**Potentiation of Photodynamic Therapy by Ursodeoxycholic Acid**

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**ABSTRACT**

Ursodeoxycholic acid (UDCA) protects cells from the apoptotic effects of hydrophobic bile acids and some other cytotoxic agents. We observed the opposite result when assessing the effects of UDCA on the apoptotic response to mitochondrial photodamage induced by photodynamic therapy (PDT). Two photosensitizers with predominantly mitochondrial specificity were used: a porphyrin we have designated CPO; and the tin etiopurpurin SnET2. UDCA potentiated the loss of mitochondrial potential, release of cytochrome c into the cytosol, activation of caspase-3, and apoptotic cell death after irradiation of photosensitized murine leukemia L1210 or hepatoma 1c1c7 cells. These effects were not observed when UDCA was added after irradiation. Glyco-UDCA and tauro-UDCA, conjugated forms of UDCA that are formed in vivo, were as effective as UDCA in promoting PDT phototoxicity. Because UDCA does not act by enhancing intracellular accumulation of the photosensitizing agents used in this study, we propose that the mode of action of UDCA involves the sensitization of mitochondrial membranes to photodamage. UDCA is used currently in gastroenterology for several indications. The drug may offer a means for promoting the efficacy of PDT with minimal adverse effects.

**INTRODUCTION**

PDT involves the preferential photodamage of neoplastic cells and has also been used for treatment of vascular diseases, e.g., atherosclerotic plaque and macular degeneration (1). Several photosensitizing agents exhibit the required selectivity for pathological versus normal tissues. These agents target a variety of subcellular organelles for photodamage (1). When mitochondria are the primary target, PDT causes a rapid loss of the mitochondrial membrane potential (ΔΨₘ), loss of cytochrome c, and initiation of a prompt apoptotic response (2). The latter is presumably mediated by activation of the Apaf-1-procasapase-9 pathway, leading to caspase-3 activation (3).

UDCA resembles its more hydrophobic analogue DCA except for a different configuration of the -OH substituents (Fig. 1). UDCA has been used clinically for the solubilization of gallstones and the treatment of biliary cirrhosis (4, 5). A variety of additional properties of the drug have been identified. UDCA has been reported to protect primary hepatocytes and hepatoma, osteogenic sarcoma, and HeLa cells from apoptosis induced by okadaic acid, hydrogen peroxide, ethanol, and DCA (6–8). UDCA was shown to reverse the toxic effects of DCA by preventing loss of the mitochondrial membrane potential and release of cytochrome c (9–12).

Because of its reported antiapoptotic effects and the results obtained with DCA, we initially thought that UDCA might suppress the apoptotic response to PDT. Instead, we found that UDCA sensitizes mitochondria to photodamage. Because UDCA has been safely used for the treatment of a variety of human conditions, the results described here suggest that UDCA may be a safe and useful agent for the promotion of PDT efficacy.

**MATERIALS AND METHODS**

**Drugs and Chemicals.** Tin etiopurpurin (SnET2; Ref. 13) was obtained from Dr. Alan Morgan, University of Louisville. The porphycene CPO (14) was obtained from Dr. Alex Cross (CytoPharm, San Francisco, CA). CPO is a highly selective agent for producing mitochondrial photodamage (2), whereas SnET2 sensitizes mitochondria more than lysosomes (15). The sensitizers were dissolved in N,N'-dimethylformamide to yield 1 mM stock solutions. Stock solutions (100 mM) of bile acids (Sigma Chemical Co., St. Louis, MO) were prepared in 0.2 mM NaOH. HO342, a probe for chromatin condensation, and MTO, a probe for the mitochondrial membrane potential, were obtained from Molecular Probes (Eugene, OR). DEVD-R110, a fluorescent substrate for caspase-3, was also provided by Molecular Probes. Ac-DEVD-AMC and a murine antibody to cytochrome c were obtained from Pharmingen (San Diego, CA). AMC was purchased from Aldrich (Milwaukee, WI).

**Cells and Cell Culture.** Murine L1210 cells were maintained in suspension culture. Hepa 1c1c7 cells are an adherent cell line. Both lines were maintained as described by Kessel et al. (16).

**PDT Protocols.** Suspensions of L1210 cells (7 mg/ml wet weight = 2 × 10⁶ cells) were incubated in growth medium containing 2 mM SnET2 or CPO for 15 min at 37°C. The cells were subsequently washed and resuspended in fresh growth medium at room temperature. For Hepa 1c1c7 cells, subconfluent cultures were exposed to 2 mM SnET2 for 20–25 min at 37°C prior to washing. Irradiation was provided by a 600 W quartz-halogen source filtered with 10 cm of water and a 800-nm cutoff filter to remove ionizing radiation. Bandwidth was further confined to 660 ± 10 nm (SnET2) or 610 ± 10 nm (CPO) by narrow-band interference filters (Oriel, Stratford, CT). The total light dose is specified for each experiment. Initial experiments indicated that a 50% loss of L1210 cell viability was produced by irradiation with 200 mJ/cm² using SnET2 or with 270 mJ/cm² with CPO. For Hepa 1c1c7 cells, an LD₅₀ dose was achieved by loading cultures with 2 mM SnET2 and irradiating with 45 mJ/cm² at 660 nm. Cell suspensions or cultures were supplemented with bile acids either before irradiation, after irradiation, or before and after irradiation as indicated in the text.

**Caspase-3 Assays.** Preliminary studies indicated that caspase-3 activation occurred very rapidly after irradiation of photosensitized L1210 cells but considerably slower with 1c1c7 cells. L1210 cells were collected 5 min after irradiation, washed, and lysed in 200 μl of buffer containing 50 mM Tris (pH 7.5), 0.03% NP40, and 1 mM DTT. The lysate was briefly sonicated, and the debris was removed by centrifugation at 10,000 × g for 1 min. The supernatant (100 μl) was mixed with 40 μl DEVD-R110, 10 mM HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM DTT in a total volume of 200 μl. The rate of increase in fluorescence emission, resulting from the release of rhodamine-110 from the fluorescent substrate, was measured using a fluorescence plate reader at room temperature.

After irradiation, Hepa 1c1c7 cells were maintained for 30–90 min at 37°C in a humidified 5% CO₂ chamber prior to being washed and harvested for caspase-3 analyses. The procedures used for preparation of lysates and the assay of caspase-3 using DEVD-AMC as substrate have been described in detail (17). Release of AMC was monitored using a fluorescence plate reader.

DEVDase activity in all cases was reported in terms of nmol product/mg protein/min. Control determinations were made on extracts of untreated cells or cells treated with drug vehicle alone. Each assay was performed with quadruplicate samples. The Bio-Rad assay, using BSA as a standard, was used to estimate protein concentrations.
Fluorescence Microscopy. Apoptotic nuclear morphology was assessed by labeling L1210 cells for 5 min at 37°C with HO342 (2 μg/ml). Nuclei were observed by fluorescence microscopy using 330–380 nm excitation and measuring fluorescence at 420–450 nm. Three fields of 100 cells were scored, and the percentage of cells exhibiting apoptotic morphology was determined. Loss of ΔΨm was measured by incubating cells for 5 min at 37°C in medium containing 300 μM MTO. Fluorescence (580–620 nm) was detected using 510–560 nm excitation.

Viability. Control and irradiated suspensions of L1210 cells were serially diluted, mixed in soft agar, plated, and cultured at 37°C in a humidified 5% CO2 chamber. Colonies were counted 5–7 days later. Subconfluent cultures of Hepa 1c1c7 cells were released from the culture dishes by trypsinization, washed, and subsequently suspended in culture medium and plated. Approximately 20 h later, cultures were treated with SnET2 and/or UDCA, washed, irradiated, and cultured at 37°C in a humidified 5% CO2 chamber. Colonies were scored 7–8 days later. Previous studies have shown that only a low percentage of Hepa 1c1c7 cells divide in the first 20 h after passing, and that this time is sufficient to replenish the glutathione lost from the cells as a consequence of trypsinization (18).

Intracellular Accumulation of CPO and SnET2. L1210 or Hepa 1c1c7 cells were incubated in growth medium containing 2 μM sensitizer ± 100 μM UDCA for 15 min (L1210) or 25 min (Hepa) at 37°C. Washed L1210 pellets were suspended in 3 ml of 10 mM Triton X-100. Hepa 1c1c7 cultures were washed with PBS, covered with 3 ml of 10 mM Triton X-100, and scraped from the plates. Levels of sensitizer in the cell lysates were assessed by fluorescence, using 400 nm excitation. A series of graded drug solutions in 10 mM Triton X-100 was used to produce a standard curve. Intracellular drug accumulations are reported as nmol/106 cells. We also examined the effect of UDCA on sites of sensitizer localization using fluorescence microscopy (15).

Western Blots for Cytochrome c. Control L1210 cells and cells after treatment with 100 μM UDCA, an LD50 PDT dose with SnET2, or the same PDT dose with 100 μM UDCA added during the incubation with SnET2 were collected by centrifugation. The procedure used for the Western blot detection of cytosolic cytochrome c has been described in detail and used 15% acrylamide-SDS gels (2).

Statistical Analyses. Data were analyzed by the Tukey HSD test. The Stata 5.0 software package (StaSoft, Inc., Tulsa, OK) was used to perform these calculations. Differences were considered statistically significant if P < 0.05.

RESULTS

Proapoptotic Effects of UDCA on Photodamaged L1210 Cells. Irradiation of L1210 cells loaded with 2 μM of the photosensitizing agents SnET2 or CPO for 15 min led to the appearance of 12 ± 4% apoptotic nuclei 60 min after irradiation and a ∼50% reduction in viability (Table 1; Fig. 2). Incubation of cells with 100 μM UDCA alone for 15 min, followed by a second incubation for 60 min in fresh medium, did not yield a detectable apoptotic response nor any loss of cell viability. In contrast, irradiation of cells loaded with either sensitizer + UDCA (extracellular concentration, 100 μM) greatly enhanced the number of apoptotic nuclei (Table 1; Fig. 2) and reduced viability to <10% (Table 1). A dose-response study revealed a relationship between the UDCA concentration and the activation of caspase-3, as measured by observing DEVDase activity after irradiation, using either sensitizer (Table 2).

Effects of UDCA on ΔΨm and Cytochrome c Release. Approximately 15% of the L1210 cells loaded with 2 μM SnET2 showed a rapid loss of ΔΨm after irradiation, as defined by a marked alteration in the MTO labeling pattern (Fig. 3, A versus B). When UDCA was included during the initial incubation with SnET2, a loss of ΔΨm was observed in >95% of cells after irradiation (Fig. 3C).

The loss of mitochondrial membrane potential is generally accompanied by release of cytochrome c into the cytosol. UDCA alone did not enhance cytochrome c release into the cytosol (Fig. 3, bottom panel). However, inclusion of UDCA at the time of loading with SnET2 markedly elevated cytochrome c release into the cytosol after irradiation (Fig. 3, bottom panel). A similar result was obtained with CPO (not shown).

Proapoptotic Effects of UDCA Conjugates. Administration of UDCA to humans results in a substantial conversion of UDCA to the glycine and taurine conjugates GUDCA and TUDCA, respectively.
Table 2: Effect of UDCA on PDT-induced DEVDase activity

<table>
<thead>
<tr>
<th>UDCA (µM)</th>
<th>No sensitizer</th>
<th>CPO</th>
<th>SnET2</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0.62 ± 0.011</td>
<td>1.69 ± 0.15</td>
<td>1.84 ± 0.28</td>
</tr>
<tr>
<td>10</td>
<td>0.074 ± 0.019</td>
<td>4.54 ± 0.72</td>
<td>3.75 ± 0.96</td>
</tr>
<tr>
<td>30</td>
<td>0.075 ± 0.013</td>
<td>6.2 ± 1.03</td>
<td>6.99 ± 1.83</td>
</tr>
<tr>
<td>100</td>
<td>0.128 ± 0.017</td>
<td>12.9 ± 5.7</td>
<td>14.68 ± 3.58</td>
</tr>
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</table>

*Significantly greater than the control value, P < 0.05.

DISCUSSION

Several earlier reports indicated that UDCA protected cells in culture from the apoptotic effects of a variety of stimuli (6–12). This protective effect was seen when the duration of irradiation was increased from 30 to 60 s. On the basis of the caspase-3 activation measurements, a concentration of 50 µM UDCA also potentiated the apoptotic response to PDT.

The results shown in Fig. 4 used a protocol in which UDCA was present both before and after irradiation. An additional experiment was carried out to assess the effect of adding UDCA only after irradiation. Data shown in Fig. 5 indicate that the addition of 20–100 µM UDCA after irradiation did not potentiate caspase-3 activation.

**Effects of UDCA on Accumulation of SnET2.** Cotreatment of either L1210 or Hepa 1c1c7 cells with 100 µM UDCA for 25 min had no effect on cell viability. Addition of varied concentrations of UDCA during the SnET2-loading incubation resulted in a concentration-dependent potentiation of cell killing by PDT (Table 3). When cultures were incubated with 100 µM UDCA, the LD₅₀ PDT dose with SnET2 became an LD₉₀ dose. Significant potentiation of cell killing was detected even with a 10 µM concentration of UDCA (Table 3).

**Table 3: Effect of UDCA on Hepa 1c1c7 viability after PDT**

<table>
<thead>
<tr>
<th>Sensitizer and UDCA (µM)</th>
<th>Colonies</th>
<th>% control</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>75 ± 5</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>74 ± 14</td>
<td>100 ± 18</td>
</tr>
<tr>
<td>SnET2</td>
<td>39 ± 16</td>
<td>52 ± 21</td>
</tr>
<tr>
<td>None</td>
<td>12 ± 5</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>100</td>
<td>9 ± 5</td>
<td>12 ± 6.5</td>
</tr>
<tr>
<td>SnET2</td>
<td>2.6 ± 1.5</td>
<td>3.5 ± 2</td>
</tr>
<tr>
<td>None</td>
<td>1.0 ± 0.7</td>
<td>1.3 ± 1</td>
</tr>
</tbody>
</table>

*Significantly less than survival measured in untreated and UDCA-treated cultures, P < 0.05.

**Fig. 3.** UDCA potentiation of PDT-mediated disruption of ΔΨm and cytochrome c release by L1210 cells. Mitochondrial membrane potential (ΔΨm) detected with MitoTracker: A, control L1210 cells; B, cells treated with 2 μM SnET2; C, cells treated with the same PDT dose but with 100 μM UDCA present during the loading incubation. Cells were labeled with MitoTracker directly after irradiation (270 mJ/cm²) at 10°C. Bar, 10 μm. Lower panel: release of cytochrome c from mitochondria into the cytosol using Western blot analysis with 10 μg of protein applied to each lane. From left: cytochrome c control (Cyto c); cytosol from control cells (Control); cells treated for 15 min at 37°C with 100 μM UDCA (UDCA); cells treated with SnET2 and light as described above (PDT); and cells given the same PDT dose with 100 μM UDCA present during the loading incubation (PDT/UDCA).

**Fig. 4.** Effects of UDCA on PDT-induced activation of caspase-3 in Hepa 1c1c7 cells. Cultures were incubated with 2 μM SnET2 and/or specified levels of UDCA for 25 min, washed, and then irradiated for 0, 30, or 60 s (0, 45, or 90 mJ/cm²). After irradiation, UDCA was added back, and the cultures were harvested 30, 60, and 90 min afterward for analyses of caspase-3 activity. Triangles, postirradiation time (30–90 min) that elapsed prior to harvesting for caspase assays. Data represent means of triplicate analyses; bars, SD.

**Fig. 5.** Effects of postirradiation addition of UDCA on PDT-induced activation of caspase-3. Hepa 1c1c7 cultures were incubated with 2 μM SnET2 for 25 min, washed, and then irradiated for 60 s (90 mJ/cm²). After irradiation, varied concentrations of UDCA were added back, and the cultures were harvested 90 min later for analyses of caspase-3 activity. Data represent means of triplicate analyses; bars, SD.
UDCA POTENTIATION OF PDT

Table 4 Effect of UDCA on sensitizer accumulation

<table>
<thead>
<tr>
<th>Photosensitizer</th>
<th>LI210</th>
<th>Hepa 1c1c7</th>
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<tbody>
<tr>
<td>CPO</td>
<td>116.4 ± 3.87</td>
<td>NA</td>
</tr>
<tr>
<td>CPO + UDCA</td>
<td>119.8 ± 5.47</td>
<td>NA</td>
</tr>
<tr>
<td>SnEt2</td>
<td>74.2 ± 4.33</td>
<td>33.8 ± 5.7</td>
</tr>
<tr>
<td>SnEt2 + UDCA</td>
<td>76.1 ± 6.26</td>
<td>27.9 ± 1.2</td>
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</tbody>
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UDCA may be a valuable addition to current protocols.

ACKNOWLEDGMENTS

We thank Patricia Mathieu and Ann Marie Santiago for excellent technical assistance.

REFERENCES