynaecological tumours, skin metastases of mammary carcinoma, cutaneous, lung, oesophagus and bladder cancer, and cervical and vulval intraepithelial neoplasias (Fisher et al., 1995).

4.1.1. Photosensitisers

PS are non-toxic substances that accumulate in the target tissue (e. g. tumour) and absorb light in the visible (preferably above 600 nm) or infrared range of the
spectrum. At the beginning of PDT, in the 1950s, a mixture of porphyrins (haematoporphyrin derivative, HpD) was used as PS. The most active components were identified as dihaematoporphyrin ethers and esters. Commercially available preparations (photofrin® and photofrin II®) contain mostly porphyrin dimers and oligomers. The drawbacks of a variable and complex mixture, low absorption above 600 nm and prolonged cutaneous sensitivity led to the development of new, so called ‘second-generation’ PS (Fisher et al., 1995). The ideal properties of a PS are defined as: chemical purity, minimal dark toxicity, high tumour selectivity, large absorption coefficient at a high wavelength, high photochemical reactivity, and high ability to induce (in)direct cell killing (Penning and Dubbelman, 1994). Absorption at a high wavelength is preferable, since light above 600 nm can penetrate tissues better due to lower absorption by water, haemoglobin etc.

PS can be classified, e.g., by their chemical structure, and include porphyrins, chlorins, bacteriochlorins, purpurins and phthalocyanines. As examples, chemical structures of PS used in this thesis are shown in Fig. 1. Also natural compounds, like a Hypericum extract used in the treatment of depression, may have photosensitising properties (Golsch et al., 1997; Bernd et al., 1999). Photodynamic effects are also observed in diseases like porphyria which results from impaired haem synthesis (for reviews, see Elder et al., 1997; Moore, 1998), or are presumed to take place, e.g., in age-related macular degeneration (Gaillard et al., 1995; Wihlmaker et al., 1997).

4.1.1.1. 5,10,15,20-Tetra(m-hydroxyphenyl)chlorin

5,10,15,20-Tetra(m-hydroxyphenyl)chlorin (mTHPC, temoporfin, Foscan®) is a neutral lipophilic second-generation PS with a high absorption at 652 nm. It inhomogenously localises in the cytoplasm around the nucleus of MCF-7 and V79 cells (Hornung et al., 1997). When cells are incubated with mTHPC and exposed to light, they are more sensitive at pH 6.8 than at pH 7.2 although the level of drug uptake is pH-independent (Ma et al., 1999). Compared to tetra(m-hydroxyphenyl)porphine and photofrin II®, incubation with mTHPC and irradiation more efficiently induces cell death when a wavelength above 600 nm is used to excite the PS (Ma et al., 1994). In vivo studies in mice bearing mammary carcinoma, tumour destruction by PDT with mTHPC was more efficient than with the corresponding porphyrin (Peng et al., 1995).
Clinical trials with mTHPC-mediated PDT, e. g., for oral cancer (Fan et al., 1997) and squamous cell carcinomas of the oesophagus (Savary et al., 1998), are ongoing.

Fig. 1. Chemical structures of 5,10,15,20-tetra(m-hydroxyphenyl)chlorin (mTHPC), meso-tetra(4-sulfonatophenyl)porphine (TPPS₄), victoria blue BO (VB-BO), δ-aminolaevulinic acid (ALA), and protoporphyrin IX (PPIX).

4.1.1.2. Meso-tetra(4-sulfonatophenyl)porphine

Meso-tetra(4-sulfonatophenyl)porphine (TPPS₄) is anionic and hydrophilic, and localises in lysosomes (Malik et al., 1997). Normal leukocytes are less sensitive to TPPS₄-mediated phototoxicity than HL60 and HEL leukaemia cells (Grebenová et al., 1997). It is, however, 50-fold less efficient as a PS than is photofrin II® in WiDr cells.
exposed to 10 μg/ml of either drug (West and Moore, 1989). In vivo, TPPS4 has been used as a PS for PDT of cutaneous metastases of breast cancer where it was applied locally to avoid skin photosensitisation (Lapes et al., 1996).

4.1.1.3. Victoria blue BO

Victoria blue BO (VB-BO) is cationic and lipophilic, and is taken up by mitochondria (Modica-Napolitano et al., 1990). Incubation of isolated mitochondria with VB-BO induces uncoupling of oxidative phosphorylation, and when followed by irradiation, it inhibits complex I of the respiratory chain (Modica-Napolitano et al., 1990). VB-BO has both a much higher dark toxicity and light-induced toxicity than TPPS4 in human leukemic cell lines K-562 and TF-1 (Fiedorowicz et al., 1993). Monocytes and leukemic cells are more sensitive than lymphocytes to VB-BO-mediated phototoxicity, suggesting a possible application of VB-BO for selective depletion of monocytes or sensitive leukemic cells (Fiedorowicz et al., 1997). A series of compounds based on the structure of VB-BO was synthesised, but none of them equalled toxicity of VB-BO in the dark or with illumination (Burrow et al., 1995).

4.1.1.4. δ-Aminolaevulinic acid

δ-Aminolaevulinic acid (ALA) is not a PS but a prodrug (Gibson et al., 1997). ALA is formed from glycine and succinyl-CoA in the first step of the synthesis of haem. When added to cells, it enhances the formation of protoporphyrin IX (PPIX) and haem (for review, see Peng et al., 1997). PPIX accumulates in the mitochondria and can serve as a PS because of the limited capacity of ferrochelatase, which incorporates iron into PPIX to form haem. In some tumours, the activity of porphobilinogen deaminase, another enzyme in the haem synthesis pathway, is increased and that of ferrochelatase decreased, so that PPIX accumulates preferentially in tumour cells (van Hillegersberg et al., 1992; Hinmen et al., 1998). Porphobilinogen deaminase activity increases when tumour cell lines are incubated with ALA due to de novo synthesis of the enzyme (Gibson et al., 1998). Iron chelators, e.g., ethylenediaminetetraacetic acid (EDTA) and desferrioxamine, enhance PPIX accumulation in cells exposed to ALA (Berg et al., 1996). Its major advantage compared to other PS is that ALA can be applied topically without the side effect of cutaneous photosensitivity, its disadvantage is a low light absorption of PPIX at wavelengths above 600 nm.
4.1.2. Mechanisms of photosensitiser accumulation

Hydrophilic PS are transported in the blood via albumin and globulins (Villanueva and Jori, 1993), and localise in the stroma of tumour and other tissues (Fisher et al., 1995). Hydrophobic PS can be incorporated into plasma lipoproteins, e.g., low density lipoproteins (LDL), and are taken up by cells partially via receptor-mediated endocytosis (Allison et al., 1994; Soncin et al., 1995). In vitro and in vivo, mTHPC binds to LDL and HDL (Michael-Titus et al., 1995). Higher selectivity in tumour targeting could be achieved by associating the PS before injection with delivery vehicles, e.g. LDL, which can interact preferentially with tumour cells (for review, see Reddi, 1997). Cells with a high content of LDL receptors, such as mitotic cells, including tumour and endothelial cells would preferentially take up the PS.

Another strategy of targeting consists in antitumour monoclonal antibodies as carriers for the PS. Tumour selectivity of antibody-conjugated mTHPC is higher in comparison with free mTHPC (Vrouenraets et al., 1999). In vitro, the efficacy of mTHPC is higher when coupled to an internalising rather than to a noninternalising antibody (Vrouenraets et al., 1999).

Lipophilic cationic PS are taken up into cells in response to the cellular and the mitochondrial transmembrane potential ($\Delta \Psi$). Carcinoma cells with their elevated $\Delta \Psi$ (Nadakavukaren et al., 1985) are sensitive to positively charged compounds, e.g., rhodamine 123 (Rh123) (Lampidis et al., 1983; Davis et al., 1985). The cause of this increase in $\Delta \Psi$, its relation to cancer and to resistance to apoptosis are not fully understood, however, this feature of tumour cells can be exploited therapeutically, i.e., by targeting them with cationic drugs (Sun et al., 1994; Koya et al., 1996; Nocentini et al., 1997). Also in vivo, a cationic phthalocyanine is a very potent PS (Cruse Sawyer et al., 1998).
4.1.3. Localisation of photosensitisers

4.1.3.1. Accumulation in tissues

To determine optimal time laps before irradiation of the target tissue after injection of the PS (drug-light interval), PS levels in tumour and normal tissues are determined either by fluorescence measurements or by extraction and HPLC analysis. In order to destroy tumour and spare normal tissues, a time point is chosen with a high tumour to normal tissue ratio of PS concentrations. For many clinically used PS this is between 24 and 72 h after injection. A fluorescence study of mTHPC in malignant and normal tissues in rats revealed high accumulation of the PS 24 h after injection in tumour tissue, small intestine, lung and liver. Forty-eight h after injection, highest mTHPC fluorescence was found in tumour tissue. PS concentrations in tumour tissue were always higher than in the surrounding muscles (Alian et al., 1994). In mice bearing mammary carcinoma, concentrations of mTHPC peaked at 24 to 48 h after injection, and only liver, urinary tract and skin took up more mTHPC than those tumours (Peng et al., 1995).

4.1.3.2. Subcellular localisation

Subcellular localisation of a PS is determined either by confocal laser scanning microscopy using the fluorescent properties of the PS or by fractionation of cells, differential centrifugation and HPLC analysis. In general, localisation depends on hydrophobicity and charge of a PS (Table 2; Woodburn et al., 1991).

The intracellular localisation of a PS determines also the sites of damage upon irradiation (for review, see Peng et al., 1996). Interestingly, Woodburn et al. (1992) found that porphyrins localised in mitochondria displayed higher phototoxicity than porphyrins with lysosomal or diffuse cytoplasmic localisation.
Table 2. Localisation of a PS depends on its hydrophobicity and charge.

<table>
<thead>
<tr>
<th>hydrophobic/hydrophilic</th>
<th>charge</th>
<th>localisation</th>
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<tr>
<td>hydrophobic</td>
<td>neutral</td>
<td>diffuse cytoplasmic plus lysosomes&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>cytoplasmic perinuclear&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>hydrophobic</td>
<td>cationic</td>
<td>mitochondria&lt;sup&gt;a,c,d&lt;/sup&gt;</td>
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<tr>
<td>hydrophobic</td>
<td>anionic</td>
<td>diffuse cytoplasmic&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>hydrophilic</td>
<td>cationic</td>
<td>nucleus&lt;sup&gt;e,f&lt;/sup&gt;</td>
</tr>
<tr>
<td>hydrophilic</td>
<td>anionic</td>
<td>diffuse cytoplasmic plus lysosomes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lysosomes&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Woodburn <i>et al.</i>, 1991; <sup>b</sup> Hornung <i>et al.</i>, 1997 (mTHPC); <sup>c</sup> Johnson <i>et al.</i>, 1981; <sup>d</sup> Modica-Napolitano <i>et al.</i>, 1990 (VB-BO); <sup>e</sup> Villanueva <i>et al.</i>, 1992 (meso-tetra(4N-methylpyridyl)porphine); <sup>f</sup> König <i>et al.</i>, 1996 (methylene blue); <sup>g</sup> Malik <i>et al.</i>, 1997 (TPPS<sub>4</sub>).

4.1.4. Fluorescence, type I and type II photochemical reactions

Light absorption excites the PS from the ground state to the first singlet state (Scheme 1). It can decay back to its ground state by emitting light (fluorescence) which is useful for tumour localisation as well as for subcellular localisation of the PS itself.

![Jablonski diagram](image)

Scheme 1. Jablonski diagram. Upon absorption of a photon, a PS in the ground state <i>S</i><sub>0</sub> is excited to <i>S</i><sub>1</sub> state. It may decay back to the ground state by emission of light (fluorescence) or undergo intersystem crossover to a triplet state <i>T</i><sub>1</sub> from where light can be emitted in the form of phosphorescence.

<i>vr</i>, vibrational relaxation; <i>isc</i>, intersystem crossover, <i>S</i><sub>0</sub> ground state; <i>S</i><sub>1</sub> first excited singlet state; <i>T</i><sub>1</sub>, first excited triplet state.
Intersystem crossover of the singlet PS ($^1$PS) yields a triplet PS ($^3$PS), which can emit light (phosphorescence), undergo type I or type II photochemical reactions (Scheme 2).

Scheme 2. Summary of events following absorption of light by a PS. The excited PS may emit fluorescence or undergo type I or type II photochemical reactions. For details, see text.

Type I photochemical reactions are characterised by electron or hydrogen atom transfer from a substrate, e.g., an unsaturated lipid (LH), to the $^3$PS (Girotti, 1990)

$$^3$PS + LH → PS$^*$ + L$^*$ + H$^+$

The radical L$^*$ may then react with O$_2$

$$L^* + O_2 → LOO^*$$

In the presence of sufficient amounts of O$_2$ and LH and in the absence of antioxidants, a chain reaction will occur resulting in the formation of lipid hydroperoxides (LOOH). The PS$^*$ radical can also react with O$_2$ forming superoxide anion (O$_2^-$)

$$PS^* + O_2 → PS + O_2^*$$

Dismutation of O$_2^*$ leads to the formation of hydrogen peroxide (H$_2$O$_2$) and in the presence of a Fenton reagent (e.g., Fe$^{2+}$) also to hydroxyl radical (OH$^*$).
In type II photochemical reactions the $^3\text{PS}$ transfers its energy to $^3\text{O}_2$

\[
^3\text{PS} + ^3\text{O}_2 \rightarrow ^1\text{PS} + ^1\text{O}_2
\]

\[
^1\text{O}_2 + \text{LH} \rightarrow \text{LOOH}
\]

To distinguish between type I and type II photochemical reactions, scavengers or traps of reactive intermediates of these reactions can be used (Girotti, 1990). An overview is given in Table 3. However, many of these scavengers lack absolute specificity, e.g., $^1\text{O}_2$ interceptors are also OH$^+$ traps. It should also be considered that for topological reasons a hydrophilic OH$^+$ trap may be inefficient against OH$^+$ generated in membranes.

Table 3. Scavengers of intermediates of type I and type II photochemical reactions. A decrease in oxidative damage (or increase with D$_2$O) in the presence of a scavenger indicates whether type I or type II photochemical reactions occurred (Girotti, 1990).

<table>
<thead>
<tr>
<th>Type</th>
<th>Reactive Intermediate</th>
<th>Scavenger</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>$^1\text{O}_2$</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>I</td>
<td>$^1\text{O}_2$</td>
<td>catalase</td>
</tr>
<tr>
<td>I</td>
<td>OH$^+$</td>
<td>iron chelators (e.g., EDTA) inhibit the formation</td>
</tr>
<tr>
<td>I</td>
<td>OH$^+$</td>
<td>mannitol, deoxyribose, butylated hydroxytoluene, $\alpha$-tocopherol</td>
</tr>
<tr>
<td>II</td>
<td>$^1\text{O}_2$</td>
<td>azide, dimethylfuran, histidine, $\beta$-carotene (no known enzymatic scavenger)</td>
</tr>
<tr>
<td>II</td>
<td>$^1\text{O}_2$</td>
<td>D$_2$O increases $^1\text{O}_2$ lifetime and accelerates reactions where $^1\text{O}_2$ is an intermediate</td>
</tr>
</tbody>
</table>
4.1.5. Oxidative damage

The formation of reactive (oxygen) species in type I and type II photochemical reactions results in oxidative damage of proteins, lipids and DNA (Scheme 2). Since the reactive oxygen species thus generated have a short lifetime and diffusion distance - singlet oxygen is assumed to diffuse less than 0.07 \( \mu \text{m} \) in cells before it reacts (Moan, 1990) - the localisation of the PS determines the topology of damage.

In order not to introduce further mutations in the DNA, PS are preferred that do not localise in the nucleus. However, many lipophilic PS also accumulate in the nuclear membrane and may cause oxidations in DNA close to the membrane. DNA damage by PDT using HpD or methylene blue but not mTHPC was detected by means of a comet assay (McNair et al., 1997). In vitro, cationic porphyrins are known to have a high affinity to DNA and induce oxidations and strand breaks when irradiated (Nicotera et al., 1994; Mettath et al., 1999). When cells are treated with either PDT or x-rays with a dose that induces equal cell killing, x-rays are more mutagenic (Evans et al., 1997; Paardekooper et al., 1997).

Oxidative modifications of proteins can be assessed by measuring the content of carbonyl groups. Carbonyl groups are reduced to the corresponding alcohol by NaB₃H₄, and a stable radioactive label is introduced into the protein. Oxidative modifications in lipids can be either aldehydes (thiobarbituric acid-reactive substances, TBARS) or LOOH. In order to determine whether type I or type II photochemical reactions have occurred, assays for TBARS and LOOH should be performed. TBARS are end-products of free radical peroxidations and should, theoretically, not be generated in pure type II reactions. LOOH as determined by a iodometric assay may or may not be end-products of photochemical reactions depending on the conditions, e. g., when ferric iron is removed by chelation or reductants are absent, LOOH will accumulate. In summary, in a type I reaction TBARS, and in a type II reaction accumulation of LOOH should be observed (Girotti, 1990).

Apart from being probes for mechanistical studies, antioxidants can reduce the oxidative damage induced by PDT. They protect red blood cells from aggregating after PDT for virus inactivation (Ben-Hur et al., 1997), and ascorbate protects coagulation.
factors from oxidation during photodynamic virus inactivation treatment of plasma (Parkkinen et al., 1996).

Upon PDT, enzymes like glutathione peroxidase, superoxide dismutase or catalase can be induced in cells (Hadjur et al., 1996), and may possibly lead to a resistance to PDT.

### 4.1.6. Direct and indirect cell killing

The major events that finally lead to cell death as a consequence of PDT are not fully understood, and may depend on the PS and protocol used.

*In vitro*, direct cell killing by oxidative damage is observed. Following PDT with lipophilic PS, damage to membrane-associated receptors and transport systems, and disturbance of membrane integrity and fluidity has been reported (Girotti, 1990). Research has focused on damage to mitochondrial enzymes, since lipophilic porphyrin PS preferentially localise in mitochondria (see below). Hydrophilic PS localising in lysosomes may cause disruption of these organelles resulting in the release of hydrolytic enzymes into the cytoplasm and ensuing cell death (Brunk et al., 1997). As discussed above, damage to DNA is believed not to be significant in PDT-induced cell death.

*In vivo*, however, indirect killing due to vascular damage is assumed to play a major role (for review, see Fingar, 1996). Vascular occlusion can be induced rapidly upon irradiation of tissue shortly after drug injection when levels of circulating PS are high. Within the first minute of irradiation, a decreased oxygen pressure was detected in murine radiation induced fibrosarcomas, which was reversible if irradiation was interrupted (Sitnik et al., 1998). Five minutes after photofrin-mediated PDT, the percentage of tumour vascular perfusion was decreased to less than 30%, inducing hypoxia in tumours (van Geel et al., 1996a). One to 4 h after PDT, reduction of partial pressure of oxygen is observed in tumours (Reed et al., 1989). In hypoxia, cells are protected from further PDT damage by the oxygen limitation of the photodynamic processes. Protocols with fractionated illumination have been developed (van Geel et al., 1996b; Müller et al., 1998) in order to address this problem. Depending on the duration of vascular occlusion, ischemic death and ischemia-reperfusion injury may
take place (Cruse-Sawyer et al., 1998), followed also by inflammatory reactions and phagocytosis of tumour cells (Krosl et al., 1995; Korbelik, 1996).

4.1.7. Resistance to or due to PDT

One major advantage of PDT over other treatment modalities is that it can be safely repeated several times, generally without inducing resistance to PDT in contrast to chemotherapy. Radiation-induced fibrosarcoma cells were subjected to 8 cycles of photofrin-mediated PDT, which resulted in only 1.8-fold resistance (Singh et al., 1991). The PDT-resistant cells were found to produce more ATP, have higher succinate dehydrogenase activity, and changes in mitochondrial structure compared to cells that were not PDT-treated (Sharkey et al., 1993). Cross-resistance to cis-diammine-dichloroplatinum (II) (1.6-fold) but not to adriamycin was reported (Moorehead et al., 1994). Chinese hamster ovary multi-drug resistant cells were also resistant to PDT possibly because they accumulated less PS (Singh et al., 1991). When cells were exposed to oxidative stress induced by PDT and subsequently incubated with adriamycin, a time-dependent decrease in adriamycin toxicity, correlated with ATP depletion and cell cycle changes, was observed (Fisher et al., 1993). A study with mTHPC-mediated PDT did not induce resistance to chemotherapy, radiotherapy or PDT in MCF-7 cells, indicating that PDT can be combined with other treatment modalities (Hormung et al., 1998).

4.1.8. Mitochondria in photodynamic therapy

Most studies on PDT effects on mitochondria were done by Salet and Moreno using HpD or photofrin as PS (for review, see Salet and Moreno, 1990). By fluorescence microscopy it was shown that porphyrins accumulate in mitochondria (Schneekenburger and Wustrow, 1988) after 24 h incubation time. Microirradiation of mitochondria but not of the nucleus or hyaloplasm leads to the death of cells incubated with HpD (Moreno and Salet, 1985). When rats were injected with haematoporphyrin,
the PS accumulated in mitochondria of hepatocytes (Cozzani et al., 1981). Also when the endogenous synthesis of porphyrins is stimulated by the addition of ALA, mitochondria are the prime target (Peng et al., 1997).

Several mechanisms may account for accumulation of PS in mitochondria. One major determinant is the partition coefficient: only lipophilic compounds are taken up by mitochondria, hydrophilic ones are localised in lysosomes (Woodburn et al., 1991). There is also evidence that porphyrins bind to benzodiazepine receptors located on mitochondrial outer membrane (Verma et al., 1987; Verma et al., 1998). Positively charged lipophilic compounds are taken up into mitochondria in response to \( \Delta \Psi \) (Johnson et al., 1981).

4.1.8.1. Studies on cells

Upon irradiation, ultrastructural changes in mitochondria are observed: the organelles appear swollen with a loss of cristae (Modica-Napolitano et al., 1996; Cernay and Zimmermann, 1996). In cells resistant to photofrin-mediated PDT, morphological changes in mitochondria were detected (Sharkey et al., 1993). When cells were photosensitised with HpD and irradiated, a decrease in cellular ATP levels paralleled by loss of viability took place (Hilf et al., 1986). To identify the site of damage, iodoacetate, an inhibitor of glycolysis, or oligomycin, an inhibitor of the mitochondrial ATPase, were added during the experiments. Only iodoacetate had an additive effect with PDT on ATP levels, indicating that haematoporphyrin and light impaired mitochondrial oxidative phosphorylation (Hilf et al., 1986). Inhibition of enzymes in cells of a R3230AC mammary carcinoma incubated with photofrin II\(^\circ\) and illuminated was determined (Gibson et al., 1989). Pyruvate kinase, a cytosolic enzyme was not inhibited, in contrast to mitochondrial enzymes. The following order of inhibition was found: cytochrome c oxidase > F\(_{0}\)F\(_{1}\) ATPase > succinate dehydrogenase > NADH dehydrogenase. Similar inhibition was found with HpD as PS (Hilf et al., 1984). Merocyanine 540, a compound that is preactivated by light before administration, also affects mitochondrial ultrastructure, Rh123 uptake and oxygen consumption in MCF-7 cells (Gulliya et al., 1995). When a porphyrin PS is coupled to boron atoms, boron neutron capture therapy can be combined with PDT. In vitro and in vivo, such a boronated porphyrin compound was localised in mitochondria of glioma cells (Hill et al., 1992). Only functional mitochondria are a target of this compound,
since it was neither cytotoxic nor phototoxic in cells lacking mitochondrial DNA (Munday et al., 1996).

Earlier studies revealed the high sensitivity of carcinoma cells compared to normal cells to lipophilic cationic compounds, e.g. Rh123 (Lampidis et al., 1983). This is a consequence of elevated mitochondrial and plasma membrane potentials of carcinoma cells (Davis et al., 1985) that progressively accumulate dyes like Rh123 in contrast to, e.g., epithelial-derived cell lines, that equilibrate with the extracellular dye (Nadakavukaren et al., 1985). Many rhodamine and cyanine dyes have been evaluated, the most effective of which was a cyanine with delocalised positive charge (Oseroff et al., 1986). The rhodacyanine MKT-077 kills carcinoma cell lines but not an epithelial cell line. It prolonged survival of nude mice bearing melanoma and reduced growth of carcinomas (Koya et al., 1996). Irradiation enhances the mitochondrial toxicity of MKT-077 (Modica-Napolitano et al., 1998). When injected into rats, MKT-077 causes reversible impairment of mitochondrial respiration (Weisberg et al., 1996). But not all cationic lipophilic compounds localise in mitochondria. When the negative charge is not delocalised as in a monocationic porphyrin, the PS does not enter the cells but accumulates at the surface membrane, and in vivo mostly shows vascular damage rather than direct tumour cell kill (Kessel et al., 1995).

4.1.8.2. Studies on isolated mitochondria

Reduction of mitochondrial functions following PDT of isolated mitochondria has been extensively studied. Not all functions are equally sensitive, the following order of events with increasing deuteroporphyrin concentration or light dose was found: uncoupling, dissipation of ΔΨ, inhibition of respiration, swelling and disruption of mitochondria (Sandberg and Romslo, 1980). Inner mitochondrial membrane enzymes are more sensitive to PDT damage than enzymes of the outer membrane, matrix or intermembrane space (Salet and Moreno, 1990). Damage to the adenine nucleotide translocator is the main cause of oxidative phosphorylation impairment when mitochondria are exposed to porphyrins and light probably due to oxidation of its thiol groups (Atlante et al., 1989). The mitochondrial permeability transition pore can also be a target of haematoporphyrin-mediated PDT (Salet et al., 1997).

Cationic PS may show effects on mitochondria even in the absence of light. Cyanines inhibited coupled respiration with β-hydroxybutyrate as substrate in a
concentration-dependent manner, but had no effect on uncoupled mitochondria (Conover and Schneider, 1981). When isolated mitochondria were incubated with Rh123 in the dark, the respiratory control ratio, ADP/ATP exchange and phosphate uptake were diminished. These effects were aggravated by irradiation (Atlante et al., 1992).

Unexpectedly, also a hydrophilic anionic porphyrin induced lipid peroxidation and inactivation of succinate dehydrogenase in isolated mitochondria (Chatterjee et al., 1997a). Relaxation of supercoiled plasmid DNA was also observed (Chatterjee et al., 1997b). It is unclear whether similar effects on mitochondria could be observed when cells are incubated with this compound, since localisation in mitochondria is unexpected.
4.2. **Apoptosis**

4.2.1. **Apoptotic cascade**

Apoptosis, also termed programmed cell death, is a highly regulated cascade of events, comprising, e.g., release of cytochrome c from mitochondria, subsequent activation of caspases, loss in ΔΨ, membrane blebbing, nuclear condensation and DNA cleavage. Morphological and biochemical changes during apoptosis lead to cell degradation, different from necrosis where a decay of the plasma membrane takes place (Fig. 2). Apoptosis is critical for development and tissue homoeostasis, and mutations in one or several proteins participating in its regulation or execution may lead to oncogenesis (Evan and Littlewood, 1998).

![Diagram of apoptotic and necrotic cell death](image)

**Fig. 2.** Apoptotic and necrotic cell death (modified from Darzynkiewicz et al., 1997). In apoptosis, morphological changes such as cell shrinkage, chromatin condensation, DNA fragmentation, and formation of apoptotic bodies take place. In necrosis, the cell and mitochondria swell and the plasma membrane ruptures.

The apoptotic pathway can be divided into three steps: induction, execution and degradation. Various stimuli, e.g., UV radiation, staurosporine or serum deprivation,
can induce apoptosis starting at different sites in the cells, but ending in a common pathway that leads to controlled degradation of the cell (for review, see Wilson, 1998).

4.2.2. Caspases

Caspases are cysteine proteases with a requirement for cleavage after aspartic acid and are involved in many apoptotic pathways (for reviews, see Núñez et al., 1998; Thornberry and Lazebnik, 1998). They are expressed as proenzymes, activated by proteolysis, and contribute to apoptosis through direct disassembly of cellular structures. It is believed that a proapoptotic signal activates an initiator caspase which, in turn, activates effector caspases, resulting in cellular disassembly. Thus, the initial signal is amplified in a cascade reaction. Different initiator caspases may mediate distinct sets of signals that finally elicit the same biochemical and morphological changes.

4.2.3. Mitochondria and apoptosis

Mitochondria play a central role in the execution of many forms of apoptosis and in the initiation of the apoptotic cascade by releasing caspase activating compounds (for review, see Green and Reed, 1998). During apoptosis, cytochrome c, a component of the respiratory chain, is released from mitochondria; this is inhibited by the anti-apoptotic protein Bcl-2 and enhanced by the pro-apoptotic protein Bax (see paragraph 4.2.4). Cytosolic cytochrome c combines with Apaf-1 (apoptotic protease-activating factor) to activate caspase-9 (Li et al., 1997). In neuronal cell culture models, caspase-9 resides inside mitochondria and is translocated to the nucleus upon addition of apoptosis-inducing agents (Krajewski et al., 1999). When the release of cytochrome c does not trigger apoptosis, e. g., in cells with mutated caspases, cells may die necrotically as a result of impaired electron transport leading to generation of oxygen radicals and decreased production of ATP. However, cells are also capable of de novo
cytochrome c synthesis and recovery when the apoptosis-inducing stimulus, e. g., growth factor deprivation, is no longer present (Martinou et al., 1999).

During apoptosis, reactive oxygen species are formed in mitochondria and $\Delta \Psi$ decreases. Initially, these were thought to be early apoptotic steps (Zamzami et al., 1995), but later it became evident that formation of reactive oxygen species is a consequence of cytochrome c loss rather than a cause for apoptosis (Cai and Jones, 1998).

### 4.2.4. Bcl-2 protein family

Bcl-2 and Bax are proteins of the same structural family, the former being anti- and the latter pro-apoptotic. The balance between pro- and anti-apoptotic proteins is believed to determine whether a cell will undergo apoptosis, since they can form hetero- and homo-dimers. All members of that family contain at least one of the four conserved motifs known as Bcl-2 homology (BH) domains essential for dimerization (for review, see Adams and Cory, 1998).

Bcl-2 has been found on the mitochondrial outer membrane, endoplasmic reticulum and nuclear membrane (Lithgow et al., 1994). Since Bcl-2 has some apparent antioxidative effects, it was initially believed to inhibit apoptosis by acting as an antioxidant (Hockenberry et al., 1993). In a cell-free system Bcl-2 prevents release of cytochrome c from mitochondria, and thus activation of caspases (Kluck et al., 1997). Caspase-3 cleaves Bcl-2, resulting in a positive feedback loop and further caspase activation (Kirsch et al., 1999).

Bcl-2, Bcl-xL and Bax are capable of forming ion channels in membranes and may thus regulate their permeability (Schendel et al., 1997; Minn et al., 1997; Antonsson et al., 1997). Bcl-2 also prevents $\Delta \Psi$ loss induced by various reagents, e. g., Ca$^{2+}$ and hydrogen peroxide, in isolated mitochondria. In this in vitro model, Bcl-2 maintained $\Delta \Psi$ by enhancing proton efflux in the presence of stimuli inducing $\Delta \Psi$ loss (Shimizu et al., 1998). However, this does not seem to be the only function of Bcl-2, since it also retards the cell cycle (O'Reilly et al., 1996) and maintains Ca$^{2+}$ homeostasis in the endoplasmic reticulum (He et al., 1997). Bax, in contrast to Bcl-2, induces
cytochrome c release from mitochondria (Manon et al., 1997; Jurgensmeier et al., 1998; Eskes et al., 1998). It has recently been proposed that pro-apoptotic proteins such as Bax accelerate opening and anti-apoptotic ones such as Bcl-x₁ close the mitochondrial voltage-dependent anion channel and thus regulate ΔѰ and the release of cytochrome c (Shimizu et al., 1999).

4.2.5. PDT and apoptosis

Upon incubation of cells with various PS and irradiation, apoptotic responses are observed, e.g., cytochrome c release into the cytosol (Varnes et al., 1999), caspase activity and poly(ADP-ribose) polymerase cleavage (He et al., 1998), DNA laddering and fragmentation of nuclei (Luo et al., 1996). Incubation of cells with a PS and irradiation can induce either necrotic or apoptotic cell death, depending on the cell line, PS and incubation protocol used.

Human prostate carcinoma cells (PC3) and rat mammary carcinoma cells (MTF7) showed an apoptotic response in contrast to human non-small cell lung carcinoma (H322a) when incubated with photofrin II® and illuminated (He et al., 1994). CV-1 cells incubated with photofrin and irradiated underwent apoptosis when incubated for 24 h with 1 μg/ml but not when incubated for 1 h with 10 μg/ml (Dellinger, 1996). P388 murine leukaemia cells showed DNA laddering when porphycene dimer or tin etiopurpurin were used as PS, but when dicationic chlorin or monocationic porphyrin were used, DNA was only cleaved to 50 kb particles (Luo et al., 1996).

The subcellular localisation of the PS seems to determine whether a cell will undergo apoptosis or not (Kessel et al., 1997). When mitochondria are the subcellular target, PDT induces apoptosis (Ball et al., 1998; Noodt et al., 1996). For lysosomal targeting contradictory results are reported (Brunk et al., 1997; Kessel and Luo, 1998). When the plasma membrane is a target, initial apoptosis can be delayed or stopped, because factors of the apoptotic cascade are lost from the cytosol (Kessel and Luo, 1998; Kessel et al., 1997).
5. AIMS OF THE STUDY

Mitochondria are critical subcellular targets of PDT. *In vitro* and *in vivo*, lipophilic PS, e. g., photofrin®, accumulate in mitochondria, and upon irradiation, structure and functions of this organelle are affected. Lipophilic cationic compounds, which are taken up into mitochondria in response to ΔΨ, are a promising class of PS for targeting carcinoma cells with elevated ΔΨ.

mTHPC is a second-generation PS undergoing clinical trials also at the University Hospital Zurich. Subcellular effects due to mTHPC-mediated PDT had not been studied, but there was evidence from microscopy studies that mTHPC also accumulates in mitochondria. In the first part of this thesis, the following questions were addressed:

- Does mTHPC accumulate in mitochondria?
- Are mitochondrial functions impaired upon incubation of isolated mitochondria with mTHPC and irradiation?
- Are mitochondrial functions also affected when cells are incubated with mTHPC and irradiated?
- Are there mechanisms other than damage to mitochondria that could elicit cell death upon mTHPC-mediated PDT?
- Which kind of photochemical reactions is mediated by mTHPC (type I or type II)?

In the second part of this thesis, cytotoxicity and phototoxicity of three, charged and uncharged, PS were compared in a pair of cell lines differing in their Bcl-2 content and ΔΨ to answer the questions:

- Are cytotoxicity and phototoxicity of a lipophilic cationic PS higher in cells with higher ΔΨ? Is this correlated with a higher PS uptake?
- Can the presence of the antiapoptotic protein Bcl-2 be used to induce higher cell kill, since Bcl-2 expression correlated with higher ΔΨ in these cells?
- Is apoptotic cell death elicited in cells after incubation with the PS and illumination? Does cytochrome c release from mitochondria take place?

In collaborations, other aspects of PDT, e. g. skin photosensitivity, were to be addressed.
6. MATERIALS AND METHODS

6.1. Materials

6.1.1. Chemicals

mTHPC was from Scotia Pharmaceuticals Ltd. (Guildford, UK), TPPS₄ from Porphyrin Products (Logan, UT, USA), and VB-BO from Aldrich (Buchs, Switzerland). mTHPC and VB-BO were dissolved in ethanol, TPPS₄ in water. ALA was obtained from ASAT AG (Zug, Switzerland). Hyperiforce comp. from Bioforce AG (Roggwil, Switzerland). A2E was prepared by C. Richter according to Parish et al. (1998). Annexin V-FLUOS staining kit was from Boehringer Mannheim (Rotkreuz, Switzerland), and nitrate/nitrite fluorometric assay kit from Cayman Chemical (Alexis, Läufelfingen, Switzerland). Dihydrorhodamine 123 and fluo-3 AM were purchased from Molecular Probes Europe (Leiden, Netherlands).

Dulbecco’s modified eagle’s medium, OptiMEM, RPMI 1640, Hank’s buffered salt solution, foetal calf serum (FCS), penicillin, streptomycin, and trypsin/EDTA were from Gibco (Basel, Switzerland), Ham’s F12 medium without phenol red from BioConcept (Basel, Switzerland). G-418, MTT and other chemicals were from Fluka (Buchs, Switzerland). Mouse monoclonal antibody clone 7H8.2C12 recognizing murine cytochrome c was purchased from RDI (Research Diagnostics, Flanders, NJ, USA), and anti-mouse Bcl-2 rabbit polyclonal antiserum from Upstate Biotechnology (Lake Placid, NY, USA).

C2 and C6 ceramide were obtained from Alexis Biochemicals (Läufelfingen, Switzerland) and DHC from Calbiochem (Luzern, Switzerland). Ceramide stock solutions were prepared in ethanol containing 1% DMSO. Daylong® 16 sunscreen and Excipial® light and dark covercreams were from Spirig AG (Egerkingen, Switzerland). Other protective substances tested were made at Kantonsapotheke (Zürich, Switzerland).

Other chemicals were purchased from standard suppliers (Fluka and Sigma, Buchs, Switzerland).
6.1.2. Cell lines

The MCF-7 cell line is derived from a human breast cancer and has a doubling time of 1.3 days. MCF-7 cells were grown in OptiMEM supplemented with 20 % FCS, 25 IU/ml penicillin, and 25 mg/ml streptomycin.

Mouse fibrosarcoma L929 cells were transfected with a vector alone (BV3) or containing the human bcl-2 gene (B22; Hennet et al., 1993). They were grown in Dulbecco’s modified eagle’s medium containing 10 % FCS, 25 IU/ml penicillin, 25 mg/ml streptomycin, and 0.5 mg/ml G-418 in every third passage. Overexpression of Bcl-2 in B22 cells was confirmed by Western blot analysis.

Small cell lung cancer cells (N417) overexpressing Bcl-2 in different levels (control, Bcl-2*2, Bcl-2*3) were obtained from Dr. U. Zangemeister-Wittke. They were grown in RPMI 1640 supplemented with 10 % FCS, 4 mM L-glutamine, 25 IU/ml penicillin, 25 mg/ml streptomycin, and 0.8 mg/ml G-418 in every second or third passage.

HaCaT cells, a human keratinocyte cell line, were grown in RPMI 1640 medium enriched with 10 % FCS, 4 mM L-glutamine, 25 IU/ml penicillin, and 25 mg/ml streptomycin.
6.2. Methods

6.2.1. Isolation of mitochondria

Female Wistar rats (200 - 250 g body weight), which had been fasted over night, were killed by decapitation. Their livers were quickly removed and washed in 30 ml of ice-cold 210 mM mannitol, 70 mM sucrose, 5 mM Hepes, 1 mM EDTA, pH 7.35 (buffer A). Fat and connective tissue were removed. The livers were cut into small pieces and homogenised in 60 ml of buffer A per liver using a glass homogeniser with a Teflon pestle. Mitochondria were isolated by differential centrifugation (Schlegel et al., 1992) as follows: All centrifugation steps were done at 4 °C. The homogenate was centrifuged for 10 min at 1’000 g (2’900 rpm) in a Sorvall centrifuge model RC-5B with an SS-34 rotor. The supernatant was centrifuged for 10 min at 10’000 g (10’000 rpm). Fat floating on top of the supernatant was removed with Linsoft tissues, and the supernatant was poured off. The mitochondrial pellet of each liver was resuspended in 80 ml of buffer B (buffer A without EDTA) using a reagent tube filled with ice. The suspension was centrifuged for 5 min at 1’000 g, and the resulting supernatant was centrifuged for 10 min at 10’000 g. The pellet was resuspended in buffer B, and mitochondria from two tubes were combined and put into one tube. Mitochondria were centrifuged again for 10 min at 10’000 g. The final pellet was suspended in buffer B to give a protein concentration of 80 - 120 mg/ml. Mitochondria were stored on ice. The protein concentration was determined by the biuret method with bovine serum albumin (BSA) as standard (Schlegel et al., 1992).

6.2.2. Incubation of mitochondria with mTHPC and irradiation

Mitochondria were suspended in 210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.35 at 4 °C (buffer B) or in 180 mM KCl, 10 mM tris-(hydroxymethyl)aminomethane, pH 7.35 at 4 °C (buffer C) at a concentration of 2 mg of protein/ml. Portions of 5 ml of suspension were placed in Petri dishes of 60 mm in diameter. mTHPC was added to final concentrations of 0, 5, 15 and 50 μg/mg mitochondrial
protein. Mitochondria were incubated for 1 h at 4 °C in the dark. They were subsequently irradiated under continuous stirring for 5 min at 652 nm with a power of 50 mW (5.3 J/cm²) using a diode laser (Applied Optronics Corp., South Plainfield, NJ). Samples for protein oxidation and lipid peroxidation measurements were frozen and stored at -80 °C. All samples were protected from light before and after irradiation.

6.2.3. Determination of protein oxidation and lipid peroxidation

6.2.3.1. Protein oxidation

Protein oxidation was determined with NaB³H₄ as described (Levine et al., 1990). Two hundred µl of mitochondrial suspension were incubated for 1 h at 37 °C with NaB³H₄ (dissolved in 0.1 M NaOH and stored at -80 °C), which reduces carbonyl groups to alcohols, thereby introducing a stable tritium label into the proteins. The reaction was stopped and the proteins were precipitated by the addition of 1 ml of cold 20 % trichloroacetic acid (w/v). The proteins were separated from the reaction mixture by filtration through glass microfibre filters (Whatman, Maidstone, UK). Unbound radioactivity was removed by thoroughly washing the filters with 10 ml of 10 % trichloroacetic acid. Radioactivity remaining on the filters was counted in a β liquid scintillation counter (Kontron Instruments). Quenching due to mTHPC was corrected for.

6.2.3.2. Thiobarbituric acid-reactive substances

Thiobarbituric acid-reactive substances (TBARS) were measured as described (Buege and Aust, 1978). Two hundred µl of mitochondrial suspension (2 mg/ml) were mixed with 1 ml of a solution containing 15 % trichloroacetic acid, 0.375 % thiobarbituric acid, 0.25 M HCl, 0.24 mM FeCl₃ and 50 µM butylated hydroxytoluene. The mixture was heated for 15 min in a boiling water bath, cooled on ice and centrifuged to remove the precipitate. The absorbance of the sample was determined at 535 nm (ε_chromophore=1.56*10⁵ M⁻¹ cm⁻¹) against a blank containing all the reagents minus the mitochondria.
6.2.3.3. Lipid hydroperoxides

Lipid hydroperoxides (LOOH) were determined by the iodometric assay as described (Buege and Aust, 1978). One ml of mitochondrial suspension (2 mg/ml) was mixed with 5 ml chloroform:methanol (2 vol:1 vol), followed by centrifugation at 1'000 g for 5 min to separate the phases. The lower layer (3 ml) was taken to dryness in a 45 °C water bath under a stream of nitrogen. One ml of acetic acid:chloroform (3:2) and 60 mg potassium iodide (0.05 ml) were then added. After mixing, the samples were placed in the dark for 5 min, followed by addition of 15 mg cadmium acetate (dissolved in 3 ml of H2O). The solution was mixed and centrifuged at 1'000 g for 10 min. The absorbance of the upper phase was determined at 353 nm against a blank containing the complete assay mixture minus the mitochondria. Unfortunately, the hydrophobic mTHPC was also extracted by the procedure described above. Since its interference in the following steps could not be prevented, only samples containing the same amount of mTHPC were compared.

6.2.4. Determination of mitochondrial oxygen consumption

Mitochondria (2 mg/ml) were incubated in buffer B under constant stirring at 25 °C in the presence of 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; 1.3 μl) in a closed chamber (1.25 ml) protected from light with aluminium foil. Mitochondrial respiration was measured with a Clark type oxygen electrode (Yellow Spring Instruments, Yellow Springs, OH), (Estabrook, 1967). Respiration dependent on complex I was induced by the addition of 2.4 mM K+-pyruvate and 1.25 mM K+-malate (5 μl) and inhibited by 5 μM rotenone (3.25 μl), respiration supported from complex II by the addition of 2.6 mM K+-succinate (6.5 μl), and respiration of complex IV by the addition of 0.5 μg antimycin A (2.5 μl; to block complex III) and 10 mM ascorbate/0.17 mM tetramethylphenylenediamine (Asc/TMPD; 21.5 μl), (Lee et al., 1967; Tyler and Gonze, 1967).
6.2.5. Determination of the mitochondrial membrane potential and of Ca\textsuperscript{2+} uptake

Mitochondria were incubated at 25 °C with continuous stirring and oxygenation at a protein content of 1 mg/ml in 2.4 ml of buffer B (pH 7.2 at 25 °C). The membrane potential (ΔΨ) was measured in the presence of 10 μM safranine T (Åkerman and Wikstroem, 1976) in an Aminco DW-2a spectrophotometer at 511 - 533 nm. The distance between the highest and the lowest absorption difference (ΔΔA) was determined after the addition of 5 μM rotenone, 2.5 mM K\textsuperscript{+}-succinate and 0.5 μM carbonyl cyanide-3-chlorophenylhydrazone (CCCP), respectively.

Ca\textsuperscript{2+} uptake and release was measured in the presence of 50 μM 3,6-bis[(2-arsenophenyl)azo]-4,5-dihydroxy-2,7-naphthalenc-disulfonic acid (arsenazo III). Ca\textsuperscript{2+} movements across the inner mitochondrial membrane were monitored in an Aminco DW-2a spectrophotometer at 675 - 685 nm (Lötscher et al., 1980; Schlegel et al., 1992). After addition of 5 μM rotenone, mitochondria were energized with 2.6 mM K\textsuperscript{+}-succinate and loaded with 40 nmol Ca\textsuperscript{2+}/mg of mitochondrial protein.

6.2.6. Extraction of mTHPC from mouse liver mitochondria

Nude mice were injected i. p. with 0.1, 0.15 or 0.3 mg mTHPC/kg body weight. After 48 h liver mitochondria were prepared as described above and stored at -80 °C. Extraction of mTHPC was performed in collaboration with Dr. W. Blodig (Institute of Biochemistry) as described (Whelpton et al., 1995) with minor modifications. The extraction solution consisted of MeOH:DMSO (4:1) to which H\textsubscript{2}O had been added at a ratio of MeOH:DMSO:H\textsubscript{2}O (40:1). Two hundred μl of mouse liver mitochondria were mixed with 400 μl of extraction solution and shaken. They were centrifuged at 14'000 rpm in an Eppendorf centrifuge for 2 min. Four hundred μl of the supernatant were mixed with 200 μl H\textsubscript{2}O. Two hundred μl were injected into the HPLC. mTHPC was detected by absorbance at 423 nm (reference 510 ± 10 nm). The mobile phase was acetonitrile:0.1% trifluoroacetic acid (77:23).
6.2.7. Confocal laser scanning microscopy

For confocal microscopy studies a Leica TCS 4D microscope (Leica, Glattbrugg, Switzerland) equipped with an ArKr laser was used.

For determination of subcellular localisation cells were grown on coverslips and incubated for 1 h with 1 μM VB-BO, for 24 h with 1 μM mTHPC or with 10 μM TPPS₄, or as indicated in the Figures. Dyes were exited at 568 nm and fluorescence above 590 nm was detected with a longpass filter.

When cells were doubly stained with dihydrorhodamine 123 (DHR) and mTHPC, excitation was at 488 nm, Rh123 fluorescence was detected with a FITC bandpass filter and mTHPC fluorescence above 590 nm with a longpass filter. There was no cross-talk between the dyes as confirmed by determination of dye fluorescence in a fluorimeter (Fig. 3).

For detection of propidium iodide (PI) and annexin V (AV) binding, cells were grown on 8 chamber microscope slides, incubated with 1 μM VB-BO for 1 h and illuminated for 6 min, with 10 μM TPPS₄ for 24 h and illuminated for 2 min or with 0.1 μM mTHPC for 24 h and illuminated for 1 min. Immediately or 30 min after illumination, cells were rinsed with HBSS and stained with AV and PI for 15 min as described by the manufacturer of the kit. Excitation was at 488 nm, emission of AV was detected with a FITC bandpass filter, emission of PI with a 590 nm longpass filter.

For estimating intracellular Ca²⁺ concentrations, cells were incubated with 4 μM fluo-3 AM for 30 min at 37 °C. Fluorescence was excited at 488 nm and detected with a FITC bandpass filter.
6.2.8. Fluorescence microscopy

For uptake studies, fluorescence of the dyes was detected using a Leitz DMRBE microscope (Leica, Glatirugg, Switzerland) equipped with a computer-controlled charge coupled device camera (Photometrics Ltd., Tucson, AZ, USA). For excitation a 530/595 nm bandpass filter, and for detection of emission a 615 nm longpass filter were used. Five micrographs per slide were taken, and 2 cells per picture were analysed. Experiments were repeated 5 times. Analysis of the pictures was done with IPLab Spectrum software, version 3.1a (Signal Analytics, Vienna, VA, USA). Uptake of positively charged dyes into cells can be determined by fluorescence microscopy and is a measurement of relative mitochondrial membrane potential (Johnson et al., 1980).

6.2.9. Irradiation of cells with laser light

Cells were irradiated using a diode laser (Applied Optronics Corp., South Plainfield, NJ, U. S. A.) which emitted light of 652 nm and was connected to a front lens light diffusor FDI (Medlight SA, Lausanne, Switzerland). The power being set to 25 mW, irradiation times of 15, 30, and 60 s corresponded to energy doses of 0.13, 0.26, and 0.53 J/cm², respectively. Power was measured with a Fieldmaster powermeter (Coherent Inc., Santa Clara, USA).

6.2.10. Illumination of cells with white light

Cells were illuminated with an Intralux® MDR 100 lamp (Volpi AG, Schlieren, Switzerland) containing a 100 W xenon lamp emitting at 400 - 780 nm. The light intensity was $4.1 \times 10^5$ lux corresponding to a power of 60.4 mW/cm² at 555 nm according to the manufacturer’s manual.
6.2.11. Colony formation assay

Per Petri dish 200 (MCF-7) or 300 (HaCaT) cells were seeded, and after 24 h the PS (mTHPC or as indicated) was added. After 24 h (effects of antioxidants) or 48 h (CF vs. oxygen consumption) incubation time the cells were irradiated. Subsequently, the medium was renewed, and cells were incubated for 7 or 8 days. They were then fixed with MeOH/acetic acid (3:1) and stained with Giemsa solution, and colonies (>50 cells) were counted.

6.2.12. MTT assay

Adherent cells (B22, BV3) were seeded into 96 well plates 24 h before addition of the PS in a volume of 200 μl. After incubation in the dark or 24 h after illumination, medium was replaced by fresh medium containing 0.5 mg MTT/ml, and cells were incubated for 3 h at 37 °C (Mosmann, 1983). Medium was replaced by DMSO and absorbance was read in an ELISA reader at 540 nm (Twentyman and Luscombe, 1987). The surviving fraction was calculated using a standard curve of untreated cells present on the same ELISA plate. Each experiment was repeated 3 to 5 times. Duplicates (illuminated cells) or triplicates of each experiment (standard curve, cells incubated in the dark) were obtained.

Suspension cells (N417) were seeded into 96 well plates 24 h before addition of the PS in a volume of 100 μl. After incubation in the dark or 24 h after illumination, 10 μl of MTT (5 mg/ml, final concentration 0.45 mg/ml) were added and cells were incubated for 3 h at 37 °C. One hundred μl lysing solution (250 ml water, 250 ml dimethylformamide, pH 4.7 adjusted with 80 % acetic acid/20 % 1 M HCl, 100 g SDS) was added. Lysis of cells took place at 37 °C overnight and absorbance was determined at 540 nm.
6.2.13. Determination of cellular oxygen consumption

About 10^6 MCF-7 cells were seeded per culture flask. Incubation with mTHPC and irradiation was performed as described above. After irradiation, the cells were trypsinised and counted. Oxygen consumption of 2x10^6 cells in their pyruvate-containing medium was measured with a Clark-type oxygen electrode at 37 °C under continuous stirring.

6.2.14. Western blot analysis

For the detection of cytochrome c release, cells grown in Petri dishes (10 cm diameter) were incubated with 1 µM VB-BO for 1 h, 0.1 µM mTHPC or 10 µM TPPS₄ for 24 h, and subsequently illuminated for 8, 2 or 5 min, respectively. One h later cells were trypsinized, and subcellular fractions were obtained as described (Liu et al., 1996) with minor modifications. Cells were resuspended in 200 µl of an extraction solution (20 mM K⁺-Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM K⁺-EDTA, 1 mM Na⁺-EGTA, 1 mM dithiothreitol, 250 mM sucrose, 0.1 mM PMSF, 5 µg/ml pepstatin and 2 µg/ml aprotinin). After sitting on ice for 15 min, they were disrupted by 20 strokes of a syringe through a 25 G needle. Suspensions were centrifuged in an Eppendorf centrifuge at 1000 g for 10 min at 4 °C. The pellet contained the nuclear fraction and was resuspended in the extraction solution. The supernatant was centrifuged at 10'000 g for 15 min. The resulting pellet contained the mitochondria and was resuspended in the extraction solution. The supernatant was centrifuged in a Beckman TL-100 ultracentrifuge at 100'000 g for 30 min. The pellet was discarded, the supernatant contained the cytosolic proteins. All samples were stored at -80 °C. The following steps were performed by S. Rocha (Department of Radiation Oncology, University Hospital Zürich): Proteins from cytosolic fractions (150 µg) were precipitated with trichloroacetic acid, resolved by SDS-polyacrylamide gel electrophoresis (15 % SDS) and blotted onto PVDF membranes. Antibody detection was achieved by ECL-enhanced chemiluminescence (Amersham, Dübendorf, Switzerland) using a horseradish peroxidase-conjugated secondary antibody, according to the manufacturer's protocol.
6.2.15. Nitrate and nitrite determination

For the detection of nitrate and nitrite in the supernatant of the cells, 5000 MCF-7 cells per well were seeded in a 96 well plate. After 24 h the OptiMEM was replaced by Ham’s F-12 medium, and 0.1 μg mTHPC/ml was added. Ham’s F-12 medium contains neither phenol red nor nitrate and nitrite that would disturb the assay. After 24 h incubation time, cells were washed with HBSS, then kept in 40 μl of Ham’s F-12 medium, and irradiated during 30 s with 25 mW (0.26 J/cm²) laser light of 652 nm wavelength. At the indicated times, 30 μl of the supernatant were taken and stored at -20 °C until analysis.

For the detection of nitrate and nitrite in the cytoplasm of the cells, 250’000 MCF-7 cells were seeded per Petri dish (diameter 6 cm). After 24 h 0.1 μg mTHPC/ml was added. After 24 h incubation time, cells were irradiated during 30 s with 25 mW (0.26 J/cm²) laser light of 652 nm wavelength. At the indicated times, the cells were rinsed with cold Ham’s F-12, scraped off, and centrifuged. The supernatant was discarded, and 50 μl of a lysis solution containing 0.1 M NaCl, 0.01 M Tris, 1 mM EDTA, 4 % SDS, 1 μg/ml aprotinin, 100 μg/ml PMSF, and 1 μg/ml pepstatin, was added. The cells were resuspended, vortexed, and centrifuged. The supernatant was kept at 20 °C until analysis.

Twenty μl of the supernatant or 10 μl of the cell extract were incubated with nitrate reductase and enzyme co-factors for 2 h according to the protocol of the kit. 2,3-diaminonphthalene was added, and after 10 min the reaction was stopped by the addition of NaOH. Fluorescence was excited at 365 nm, and emission at 405 or 408 nm was recorded (samples from supernatant or cell extract, respectively).

6.2.16. Statistical analysis

Statistical analysis of experiments with isolated mitochondria or MCF-7 cells (CF, oxygen consumption) was performed using a Student’s t-test. Data are expressed as means ± standard errors (SE) of 3 to 4 experiments. Statistical significance is indicated as: *, 0.05 ≥ P > 0.01; **, 0.01 ≥ P > 0.001; ***, 0.001 ≥ P.
Statistical analysis of experiments with B22 and BV3 cells (dye uptake, survival) was done using a Wilcoxon Mann Whitney test, $\alpha = 0.05$. Data are expressed as means ± SE of 3 to 5 experiments.
7. RESULTS

7.1. Photosensitisation of isolated mitochondria by mTHPC

7.1.1. Protein oxidation and lipid peroxidation

Oxidative modifications in proteins and lipids of isolated rat liver mitochondria incubated with increasing concentrations of mTHPC and irradiated with laser light were assessed by measuring the amount of carbonyl groups and TBARS, respectively. Neither incubation with mTHPC nor irradiation alone affected these parameters, but when applied in combination an increase in carbonyl groups (Fig. 4) as well as TBARS (Fig. 5) was found. The extent of oxidative modifications depended on the concentration of mTHPC present.

Fig. 4. Protein oxidation in rat liver mitochondria incubated in buffer B with increasing mTHPC concentrations and irradiated with laser light (5.3 J/cm²). The carbonyl content was determined by reduction of carbonyl groups with NaB₃H₄. Values are means ± SE from four experiments.
In order to investigate the mechanism - type I or type II - of mTHPC-mediated phototoxicity, the effects of antioxidants as well as D$_2$O on the formation of LOOH, TBARS and carbonyl groups were determined (Table 4). The hydroxyl radical trap mannitol had no effect on the oxidation of proteins and lipids, possibly because the reactive oxygen species were produced inside the membranes, to which mannitol has no access. In contrast, the lipophilic antioxidant butylated hydroxytoluene (BHT) decreased the amount of all oxidation products investigated. Preparing the buffer with D$_2$O instead of H$_2$O clearly, although statistically not significantly (due to the limitations of this assay as mentioned in Materials and methods), stimulated the formation of LOOH. Finally, the singlet oxygen scavenger dimethylfuran (DMF) diminished the amount of LOOH. Taken together, these results indicate a mechanism composed of type I as well as type II reactions.
Table 4. Effects of inhibitors of reactive oxygen species and effect of D₂O on the formation of lipid hydroperoxides (LOOH)\(^a\), thiobarbituric reactive substances (TBARS)\(^b\) and carbonyl groups\(^b\) induced by incubation of mitochondria in buffer C with 15 μg mTHPC/mg protein for 1 h and irradiation with 5.3 J/cm\(^2\). As indicated, 100 mM mannitol, 100 μM butylated hydroxytoluene (BHT) or 100 μM dimethylfuran (DMF) were present during the irradiation, or the incubation buffer (buffer C) was prepared using D₂O.

<table>
<thead>
<tr>
<th>LOOH</th>
<th>TBARS</th>
<th>carbonyl groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of control ± SE</td>
<td>% of control ± SE</td>
<td>% of control ± SE</td>
</tr>
<tr>
<td>control</td>
<td>100.0 ± 45.3</td>
<td>100.0 ± 11.7</td>
</tr>
<tr>
<td>100 mM mannitol</td>
<td>101.7 ± 35.9</td>
<td>101.9 ± 16.2</td>
</tr>
<tr>
<td>100 μM BHT</td>
<td>78.6 ± 35.7</td>
<td>35.8 ± 2.4</td>
</tr>
<tr>
<td>in D₂O</td>
<td>118.5 ± 56.3</td>
<td>91.3 ± 9.8</td>
</tr>
<tr>
<td>100 μM DMF</td>
<td>75.0 ± 16.5</td>
<td>103.3 ± 13.3</td>
</tr>
</tbody>
</table>

\(^a\) \(n=3\).
\(^b\) \(n=4\).

7.1.2. Decreased oxygen consumption

Table 5 shows the effects on respiration of incubation with different concentrations of mTHPC and irradiation. Oxygen consumption stimulated by 2.4 mM K⁺-pyruvate/1.25 mM K⁺-malate via complex I was increased at the lowest mTHPC concentration used, but decreased at higher concentrations. Oxygen consumption stimulated by 2.6 mM K⁺-succinate via complex II and 10 mM ascorbate/0.17 mM tetramethylphenylenediamine via complex IV decreased with raising mTHPC concentrations; complex II was more sensitive than complex IV. Addition of 0.8 μM CCCP, an uncoupler of mitochondria, showed little effect on oxygen consumption of mitochondria that had received mTHPC plus irradiation, obviously because they were already uncoupled. The ratio of the rate of oxygen consumption during (state 3) and after (state 4) ADP-stimulated respiration (respiratory control index) was also determined for complex II. Mitochondria were incubated in the presence of 1 mM KH₂PO₄. Addition of 75 nmol ADP did not result in an increased oxygen consumption in any of the photodynamically treated samples (Fig. 6, only the sample with lowest mTHPC concentration shown).
Table 5. Oxygen consumption supported from different respiratory complexes of mitochondria incubated with mTHPC and irradiated with laser light (5.3 J/cm²). For experimental details see Materials and Methods. n=4. Statistically significant differences (not irradiated vs. irradiated): *, 0.05 ≥ P > 0.01; **, 0.01 ≥ P > 0.001; ***, 0.001 ≥ P.

| µg mTHPC/ | not irradiated | irradiated | statistical significance |
| mg protein | % of control ± SE | % of control ± SE | |
| Complex I | | | |
| 0 | 100.0 ± 8.5 | 119.6 ± 15.9 | |
| 5 | 103.0 ± 14.3 | 160.8 ± 8.9 | * |
| 15 | 109.8 ± 12.8 | 112.8 ± 23.9 | |
| 50 | 103.6 ± 15.6 | 80.7 ± 7.0 | |
| Complex II | | | |
| 0 | 100.0 ± 7.3 | 101.7 ± 9.6 | |
| 5 | 86.3 ± 7.7 | 77.3 ± 9.6 | |
| 15 | 100.4 ± 11.2 | 32.8 ± 4.2 | *** |
| 50 | 94.6 ± 7.8 | 20.1 ± 1.8 | *** |
| Complex IV | | | |
| 0 | 100.0 ± 5.1 | 102.5 ± 2.5 | |
| 5 | 99.2 ± 7.1 | 94.9 ± 7.4 | |
| 15 | 94.3 ± 5.9 | 91.1 ± 4.7 | |
| 50 | 95.2 ± 4.5 | 53.3 ± 7.7 | ** |

Fig. 6. ADP-stimulated oxygen consumption in rat liver mitochondria. Mitochondria were incubated in buffer B at 25 °C in the presence of 1 mM EGTA, 5 µM rotenone, 2.6 mM K⁺-succinate, and 1 mM KH₂PO₄. At the solid arrows, 75 nmol ADP was added, and at the dashed arrows, 0.8 µM CCCP was added. (a) Untreated mitochondria. (b) Mitochondria irradiated during 5 min with 50 mW of light of 652 nm (5.3 J/cm²). (c) Mitochondria incubated with 5 µg mTHPC/mg protein for 1 h in the dark. (d) Mitochondria incubated as in (c) and irradiated as in (b). One experiment typical of four is shown.
7.1.3. Decreased mitochondrial membrane potential and Ca\textsuperscript{2+} uptake

Since ATP production in mitochondria depends on ΔΨ, we were interested in this parameter of membrane intactness. Fig. 7A and B show how ΔΨ is affected by PDT: irradiation in the presence of as little as 5 μg mTHPC/mg protein diminished the distance between the highest and the lowest absorption difference by about half, and irradiation in the presence of 15 μg mTHPC or more/mg protein completely prohibited build-up of ΔΨ. Both the rate and the total amount of Ca\textsuperscript{2+} taken up by mitochondria incubated with 5 μg mTHPC/mg protein and irradiated were clearly diminished as compared to controls (Fig. 8).
Fig. 7. Changes in the membrane potential of rat liver mitochondria incubated with mTHPC and irradiated with laser light. (A, a) Untreated mitochondria. (b) Mitochondria incubated with 5 μg mTHPC/mg protein for 1 h and irradiated during 5 min with 50 mW of light of 652 nm (5.3 J/cm²). For details see Materials and methods. One experiment typical of four is shown. (B) Distance between the highest and the lowest absorption difference (ΔΔA) after the addition of rotenone/K⁺-succinate and CCCP, respectively. Values are means ± SE from three (15 an 50 μg mTHPC/mg protein) or four (0 and 5 μg mTHPC/mg protein) experiments.

Fig. 8. Ca²⁺ uptake by mitochondria incubated with mTHPC and irradiated with laser light. At the arrows, 5 μM rotenone, 2.6 mM K⁺-succinate, and 40 nmol Ca²⁺/mg of mitochondrial protein was added. (a) Untreated mitochondria. (b) Mitochondria irradiated during 5 min with 50 mW of light of 652 nm (5.3 J/cm²). (c) Mitochondria incubated with 5 μg mTHPC/mg protein for 1 h in the dark. (d) Mitochondria incubated as in (c) and irradiated as in (b). One experiment typical of three is shown.
7.1.4. Accumulation of mTHPC in mouse liver mitochondria

To answer the question whether mTHPC would also in vivo accumulate in liver mitochondria, nude mice were injected i. p. with 0.15 or 0.3 mg mTHPC/kg body weight 48 h before preparation of liver mitochondria. mTHPC contents were determined by HPLC (Fig. 9). Retention time of pure mTHPC was 7.3 min, and of mTHPC extracted from mitochondria 8.0 min under these conditions. The peak shortly before 3 min in Fig. 9b may be a degradation product of mTHPC, since it is not present in pure mTHPC (a) or when mTHPC was added to isolated mitochondria immediately before extraction (d).

Fig. 9. Detection of mTHPC by HPLC. (a) 600 ng mTHPC. (b) mTHPC extracted from liver mitochondria of a mouse injected with 0.1 mg mTHPC/kg body weight 48 h before isolation of mitochondria. (c) Extract from liver mitochondria of a control mouse. (d) As (c), but before extraction 50 ng mTHPC were added. Arrows indicate mTHPC peak, dashed arrow indicates peak of possible degradation product.
In another set of experiments, mitochondria from 5 mice injected with 0, 0.15 or 0.3 mg mTHPC/kg body weight 48 h before isolation of mitochondria were obtained and analysed (Table 6). Unfortunately, the yield from the extraction procedure was low, and the HPLC peaks were too small to be quantitatively analysed. Therefore, this study was not continued.

Table 6. Extraction of mTHPC from mouse liver mitochondria. Mice were injected with 0, 0.15 or 0.3 mg mTHPC/kg body weight 48 h before isolation of mitochondria. For details of extraction procedure, see Materials and methods.

<table>
<thead>
<tr>
<th>mouse</th>
<th>mg mTHPC/kg body weight</th>
<th>calculated amount of mTHPC in liver mitochondria (ng mTHPC/mg mitochondrial protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.15</td>
<td>(peak area too small)</td>
</tr>
<tr>
<td>C</td>
<td>0.15</td>
<td>(peak area too small)</td>
</tr>
<tr>
<td>D</td>
<td>0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>E</td>
<td>0.3</td>
<td>0.55</td>
</tr>
</tbody>
</table>
7.2. Subcellular events after photosensitisation of MCF-7 cells by mTHPC and irradiation

7.2.1. Subcellular localisation of mTHPC

Incubation of MCF-7 cells for 6 h with mTHPC gave weak fluorescence in the cytoplasm (Fig. 10a) as determined by confocal laser scanning microscopy (CLSM). After 24 h the fluorescence became brighter and the nuclear membrane was distinctly stained (Fig. 10b). After incubation for 48 and 72 h membranous structures in the cytoplasm were more clearly seen (Fig. 10c and d). No fluorescence signals were

Fig. 10. mTHPC fluorescence in MCF-7 cells. MCF-7 cells were incubated with 1 μg mTHPC/ml for 6 h (a; 64 μm x 64 μm), 24 h (b; 64 μm x 64 μm), 48 h (c; 62 μm x 62 μm) or 72 h (d; 100 μm x 100 μm). Fluorescence was detected by CLSM, excitation was at 488 nm, emission was detected with a 590 nm longpass filter.
detected within the nucleus at any time during these experiments.

To investigate the possible localisation of mTHPC in mitochondria, MCF-7 cells were also stained with dihydrorhodamine 123 (DHR). The fluorescence patterns of rhodamine 123 (Rh123), the oxidation product of DHR, and mTHPC were not identical. Rh123 localised around the nucleus (Fig. 11a), but mTHPC also in the outer regions of the cells (Fig. 11b). Some of the structures stained with mTHPC were identified as mitochondria by simultaneous staining with Rh123 (Fig. 11c).

![Images](image.png)

Fig. 11. MCF-7 cells incubated with 1 μg mTHPC/ml for 68 h and with 3 μM Rh123 for 15 min. Green (Rh123; a) and red (mTHPC; b) fluorescence was simultaneously detected by CLSM on two channels. c: a and b superimposed.

In sperm cells mTHPC accumulated mainly in the midpiece where the mitochondria are located. Weak fluorescence was also detected in the cytoplasm of both head and tail (Fig. 12a and b).

![Images](image.png)

Fig. 12. Human sperm cells incubated with 1 μg mTHPC/ml for 0.5 (a) and 5 h (b). Fluorescence was detected by CLSM.
7.2.2. Colony formation

Incubation with mTHPC and irradiation with laser light diminished the CF of MCF-7 cells (Table 7). It decreased with increasing mTHPC concentrations and energy doses.

Table 7. Effect of incubation with mTHPC and irradiation with laser light on CF of MCF-7 cells. Data are expressed in % of the untreated control. *n = 3.* Statistically significant differences (vs. control): ***, 0.001 ≥ P.

<table>
<thead>
<tr>
<th>mTHPC (µg/ml)</th>
<th>irradiation dose (J/cm²)</th>
<th>CF ± SE</th>
<th>significance</th>
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<tr>
<td>0</td>
<td>0</td>
<td>100.0 ± 3.1</td>
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</tr>
<tr>
<td>0</td>
<td>0.13</td>
<td>97.2 ± 2.1</td>
<td></td>
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<tr>
<td>0</td>
<td>0.26</td>
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<tr>
<td>0</td>
<td>0.53</td>
<td>94.1 ± 4.1</td>
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<td>0.1</td>
<td>0</td>
<td>94.9 ± 5.0</td>
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<td>0.13</td>
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<tr>
<td>0.1</td>
<td>0.26</td>
<td>64.5 ± 3.5</td>
<td>***</td>
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<tr>
<td>0.1</td>
<td>0.53</td>
<td>6.2 ± 2.2</td>
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<td>0.13</td>
<td>34.9 ± 8.2</td>
<td>***</td>
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<tr>
<td>0.25</td>
<td>0.26</td>
<td>0.8 ± 0.4</td>
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<tr>
<td>0.25</td>
<td>0.53</td>
<td>0.1 ± 0.1</td>
<td>***</td>
</tr>
</tbody>
</table>
7.2.3. Decreased oxygen consumption

After incubation with 0.1 µg mTHPC/ml and irradiation during 30 s, the oxygen consumption of MCF-7 cells was decreased to 79 % in the normal medium (Fig. 13a), and to 92 % after addition of carbonyl cyanide-4-trifluoromethoxyphenylhydrazone (FCCP; Fig. 13b). Incubation with 0.25 µg mTHPC/ml and irradiation with 0.26 J/cm² resulted in a reduction of oxygen consumption to 69 % and 72 %, respectively.

Fig. 13. Oxygen consumption of MCF-7 cells after incubation with mTHPC for 48 h and irradiation with laser light during 30 s (0.26 J/cm²) expressed in % of the untreated cells. a: Basal oxygen consumption in the pyruvate-containing medium. b: Oxygen consumption after addition of 8 µM FCCP.

☐ not irradiated, ■ irradiated. Values are means ± SE of three experiments. *, 0.05 ≥ P > 0.01 (vs. control).
7.2.4. Decreased mitochondrial transmembrane potential

To determine whether also ΔΨ' was affected by incubation of MCF-7 cells with mTHPC and irradiation with laser light, cells were incubated with Rh123, that is taken up by mitochondria in response to ΔΨ', before or after irradiation.

When cells were incubated with 0.1 μg mTHPC/ml for 48 h and subsequently irradiated during 30 s at 25 mW (0.26 J/cm²), a diminished Rh123 fluorescence was seen after 6 h (not shown). When cells were incubated with 1 μg mTHPC/ml for 68 h and irradiated during 4 min at 25 mW (2.1 J/cm²), some cells clearly took up less Rh123 already 1 h after irradiation (Fig. 14a). Those cells and their nuclei appeared swollen and the plasma membranes disrupted, resembling rapid necrotic cell death (Fig. 14b).

Fig. 14. Rh123 uptake into MCF-7 cells after incubation with mTHPC and irradiation. MCF-7 cells were incubated with 1 μg mTHPC/ml for 68 h and irradiated during 4 min. 45 min later they were stained with 4 μM Rh123 for 15 min. Fluorescence was detected by CLSM on two channels. a: Rh123 fluorescence. b: mTHPC fluorescence. Picture size is 100 μm x 100 μm.
7.2.5. Effects of antioxidants

Addition of 100 mM mannitol, 100 μM butylated hydroxytoluene (BHT) or 100 μM dimethylfuran (DMF) to the cells 30 min before irradiation slightly increased the CF as compared to the cells to which no antioxidant had been added (Fig. 15). Referring to the antioxidant added, the CF decreased in the following order: mannitol > DMF > BHT.

Fig. 15. Effects of antioxidants on the survival of MCF-7 cells after incubation with 0.25 μg mTHPC/ml for 24 h and irradiation with laser light (0.13 J/cm²). 100 mM mannitol, 100 μM BHT and 100 μM DMF, respectively, were added to the cells 30 min before irradiation. Values are means ± SE of three experiments and are expressed as a fraction of the number of colonies that were formed by the cells to which no antioxidant had been added.
7. Results

7.2.6. Increased intracellular calcium levels and nitric oxide formation

mTHPC also localises in the cellular membrane, and the site of PS localisation determines the site of damage upon irradiation. It was investigated whether upon incubation with mTHPC and irradiation with laser light the cytoplasmic Ca\(^{2+}\) concentration is elevated as a consequence of membrane damage as it had been reported for other PS and cells. MCF-7 cells were incubated with 0.1 µg mTHPC/ml for 24 h and irradiated with 0.26 J/cm\(^2\). They were incubated at various times after irradiation with fluo-3 AM, a cell permeable probe that fluoresces when Ca\(^{2+}\) is bound. Thirty min and 1

Fig. 16. Increase in intracellular Ca\(^{2+}\) concentrations of MCF-7 cells after incubation with mTHPC and irradiation with laser light. MCF-7 cells were incubated with 0.1 µg mTHPC/ml for 24 h, and irradiated during 30 s with 25 mW light of 652 nm (0.26 J/cm\(^2\)). For half an hour before CLSM pictures were taken, cells were incubated with fluo-3 AM. (a) control cells. (b) cells 30 min after irradiation. (c) cells 1 h after irradiation. (d) cells 2 h after irradiation. Picture size is 250 µm x 250 µm. Typical pictures from three experiments are shown.
h after irradiation a slight increase in intracellular $\text{Ca}^{2+}$ is detectable as a punctuate staining (Fig. 16b and c). Two h after irradiation $\text{Ca}^{2+}$ is elevated in the whole cells, especially in the nuclei (Fig. 16d).

The activity of constitutive nitric oxide synthase (NOS) is $\text{Ca}^{2+}$-dependent. Therefore, it was investigated, whether an increase in cytoplasmic $\text{Ca}^{2+}$ concentration was followed by nitric oxide (NO) formation. After incubation with mTHPC and irradiation of MCF-7 cells, nitrate and nitrite concentrations in the supernatant and cytoplasm were determined (Fig. 17).

![Graph showing nitrate and nitrite formation](image)

Fig. 17. Nitrate and nitrite formation in MCF-7 cells after incubation with mTHPC and irradiation with laser light. MCF-7 cells were incubated with 0.1 µg mTHPC/ml for 24 h and irradiated during 30 s with 25 mW laser light of 652 nm (0.26 J/cm²). At the indicated times, samples were taken, and nitrate and nitrite concentrations were fluorometrically determined as described in Materials and methods.

Probably due to the presence of quenching substances in the samples (supernatant or cytoplasm), their fluorescence was always lower than that of the standard curve. Therefore, nitrate and nitrite concentrations could only be qualitatively determined.

As it can be seen in Fig. 17, the increase in nitrate and nitrite concentrations in the cytoplasm preceeded the increase in the supernatant. Interestingly, there are two phases of nitrate and nitrite formation. Whereas one phase could be attributed to the increase in intracellular $\text{Ca}^{2+}$, the other one might reflect iNOS expression.
7. Results

7.3. Protection against and sensitisation to in vitro photodynamic therapy by overexpression of Bcl-2

7.3.1. Subcellular localisation of VB-BO, mTHPC and TPPS₄ in BV3 and B22 cells

Firstly, the localisation of the three PS in BV3 and B22 cells was analysed. The positively charged VB-BO accumulated in mitochondria (Fig. 18a and d), the neutral, lipophilic mTHPC was detected in membranous structures (Fig. 18b and e), and the negatively charged TPPS₄ was localised in organelles resembling lysosomes (Fig. 18c and f) in accordance with previous findings (Modica-Napolitano et al., 1990; Hornung et al., 1997; Malik et al., 1997). Localisation patterns were identical in both cell lines.

Fig. 18. Intracellular localisation of VB-BO (a, d), mTHPC (b, e) and TPPS₄ (c, f) in BV3 (a, b, c) and B22 cells (d, e, f) determined by CLSM. BV3 and B22 cells were incubated with 1 μM VB BO for 1 h, 1 μM mTHPC or 10 μM TPPS₄ for 24 h. Typical pictures (100 μm x 100 μm) are shown.
7.3.2. Uptake of VB-BO, mTHPC and TPPS₄ into BV3 and B22 cells

Uptake of mTHPC and TPPS₄ into BV3 and B22 cells, as quantitatively measured by fluorescence intensity, was equal (Table 8). B22 cells, however, took up 18% more VB-BO as compared to BV3 cells after only 15 min incubation with 1 μM VB-BO (Table 8). VB-BO uptake depended on ΔΨ since it was reduced to a great extent in the presence of 25 μg/ml antimycin A and 1.25 μM FCCP (not shown). In all experiments, much shorter incubation times were chosen for VB-BO (15 min to 4 h) than for mTHPC and TPPS₄ (24 h), because VB-BO is taken up rapidly in response to ΔΨ, and not via diffusion or endocytosis.

Table 8. Uptake of VB-BO, mTHPC and TPPS₄. B22 and BV3 cells were incubated with 1 μM VB-BO for 15 min, 1 μM mTHPC or 10 μM TPPS₄ for 24 h, micrographs were taken and relative fluorescence was determined. Values are presented as means ± SE, n=5, * statistically significant difference (B22 vs. BV3, α=0.05).

<table>
<thead>
<tr>
<th></th>
<th>VB-BO</th>
<th>mTHPC</th>
<th>TPPS₄</th>
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<tbody>
<tr>
<td>BV3</td>
<td>136 ± 17</td>
<td>377 ± 34</td>
<td>34 ± 18</td>
</tr>
<tr>
<td>B22</td>
<td>161 ± 15*</td>
<td>358 ± 25</td>
<td>31 ± 16</td>
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7.3.3. Dark toxicity and light-induced toxicity of VB-BO, mTHPC and TPPS₄ in BV3 and B22 cells

Cells were incubated with various concentrations of the three PS in the dark. Incubation of the cells with the highest non-toxic concentrations was then followed by illumination to elicit phototoxic reactions.
Fig. 19. Toxicity of VB-BO in B22 and BV3 cells. Cells were incubated with the indicated concentrations (a) or 1 μM VB-BO (b) for 4 h in the dark, or with 1 μM VB-BO for 1 h followed by illumination (c). Illumination times of 2, 4, 6, and 8 min correspond to doses of 7.25, 14.5, 21.75 and 29 J/cm², respectively. Cell survival was determined by MTT assay (a, c) or by counting the cells up to 3 days after treatment (b). ○ BV3 cells incubated with VB-BO, ■ B22 cells incubated with VB-BO, ○ BV3 cells without VB-BO, □ B22 cells without VB-BO, * statistically significant difference (BV3 vs. B22, α=0.05), n = 4.
Incubation of the cells for 4 h in the dark with 10 μM or 30 μM VB-BO resulted in higher toxicity in B22 cells than in BV3 cells (Fig. 19a). Since VB-BO targets mitochondria and the MTT cell survival assay is based on mitochondrial dehydrogenase activity, a proliferation assay was used to confirm this finding. When incubated even only with 1 μM VB-BO for 4 h, a decrease in proliferation of B22 cells as compared to BV3 cells was found after 48 and 72 h (Fig. 19b). At this concentration of the PS, no difference in survival between the two cell lines was found after 4 h with the MTT assay. B22 cells also showed higher sensitivity when incubation with 1 μM of the dye was followed by illumination with white light of 400 to 780 nm (Fig. 19c).

Fig. 20 shows dark toxicity (Fig. 20a) and light-induced toxicity (Fig. 20b) of mTHPC. No statistically significant differences between BV3 and B22 cells were found. TPPS₄ was not toxic up to 0.3 mM and was equally toxic to both cell lines at 1 mM in the dark (Fig. 21a). The surviving fraction of BV3 cells was lower than that of B22 cells when incubation with TPPS₄ was followed by illumination (Fig. 21b).

Fig. 20. Toxicity of mTHPC in B22 and BV3 cells. Cells were incubated with the indicated concentrations of mTHPC for 24 h in the dark (a) or with 0.1 μM mTHPC followed by illumination (b), and cell survival was determined by MTT assay (● BV3 cells, ■ B22 cells). Illumination times of 0.25, 0.5, 1 and 2 min correspond to doses of 0.91, 1.81, 3.62 and 7.25 J/cm², respectively. n = 3.
7. Results

Fig. 21. Toxicity of TPPS₄ in B22 and BV3 cells. Cells were incubated with the indicated concentrations of TPPS₄ for 24 h in the dark (a) or with 10 μM TPPS₄ followed by illumination (b), and cell survival was determined by MTT assay. Illumination times of 0.5, 1, 2 and 3 min correspond to doses of 1.81, 3.62, 7.25 and 10.87 J/cm², respectively. ● BV3 cells, ■ B22 cells, * statistically significant difference (BV3 vs. B22, α=0.05). n = 4.

7.3.4. Cytochrome c release, membrane blebbing and annexin V binding in BV3 and B22 cells

It was also investigated whether phototoxic reactions would induce apoptosis. Fig. 22 shows cytochrome c release into the cytosol 1 h after incubation of the cells with PS and illumination. Cytochrome c release in B22 was very pronounced after incubation with VB-BO and illumination as compared to BV3 cells in accordance with the higher light-induced toxicity of VB-BO in B22 than in BV3 cells. In contrast, incubation with mTHPC and illumination induced more cytochrome c release in BV3 cells. Despite higher survival, cytochrome c release in B22 cells after incubation with TPPS₄ and illumination was slightly increased compared to BV3 cells.

An early change during apoptosis is the relocalisation of phosphatidylserine from the inner to the outer surface of the cell membrane. Therefore, binding of AV to phosphatidylserine on the cell membrane in the absence of nuclear staining with PI is a marker for early apoptosis. Late apoptosis cannot be distinguished from necrosis by this method. After incubation with the PS and illumination, cells were stained with AV and PI. BV3 and B22 showed similar reactions, and examples of both cell lines are shown.
Results

Control cells had an elongated shape (Fig. 23a), and poorly bound AV (Fig. 23b). Thirty min after incubation with 10 μM TPPS₄ and illumination for 2 min BV3 cells showed extensive membrane blebbing (Fig. 23c) and increased AV staining (Fig. 23d) in the absence of PI staining (not shown). Cellular changes were similar when cells were incubated with 0.1 μM mTHPC for 24 h and illuminated for 1 min (not shown). Figs. 23e and f show BV3 cells 30 min after incubation with 1 μM VB-BO and illumination for 6 min. Cells were rounded off, membrane blebbing and AV binding were slightly increased as compared to control cells. Damage was more pronounced with time. B22 cells 1 h after incubation with VB-BO and irradiation were already disintegrated (Fig. 23g), bound to AV (Fig. 23h) and were positive for PI (Fig. 23i). Despite slight cross-talk between PI and PS fluorescence, PI staining could clearly be identified since none of the PS localised in the nucleus.

Fig. 23. Membrane blebbing and AV binding of B22 and BV3 cells after incubation with photosensitisers and illumination. Cells were stained with AV and PI. a, c, e, g: bright field images, b, d, f, h: AV fluorescence, i: PI fluorescence. Typical pictures (250 μm x 250 μm) of B22 control cells (a, b), BV3 cells 30 min after incubation with 10 μM TPPS₄ and illumination for 2 min (c, d), BV3 cells 30 min after incubation with 1 μM VB-BO and illumination for 6 min (e, f) and B22 cells 1 h after incubation with VB-BO and illumination (g, h, i) are shown. Arrowheads indicate membrane blebbing (c) or cells rounding off (e).
7. Results
7.3.5. Dark toxicity and light-induced toxicity of A_{2E} in BV3 and B22 cells

A_{2E} is a lipophilic cationic fluorophore present in lipofuscin granules. As determined by fluorescence microscopy after 24 h incubation time, A_{2E} localised in BV3 and B22 cells in organelles resembling mitochondria and/or lysosomes (Fig. 24).

Fig. 24. Localisation of A_{2E} fluorescence in BV3 and B22 cells. BV3 and B22 cells were incubated with 10 \mu M A_{2E} for 24 h. Fluorescence was excited with a 450/490 nm bandpass filter, and detected with a 515 nm longpass filter. a, c: autofluorescence of BV3 and B22 cells, respectively. b, d: A_{2E} fluorescence in BV3 and B22 cells, respectively. Picture size is 220 \mu m x 175 \mu m.

Fig. 25 shows dark toxicity of A_{2E} in BV3 and B22 cells. Both cell lines showed comparable sensitivity.

Illumination of BV3 and B22 cells showed an additional rather than a synergistic effect with incubation with A_{2E} (Fig. 26a). Since this is probably due to a poor absorbance of A_{2E} of the white light emitted by the xenon lamp, a filter for blue light
was used (Fig. 26b). Also under these conditions no synergistic effect on cell survival of light and A$_2$E was observed. This study was not followed further since no appropriate light source was available.

Fig. 25. Dark toxicity of A$_2$E in BV3 and B22 cells. Cells were incubated with the indicated concentrations for 24 h in the dark. Cell survival was determined by MTT assay. ▼ BV3 cells, ■ B22 cells.

Fig. 26. Light-induced toxicity of A$_2$E in BV3 and B22 cells. Cells were incubated for 24 h in the presence of 10 µM A$_2$E and illuminated for the indicated times. Illumination times of 2, 4, 6, and 8 min correspond to doses of 7.25, 14.5, 21.75 and 29 J/cm$^2$, respectively. In a, white light, and in b, blue light was used. ▼ BV3 cells incubated with A$_2$E, ■ B22 cells incubated with A$_2$E, ○ BV3 cells without A$_2$E, □ B22 cells without A$_2$E.
7.3.6. Uptake and dark toxicity of VB-BO in N417 cells

To further test the hypothesis that Bcl-2 overexpressing cells show enhanced sensitivity to lipophilic cationic PS due to higher ΔΨ and, therefore, increased uptake of these compounds, three cell lines derived from N417 cells with different levels of Bcl-2 expression (control, Bcl-2*2, Bcl-2*3) were used. Uptake of VB-BO into control N417 cells was slightly higher than in Bcl-2 overexpressing cells, although the difference was statistically not significant (Fig. 27).

![Graph showing VB-BO fluorescence](image)

Fig. 27. Uptake of VB-BO into control and Bcl-2 overexpressing N417 cells. Cells were incubated with 1 μM VB-BO for 15 min, centrifuged, resuspended, micrographs were taken and fluorescence was determined. Values are presented as means ± SE, n = 3.
Dark toxicity of VB-BO in N417 cells was also determined. In contrast to BV3 and B22 cells, N417 cells grow in suspension. Therefore, the MTT assay had to be modified. As a consequence of this, lower concentrations of VB-BO and a longer incubation time were used. Fig. 28 shows dark toxicity of VB-BO in N417 cells. In contrast to the results with BV3 and B22 cells reported above, Bcl-2 overexpressing N417 cells were less sensitive to VB-BO. Bcl-2*2 cells showed a higher survival compared to control cells when incubated with 0.3 μM VB-BO for 20 h. Light-induced toxicity of VB-BO could not be determined, since N417 cells showed a high sensitivity to white light even in the absence of a PS.

Fig. 28. Dark toxicity of VB-BO in N417 cells. Cells were incubated with the indicated concentrations for 20 h, and survival was determined by MTT assay. n=4 * statistically significant difference (control vs. Bcl-2*2, α=0.05).
7.4. *Bcl-2 protects isolated mitochondria but not cells from ceramide-induced effects*

In a study with isolated mitochondria, we showed that N-acetylsphingosine (C2) and N-hexanoylsphingosine (C6) ceramide and, to a much lesser extent, dihydroceramide (DHC) induce cytochrome c release. This could be prevented by addition of human recombinant Bcl-2. Upon cytochrome c loss, mitochondrial oxygen consumption, ΔΨ and Ca^{2+} retention were diminished. It was investigated whether Bcl-2 overexpressing cells were protected against ceramide-induced cell death. Fig. 29 shows survival of B22 and BV3 cells incubated with increasing C2, C6 or DHC concentrations.

Fig. 29. Ceramide-induced cell death of BV3 and B22 cells. Cells were incubated with the indicated concentrations of C2 or C6 ceramide and DHC for 16 h. Survival was determined by MTT assay. Results are presented as means ± SE from 5 independent experiments. ● BV3, ■ B22.
The order of toxicity was: $C_6 > C_2 >> DHC$. Although single experiments indicated protection by Bcl-2 of ceramide induced cell death (not shown), on average no significant differences between BV3 and B22 survival were found.

To determine whether ceramide would induce apoptotic cell death in BV3 and B22 cells, they were stained with AV and PI. Fig. 30 shows BV3 cells incubated with 50 $\mu$M $C_2$ for 30 min. Enhanced binding of AV to cells incubated with $C_2$ (60 % AV positive cells) compared to control cells (26 %) was found. When cells were incubated with 100 $M C_6$ for 2 h, they did not only show AV binding but also nuclear staining with PI, indicating either late apoptosis or necrosis (not shown). No differences between BV3 and B22 cells were seen.

Fig. 30. AV binding to BV3 cells after incubation with $C_2$. BV3 cells were incubated with 50 $\mu$M $C_2$ for 30 min and stained with AV and PI. Fluorescence was detected by CLSM. a, b: control cells. c, d: cells incubated with $C_2$. a, c: AV fluorescence. b, d: PI fluorescence. Picture size is 250 $\mu$m x 250 $\mu$m.
7.5. *Skin photosensitivity in PDT*

### 7.5.1. Protective substances

The efficacy of several protective substances to reduce transmission of laser light of 652 nm had been determined. The findings were to be confirmed with an *in vitro* model consisting of human keratinocytes, mTHPC as the PS, and a xenon lamp mimicking sun light. Fig. 31 shows the survival expressed as % CF of cells incubated with mTHPC and illuminated directly or through a layer of the protective substances indicated.

![Graph](image-url)  
*Fig. 31. Protective substances against skin photosensitivity. HaCaT cells were incubated with 0.1 μg mTHPC/ml for 24 h and illuminated during 1 min (3.62 J/cm²). When indicated, 2 mg/cm² of the protective substance was applied on Transpore tape through which the light was administered. Results are presented as means ± SE of 3 independent experiments. # significant difference (substance vs. Transpore), * significant difference (substances as indicated). Statistical analysis was done with a one-sided Wilcoxon-Mann-Whitney test. α=0.05.*
25 % TiO₂, 9 % TiO₂ + 1 % Fe₂O₃, and light and dark cover cream protected HaCaT cells significantly from mTHPC-mediated light toxicity. Addition of 1 % Fe₂O₃ increased the protective effect of ZnO and TiO₂. Dark cover cream was more protective than light cover cream and was the best protective substance tested.

7.5.2. Hyperiforce, a Hypericum extract, displays characteristics of a PS

Prompted by a case report of a woman showing severe skin reactions upon intake of hyperiforce and ALA, we sought to investigate a possible synergistic effect of these two drugs. Hyperiforce contains photosensitising compounds that fluoresce and accumulate in HaCaT cells mostly in membranes and perinuclear organelles when cells are incubated with 1 % hyperiforce (v/v medium) for 24 h (Fig. 32).

Dark toxicity of hyperiforce was determined by a CF assay. It exceeded 10 % for hyperiforce concentrations above 1 % (v/v medium) and 99 % for concentrations above 6.25 % (Fig. 33).

Fig. 32. Hyperiforce fluorescence in HaCaT cells. HaCaT cells were incubated for 24 h with 1 % hyperiforce. Fluorescence was excited with a 450/490 nm bandpass filter, and detected with a 515 nm longpass filter. a: autofluorescence of HaCaT cells, b, c: hyperiforce fluorescence.
Fig. 33. Dark toxicity of hyperiforce. HaCaT cells were incubated with increasing concentrations of hyperiforce for 24 h, and survival was determined by a CF assay. Values are means from one experiment ± SE.

Incubation of HaCaT cells with 1 mM ALA for 3 h resulted in no dark toxicity, and only minor light-induced toxicity was observed (Fig. 34). Incubation of the cells with 1 % hyperiforce for 24 h resulted in 5 % reduction of cell survival in the dark, and
upon illumination the CF was further diminished. Incubation with ALA and hyperiforce in the dark and upon illumination resulted in a synergistic effect of these two compounds, i.e., CF was lower than expected from adding up toxicity of both compounds alone.
8. DISCUSSION

8.1. Mitochondria as a target of mTHPC photosensitisation

8.1.1. Localisation of mTHPC

There is no doubt that mitochondria are sensitive targets in PDT. Lipophilic, and especially cationic, PS accumulate at least partially in mitochondria (Woodburn et al., 1991). Although confocal laser scanning microscopy showed a diffuse cytoplasmic localisation of HpD in C6 and V79 cells (Woodburn et al., 1991), mitochondrial enzymes were inhibited by HpD-mediated PDT of R3230AC mammary adenocarcinoma cells in contrast to cytosolic enzymes, which remained largely unaffected (Hilf et al., 1984). Also the most effective PS (in terms of cell killing) in a group of porphyrins tested were the ones localising in mitochondria (Woodburn et al., 1992). mTHPC is a neutral lipophilic PS that localises in membranes and organelles around the nucleus of MCF-7 and V-79 cells (Hornung et al., 1997; Fig. 10). By double staining with Rh123 we could show that some of these organelles correspond to mitochondria (Fig. 11). In human sperm cells mTHPC also accumulated in mitochondria, that are located in their midpiece (Fig. 12). Additionally, in vivo mTHPC accumulated in mitochondria as measured in liver mitochondria of mice injected with mTHPC 48 h before (Fig. 9, Table 6). Not considering excretion of the PS, mTHPC accumulation was 2- to 3-fold in liver mitochondria (0.55 to 0.9 ng mTHPC/mg protein) as compared to the concentration expected for a uniform distribution in all tissues (0.3 mg mTHPC/kg body weight injected). In a study by Peng et al. (1995), mice were injected with 1 mg/kg body weight mTHPC, and concentrations of mTHPC in various tissues were determined at different time points. Highest concentrations were found in the urinary tract, skin, tumour (mammary carcinoma) and liver, and were about 3 and 1.2 ng/mg in liver (24 and 48 h after injection, respectively). Although more experiments are needed to support these findings, these data suggest an accumulation of mTHPC in liver mitochondria as compared to total liver or other tissues.
8. Discussion

8.1.2. Photosensitisation of isolated mitochondria

Damage to isolated rat liver mitochondria by deuteroporphyrin as a PS plus irradiation was shown by Sandberg and Romslo (1980). They found the following sequence of reactions: uncoupling and inhibition of oxidative phosphorylation, decreased ΔΨ, inhibition of respiration, swelling, and disruption of mitochondria. These findings correspond quite well with our results for PDT using mTHPC: Already at a low mTHPC concentration mitochondrial oxygen consumption was no longer stimulated by ADP addition (Fig. 6), although at this concentration the mitochondria were still able to build up an, albeit reduced, ΔΨ (Fig. 7). This is in agreement with the study of Salet et al. (1991) who observed that ΔΨ was only slightly affected at a PDT dose that completely abolished coupling, and concluded that uncoupling was not simply a consequence of membrane damage. At the lowest mTHPC concentration we used, oxygen consumption supported by pyruvate and malate was stimulated after irradiation, also indicating uncoupling (Table 5). Similar results with haematoporphyrin as the PS later confirmed our findings (Salet et al., 1998). Inhibition of oxidative phosphorylation has been explained by noncompetitive inhibition of the adenine nucleotide translocator after photofrin I®-mediated PDT (Atlante et al., 1989). Dissipation of ΔΨ and inhibition of respiration were found when higher mTHPC concentrations were present during irradiation. Increased respiration of complex I after a mild assault using haematoporphyrin as a PS had been previously observed (Salet and Moreno, 1981; Salet et al., 1983). Impaired Ca²⁺ homeostasis of photodynamically treated mitochondria was reported by the same group, which is in accordance with our study (Fig. 8). Later it was suggested that oxidative damage to mitochondria induced by PDT also includes inactivation of the mitochondrial permeability transition pore, possibly by the degradation of critical histidines (Salet et al., 1997).
8.1.3. Photosensitisation of MCF-7 cells

Following the investigations on isolated mitochondria described above, we were interested in the question whether mitochondria are the prime cellular targets in the photosensitisation with mTHPC. When MCF-7 cells were incubated with mTHPC and irradiated, oxygen consumption in a pyruvate-containing medium in the absence or presence of an uncoupler was diminished compared to control cells (Fig. 13). A decrease to 69% of the control values was observed under conditions that yield only 0.8% colony formation (Table 7). However, it should be considered that a decrease in oxygen consumption, as seen with isolated mitochondria, reflects mostly oxidative damage to the proteins of the respiratory chain, and is not the most sensitive parameter to determine impairment of mitochondria. $\Delta W$' was also diminished in MCF-7 cells after incubation with mTHPC and irradiation (Fig. 14), but was not quantitatively assessed. Thus, mitochondria seem to be a target in mTHPC-mediated PDT, but possibly not the only target, which is expected considering the subcellular distribution of mTHPC in various organelles and membranes. Similar results were also reported for another dye, merocyanine 540, that is preactivated by light before addition to the cells. During incubation with the PS, MCF-7 cells released Rh123, with which they had been loaded, and ATP levels and the activity of succinate dehydrogenase decreased (Gulliya et al., 1995). As discussed above, the lipophilic mTHPC accumulates mainly in membranes, but not in the nucleus. Thus, it is not astonishing that after mTHPC-mediated PDT of human myeloid leukemia cells no DNA damage was detectable using a comet assay (McNair et al., 1997). The integrity of the plasma membrane, however, was diminished as shown by the release of lactate dehydrogenase from normal fibroblastic and murine hepatoma cells (Kirveliene et al., 1997). Damage to mitochondria can also be measured by MTT assay, that reflects activity of mitochondrial oxidoreductases. After incubation of BHK and MH22 cells with 0.5 μg/ml mTHPC and irradiation, MTT activity decreased during 5 h after irradiation. Interestingly, the ATP level in these cells decreased only in a medium depleted of energy sources, but not in a glucose-containing medium, where the cells seemed to compensate the failure of mitochondrial ATP production by enhanced glycolysis (Kirveliene et al., 1997). Similar results had been reported for HpD-mediated PDT (Hilf et al., 1986), and further support the sensitivity of mitochondria in PDT.
8.2. Oxidative damage by mTHPC-mediated in vitro PDT

When isolated rat liver mitochondria were incubated with mTHPC and subsequently irradiated, the amount of carbonyl groups and TBARS was significantly enhanced, dependent on the mTHPC concentration present (Figs. 4 and 5). Presumably as a consequence of these oxidative modifications, mitochondrial functions were diminished as discussed above.

The use of scavengers of different oxygen radicals is one possibility to distinguish whether a photosensitiser reacts via a type I or type II mechanism (Girotti, 1990). Since the addition of 1,3-diphenylisobenzofuran before irradiation reduces cell inactivation during PDT, mTHPC is thought to act partially via type II reactions (Ma et al., 1994). We also observed a decreased formation of LOOH in the presence of DMF, indicating type II reactions (Table 4). This is supported by the finding that LOOH formation was increased when the incubation and irradiation took place in D$_2$O instead of H$_2$O. On the other hand, BHT reduced the amount of all oxidative modifications measured, therefore type I reactions could also play a role in mTHPC-mediated PDT. However, as discussed by Girotti (1990), there are several limitations in using scavengers of reactive oxygen species to investigate the reaction type, since many of them lack absolute specificity. According to theoretical considerations, TBARS should not be found as a result of type II reactions, but in the presence of iron decomposition of LOOH generated may occur and lead to TBARS formation, i.e., low amounts of LOOH and high amounts of TBARS do not rule out the involvement of type II reactions.

Studying oxidative damage in MCF-7 cells, increase in CF rather than oxidative modifications were measured in order to determine whether a given antioxidant had an effect (Fig. 15). Although mannitol showed the strongest ability to increase CF, the data still suggest a mixed type I/type II mechanism. In contrast to these experiments, mannitol had no effect on oxidative damage in isolated mitochondria that were incubated with mTHPC and irradiated (Table 4). The minor increase in CF (10 to 20%) seen here when using antioxidants could be explained by the finding that cells are able to repair damage such as lipid peroxidation induced by PDT (Kirveliene et al., 1997).
8.3. Protection against and sensitisation to in vitro PDT by over-expression of Bcl-2

8.3.1. Enhanced sensitivity of Bcl-2 overexpressing L929 cells to the lipophilic cationic photosensitiser Victoria blue BO

The sensitivity of control (BV3) and Bcl-2 overexpressing (B22) L929 cells to three PS, alone or in combination with light were investigated. It is known that overexpression of Bcl-2 or Bcl-xL can protect cells against PDT (He et al., 1996; Granville et al., 1998), whereas transfection with bcl-2 antisense oligonucleotide increases the sensitivity of cells to PDT (Zhang et al., 1999). Here we show that, depending on the PS used, Bcl-2 expression has a positive, none or even a negative effect on survival of cells after in vitro PDT.

TPPS₄, a hydrophilic anionic PS, accumulated in organelles resembling lysosomes (Fig. 18c and f), was taken up equally into both cell lines (Table 8), and displayed equal dark toxicity to BV3 and B22 cells (Fig. 21a). When incubation with the PS was followed by illumination, BV3 cells had a lower surviving fraction than B22 cells (Fig. 21b). mTHPC, a lipophilic neutral PS, was localised in membranous structures (Fig. 18b and c), and was also taken up equally into both cell lines (Table 8). Overexpression of Bcl-2 in B22 cells had no effect on the level of toxicity of the PS alone or in combination with light (Fig. 20). In contrast, Bcl-2 overexpressing cells showed an increased uptake of the lipophilic cationic PS VB-BO (Table 8), that accumulated in mitochondria (Fig. 18a and d). Accordingly, they were more sensitive to VB-BO in the dark (Fig. 19a and b) and combined with illumination (Fig. 19c). The enhanced sensitivity of B22 cells to VB-BO is specific, B22 cells are not generally more sensitive than BV3 cells as shown by their equal or diminished sensitivity to mTHPC and TPPS₄ phototoxicity, respectively.

The antiapoptotic protein Bcl-2 inhibits cytochrome c release from mitochondria (Kluck et al., 1997), but it is not fully understood if and how Bcl-2 affects ΔΨ in cells not undergoing programmed cell death, e.g., in the Bcl-2 overexpressing L929 cells used in this study. Smets et al. (1994) investigated several leukemic cells with different
sensitivity to glucocorticoid-induced apoptosis. The cells with highest resistance had the longest cell cycle, highest content of Bcl-2 and highest ATP levels, which could reflect increased mitochondrial activity or tighter coupling resulting in increased ΔΨ'. This may be due to the ability of Bcl-2 and related proteins to form ion channels (Schendel et al., 1997; Minn et al., 1997; Antonsson et al., 1997). A study with isolated mitochondria gave evidence that Bcl-2 regulates proton flux, since mitochondria with an elevated Bcl-2 content were able to maintain ΔΨ' by enhancing proton efflux upon addition of ΔΨ'-loss inducing stimuli, e.g., Ca^{2+}, a protonophore, or valinomycin (Shimizu et al., 1998). Addition of human recombinant Bcl-2 to isolated rat liver mitochondria can increase ΔΨ' as measured with the probe safranine (unpublished observation).

PDT kills cells via necrosis or apoptosis, depending on the PS, cell type and treatment protocol (Dellinger, 1996; Luo and Kessel, 1997). When mitochondria are the subcellular target, PDT induces apoptosis (Ball et al., 1998; Noodt et al., 1998; Kessel and Luo, 1999), but for lysosomal targeting contradictory results were reported (Brunk et al., 1997; Kessel and Luo, 1998). It has been shown that various PS can elicit different apoptotic pathways depending on their intracellular localisation (Noodt et al., 1999). In our study, analysis of ΔΨ' loss or stabilisation upon PDT was technically not possible because of interference of PS fluorescence with probes for FACS analysis. Therefore, AV binding, a marker of apoptotic cell death, was qualitatively determined by confocal laser scanning microscopy (Fig. 23). Enhanced AV binding and extensive membrane blebbing indicate the onset of programmed cell death in both cell lines and with all three PS used. However, lipophilic PS are likely to target the plasma membrane resulting in blebbing independent of the mode of cell death. Therefore, we additionally investigated cytochrome c release from mitochondria (Fig. 22), another hallmark of apoptosis known to occur after PDT (Varnes et al., 1999). Cytochrome c release is expected to happen when mitochondria are a target of the PS (Kessel and Luo, 1999), as for VB-BO in our study, where mitochondria are the exclusive, or for mTHPC, where they are one of the targets. As expected, when incubated with VB-BO and illuminated, B22 cells showed a marked cytochrome c release in contrast to BV3 cells despite the higher Bcl-2 content of B22 cells (Fig. 22). B22 and BV3 cells were equally sensitive to mTHPC-mediated toxicity (Fig. 20). Cytochrome c release from B22 cells was less pronounced with the PS mTHPC (Fig. 22) than with VB-BO, although conditions for PDT with VB-BO and mTHPC were used that induced comparable cell death in B22
cells. Upon illumination, reactive oxygen species are formed at the sites of mTHPC localisation, including cell membranes. Initial apoptosis induced by PDT can be delayed or stopped when the cell membrane is destroyed, because factors of the apoptotic cascade are lost from the cytosol (Kessel and Luo, 1998; Kessel et al., 1997), and it might be interesting to study later events in apoptosis upon mTHPC-mediated PDT. B22 cells were more resistant than BV3 cells to TPPS4 phototoxicity mediated via lysosome destruction (Fig. 21), although in B22 cells cytochrome c release was slightly higher (Fig. 22).

In a very recent study, it was found that overexpression of Bcl-2 was paralleled by enhanced expression of Bax in MCF10A cells (Kim et al., 1999). PDT with aluminium phthalocyanine selectively destroyed Bcl-2, resulting in an enhanced apoptotic response in the cell line transfected with Bcl-2. This is supported by the finding that also in chemotherapy-induced apoptosis Bcl-2 cleavage is part of the apoptotic cascade (Fadcel et al., 1999). It cannot be excluded that Bcl-2 was destroyed when B22 and BV3 cells were incubated with VB-BO and illuminated. However, there are several differences in the study by Kim et al. (1999) and ours, so that direct comparison is difficult. Firstly, aluminium phthalocyanine is localised diffusely in the cytoplasm (Luo and Kessel, 1997) whereas VB-BO accumulates in mitochondria (Fig. 18a and d). Secondly, control and Bcl-2 overexpressing MCF10A cells took up equal amounts of aluminium phthalocyanine, whereas B22 cells took up more VB-BO than BV3 cells (Table 8). The difference in PS uptake is believed to be the reason for enhanced killing of B22 cells by incubation with VB-BO, but destruction of Bcl-2 resulting in enhanced apoptotic response may also have taken place.

The B22 cells were used as a model for cancer cells with which they share two characteristics: they are protected against apoptosis, and they show higher ΔΨ (Nadakavukaren et al., 1985) as compared to normal cells, or BV3 cells in this study. Lipophilic cationic drugs, e.g., VB-BO, accumulate in response to ΔΨ preferentially in tumour cells and may display high toxicity despite the increased resistance of the cells to other stress factors. It remains to be investigated whether this promising approach is also a successful strategy for targeting and killing cancer cells in vivo.

Small cell lung cancer cells (N417) with different levels of Bcl-2 expression were also used to further support our hypothesis. However, the Bcl-2 overexpressing N417 cells took up less VB-BO (Fig. 27) and were, accordingly, less sensitive to this PS
in the dark (Fig. 28). Light-induced cell killing could not be determined, since these cells were very sensitive to light even in the absence of a PS.

### 8.3.2. Effects of ceramide

Many pathways are elicited by PDT that finally contribute to (apoptotic) cell death, amongst the formation of the second messenger ceramide (Separovic et al., 1997; Separovic et al., 1998). In a study with isolated rat liver mitochondria, we observed that ceramide induced cytochrome c release resulting in decreased mitochondrial functions, and that these effects were inhibitable by Bcl-2. The Bcl-2 overexpressing B22 cells are more resistant to tumour necrosis factor-α-mediated cytotoxicity than BV3 cells (Hennet et al., 1993). Since tumour necrosis factor-α receptor stimulation induces de novo formation of ceramide, B22 cells were expected to be also protected against ceramide-mediated cytotoxicity, as is reported in the literature for other cells overexpressing Bcl-2 (Zhang et al., 1997). However, although ceramide induced an apoptotic response in BV3 and B22 cells as shown by enhanced binding of AV (Fig. 30), no significant differences in cell survival upon incubation with C2, C6 or DIIIC were found (Fig. 29). This might be due to the finding that Bcl-2 not only prevents cytochrome c release from mitochondria, but also inhibits ceramide formation (Yoshimura et al., 1998). Thus, Bcl-2 might be twofold protective against tumour necrosis factor-α but not ceramide, and, therefore, its effect on cell survival would be more pronounced when tumour necrosis factor-α is the cytotoxic agent.

### 8.3.3. A₃E, a component of lipofuscin, as a photosensitiser

With age, lipofuscin granules accumulate in retinal pigment epithelial cells of humans (Feeney-Burns et al., 1980). One of the major fluorophores present in lipofuscin is A₃E, a lipophilic cationic compound formed of two molecules of retinal and one molecule of ethanolamine (Sakai et al., 1996). Lipofuscin has properties of a
PS, namely upon light absorption formation of $^1\text{O}_2$ occurs (Gaillard et al., 1995). $\text{A}_2\text{E}$ coupled to LDL has been found to accumulate in lysosomes of human retinal pigment epithelial cells and to inhibit lysosomal functions (Holz et al., 1999). Since $\text{A}_2\text{E}$ is a lipophilic cationic compound, one would expect it to be taken up by mitochondria. Our microscopy study can neither confirm nor discard this hypothesis. The observed fluorescence in BV3 and B22 cells could be attributed to mitochondrial or lysosomal structures (Fig. 24). B22 and BV3 cells showed no significant difference in sensitivity towards $\text{A}_2\text{E}$, although BV3 cells seemed to be slightly more sensitive (Figs. 25 and 26). Only a minor light-induced effect by $\text{A}_2\text{E}$ on cells was observed (Fig. 26). It was impossible to illuminate the cells for longer periods of time, since light alone in the absence of a PS already reduced cell survival under these conditions. The emission spectrum of the xenon lamp (400 to 780 nm) does not well correspond to the absorbance spectrum of $\text{A}_2\text{E}$ (absorption maxima at 336 and 436 nm). However, with an appropriate light source $\text{A}_2\text{E}$-mediated phototoxicity is very likely to occur, since irradiation with blue light of lipofuscin-loaded cells has been shown to decrease cell viability and lysosomal stability (Wihlmark et al., 1997).

8.4. Role of $\text{Ca}^{2+}$ and NO in PDT

When the plasma membrane is a target of PDT, as in mTHPC-mediated PDT, lipids and proteins will be oxidatively damaged. As a result, the membrane becomes permeable for ions, e. g. $\text{Ca}^{2+}$. When mouse myeloma cells were photosensitised with zinc phthalocyanine, plasma membrane depolarisation was observed (Specht and Rodgers, 1991). This depolarisation depended on the extracellular concentration of $\text{Na}^+$, was diminished in the presence of a $\text{Na}^+$ channel blocker, and was accompanied by an increase in cytosolic $\text{Ca}^{2+}$ concentration. After aluminium phthalocyanine-mediated PDT, Chinese hamster ovary cells transiently had increased cytosolic $\text{Ca}^{2+}$ concentrations (Penning et al., 1992). This response was only eliminated in the presence
of 1 mM EGTA in the medium, but not by 20 μM verapamil. Interestingly, chelating intracellular Ca$^{2+}$ enhanced cell killing, indicating a role of Ca$^{2+}$ in cell rescue after PDT. Similar results were found in human skin fibroblasts (Hubner et al., 1996). Increase of cytosolic Ca$^{2+}$ in human cerebral glioma cells incubated with HpD and irradiated was due to influx of extracellular Ca$^{2+}$, and was inhibited by the Ca$^{2+}$ channel blocker diltiazem (Joshi et al., 1994). When incubation with the PS is long enough so that the compound localises in intracellular organelles, endoplasmic uptake of Ca$^{2+}$ is inhibited upon irradiation which could also contribute to elevated cytoplasmic Ca$^{2+}$ levels (Dellinger et al., 1994). Influx of Ca$^{2+}$ does not reflect a general membrane damage upon PDT, and although it is not per se a cause of cell death (Gederaas et al., 1996), it can be paralleled by DNA fragmentation (Tajiri et al., 1998). Also in our study, incubation of MCF-7 cells with mTHPC and irradiation led to an increase in intracellular Ca$^{2+}$ levels (Fig. 16).

Since the constitutive isoformes of NOS are Ca$^{2+}$-dependend, we expected an increase in NO formation following PDT. Interestingly, two phases of increased nitrite and nitrate formation were found (Fig. 17). This could reflect two phases of intracellular Ca$^{2+}$ accumulation, as one study reports transient elevated Ca$^{2+}$ levels already 5 min after irradiation (Penning et al., 1992), but another only after 1 to 2 h (Tajiri et al., 1998). Alternatively, inducible NOS could be expressed. In human epidermoid carcinoma cells photosensitised with a silicon phthalocyanine, increased expression of constitutive NOS was found as early as 15 s after PDT (Gupta et al., 1998). It remains to be addressed - by modulation of extracellular Ca$^{2+}$ concentration and Western blot analysis - which are the exact mechanisms of for the biphasic nitrite and nitrate formation observed in mTHPC-mediated in vitro PDT of MCF-7 cells.
8.5. Skin photosensitivity

8.5.1. Protection against skin photosensitivity

One of the major side effects of PDT is prolonged cutaneous sensitivity. For example, when photosensitised with photofrin, patients show skin reactions upon light exposure up to 13 weeks after injection of the PS (Wagnieres et al., 1998). One of the goals in the development of new PS is to reduce skin sensitivity. Skin reactions in patients injected with the second-generation PS mTHPC were most pronounced 1 week after administration, and lasted only up to 6 weeks (Wagnieres et al., 1998). In general, prevention of skin damage may take place at several steps in the process of photodynamic action: i) prevention of PS accumulation in the skin, ii) prevention of excitation of the PS by light, and iii) prevention of oxidative damage of cellular components.

Before our study, patients at the University Hospital were advised to avoid strong sunlight and to protect themselves, e. g., by using sunscreens. Sunscreens, however, are meant to absorb light in the UV range (290 to 380 nm), but PS strongly absorb light in the visible (380 to 780 nm) and near infrared (780 nm to 2.5 μm) range of the spectrum. In our in vitro study, sunscreens showed no protection of HaCaT cells that were incubated with mTHPC and illuminated (Fig. 31) and only minor effects in the following in vivo study (by V. A. Schwarz) compared to creams containing pigments such as TiO₂ and Fe₂O₃. While the protective creams tested are meant to be applied on face and hands, special fabric materials might be used to protect other parts of the body from visible light (Menter et al., 1998). Many in vitro and in vivo studies have investigated the protective effect of antioxidants, e. g., β-carotene (Moshell and Bjornson, 1977), N-acetylcysteine (Baas et al., 1994; Baas et al., 1995; Yusof et al., 1999), 1,3-diphenylisobenzofuran (McLear and Hayden, 1989), ascorbic acid (Bolla et al., 1996), and melatonin (Zang et al., 1998), in order to protect patients suffering from porphyria or undergoing PDT.
8.5.2. Synergistic effects of two photosensitisers

Hypericum extracts for treatment of depression contain the photosensitising compound hypericin and are known to induce skin photosensitivity (Golsch et al., 1997; Bernd et al., 1999). Hypericin itself is used as a PS (Koren et al., 1996), and has been shown to induce apoptosis (Assefa et al., 1999). Incubation with hypericin and irradiation of HeLa cells caused cytochrome c release and activation of caspase-3 (Vantieghem et al., 1998). Hypericin has been reported to localise in the nucleus of T47D mammary tumour cells (Miskovsky et al., 1995). In contrast, we observed fluorescence in organelles around the nucleus and in membranes of HaCaT cells incubated with hyperiforce, but never in the nucleus (Fig. 32). More consistent with our observations, hypericin-mediated PDT decreased cellular ATP levels and respiration in EMT6 mouse mammary carcinoma cells, indicating that mitochondria were a major subcellular target (Johnson et al., 1998).

Our aim was to study a possible synergistic effect of ALA and hyperiforce, and to confirm an observation in a patient with severe skin reactions, who had received both drugs (in collaboration with D. Ladner). When HaCaT cells were incubated with hyperiforce and ALA, the two PS acted in fact synergistically, i.e., the toxicity in the dark and upon light exposure was more pronounced than expected for additive effects (Fig. 34). In the literature, synergistic as well as antagonistic effects for the combination of treatments with hypericin have been described. Hypericin significantly enhanced radiosensitivity of glioblastoma cell lines (Zhang et al., 1996), but caused stimulated growth of Staphylococcus aureus when combined with photofrin II® or mTHPC (Kubin et al., 1999).

We conclude from our data that until PS with improved properties are available, protection against light with cover creams is a useful tool for avoiding skin reactions such as oedema and erythema in patients. Furthermore, possible photosensitising properties of other drugs, especially natural compounds such as hypericum extracts, should not be underestimated.
8.6. Conclusions

Selective destruction of tumour cells is the aim of anti-cancer therapies. In PDT this is accomplished by enhanced accumulation of a PS in the target tissue followed by light activation. A variety of compounds is being tested in vitro and in vivo as possible new PS with improved properties. Amongst many parameters, the intracellular localisation of a PS influences its capacity to kill a cell. Special attention has been paid to mitochondria as a subcellular target, not only because they are mainly responsible for energy supply in most cell types but also because their major role in apoptotic cell death has recently become evident.

In the first part of this thesis, destruction of isolated mitochondria or mitochondria in MCF-7 cells by mTHPC-mediated PDT was investigated. Oxygen consumption and ΔΨ of mitochondria and cells were diminished upon incubation with mTHPC and irradiation. However, it cannot be excluded that damage to other organelles contributes to cell death induced by PDT with mTHPC, since this PS is not only localised in mitochondria.

In the second part of this thesis, it was investigated whether the relatively higher ΔΨ of carcinoma cells could be used for targeting these cells with cationic PS. As a model system, cells overexpressing the anti-apoptotic protein Bcl-2, that displayed higher ΔΨ than control cells, were used. Interestingly, overexpression of Bcl-2 had a positive, none or even a negative effect on cell survival after in vitro-PDT with three different PS.

In summary, PDT is able to induce cell death even in cells which are protected against apoptosis or display resistance to other therapies, provided that an appropriate PS and corresponding protocol are used.
9. References


References


9. References


References


References


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11. Publications


12. CONGRESSES


First World Congress of Photomedicine in Gynecology, Zürich, Switzerland, 1998. Oral presentation: Klein, S. D. Photosensitization of isolated rat liver mitochondria by tetra(m-hydroxyphenyl)chlorin.


13. CURRICULUM VITAE

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