

Potential of Photodynamic Therapy by Ursodeoxycholic Acid¹

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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 porphycene 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

PDT³ 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 ($\Delta\Psi_m$), loss of cytochrome *c*, and initiation of a prompt apoptotic response (2). The latter is presumably mediated by activation of the Apaf-1-procaspase-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 M 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 fluorogenic 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 = 2×10^6 cells) were incubated in growth medium containing 2 μ M 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 μ M 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 μ M 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 μ l 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 $\times g$ for 1 min. The supernatant fluid (100 μ l) was mixed with 40 μ M DEVD-R110, 10 mM HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM DTT in a total volume of 200 μ l. The rate of increase in fluorescence emission, resulting from the release of rhodamine-110 from the fluorogenic substrate, was measured using a fluorescent 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 is 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.

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³ The abbreviations used are: PDT, photodynamic therapy; UDCA, ursodeoxycholic acid; TUDCA, tauro-UDCA; AMC, 7-amino-4-methylcoumarin; CPO, 9-capronyloxyl-tetrakis(methoxyethyl) porphycene; DEVD-AMC, asp-glu-val-asp-aminomethylcoumarin; DCA, deoxycholic acid; GUDCA, glyco-UDCA; HO342, Höchst dye HO33342; $\Delta\Psi_m$, mitochondrial membrane potential; MTO, MitoTracker Orange; SnET2, tin etiopurpurin.

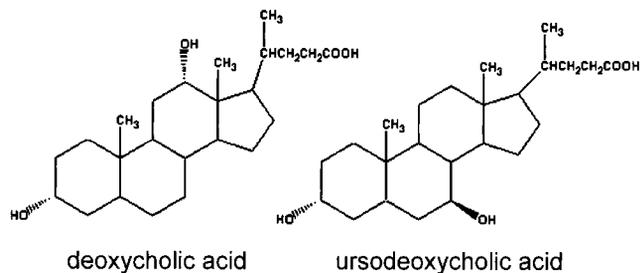


Fig. 1. Structures of DCA and UDCA.

Fluorescence Microscopy. Apoptotic nuclear morphology was assessed by labeling L1210 cells for 5 min at 37°C with HO342 (2 $\mu\text{g}/\text{ml}$). 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 $\Delta\Psi_m$ was measured by incubating cells for 5 min at 37°C in medium containing 300 nm 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% CO_2 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% CO_2 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 passaging, 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 \pm 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/ 10^6 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 LD_{50} 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 Statistica 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 $12 \pm 4\%$ apoptotic nuclei 60 min after irradiation and a $\sim 50\%$ 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 $\Delta\Psi_m$ and Cytochrome *c* Release. Approximately 15% of the L1210 cells loaded with 2 μM SnET2 showed a rapid loss of $\Delta\Psi_m$ 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 $\Delta\Psi_m$ 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 1 Effect of bile acids on viability after PDT

L1210 cells were incubated with 2 μM SnET2 or CPO and 100 μM UDCA or the corresponding glycine (GUDCA) or taurine (TUDCA) conjugates for 15 min at 37°C, washed, suspended in fresh medium, and irradiated at 660 or 610 nm as described in the legend to Fig. 2. Colonies were counted 5–7 days later. Numbers represent the mean \pm SD for three determinations.

Sensitizer and additions	Colonies	Apoptotic nuclei
None		
None	163 \pm 12	<2
UDCA	158 \pm 15	<2
GUDCA	167 \pm 8	<2
TUDCA	148 \pm 11	<2
SnET2		
None	91 \pm 5	12 \pm 4
UDCA	12 \pm 3 ^a	68 \pm 3 ^b
GUDCA	16 \pm 2 ^a	72 \pm 3 ^b
TUDCA	14 \pm 5 ^a	65 \pm 5 ^b
CPO		
None	83 \pm 5	14 \pm 6
UDCA	9 \pm 3 ^a	69 \pm 3 ^b
GUDCA	11 \pm 2 ^a	66 \pm 5 ^b
TUDCA	13 \pm 4 ^a	63 \pm 7 ^b

^aSignificantly less than cell survival in cultures irradiated in the absence of UDCA or a conjugate, $P < 0.05$.

^bSignificantly greater than the number of apoptotic nuclei in cells irradiated in the absence of UDCA or a conjugate, $P < 0.05$.

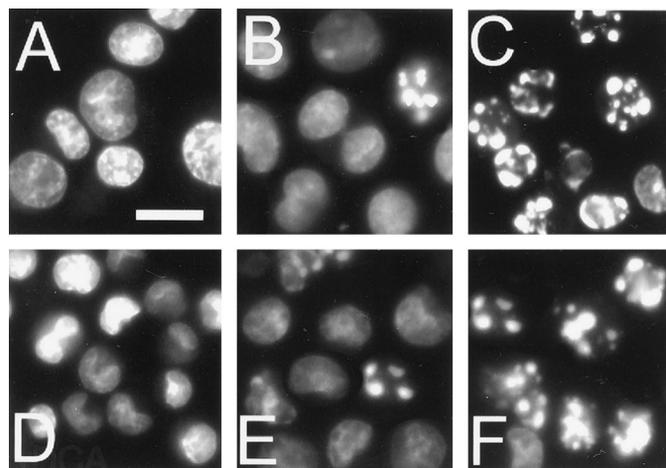


Fig. 2. Effect of UDCA on PDT-induced apoptosis in L1210 cells as detected by HO342 labeling. Cells were incubated with sensitizer and/or 100 μM UDCA for 15 min prior to being washed, refed, and irradiated. Cultures were incubated for an additional 60 min at 37°C before HO342 labeling. A, control cells; B, cells incubated with 2 μM CPO and irradiated (270 mJ/cm²); C, cells incubated with CPO + 100 μM UDCA and irradiated; D, control cells incubated with 100 μM UDCA; E, cells incubated with 2 μM SnET2 and irradiated (200 mJ/cm²); F, cells incubated with SnET2 + 100 μM UDCA and irradiated. Bar, 10 μm .

Table 2 Effect of UDCA on PDT-induced DEVDase activity

L1210 cells were incubated with no sensitizer, 2 μM CPO, or SnET2 plus specified concentrations of UDCA for 15 min at 37°C, resuspended in fresh medium, irradiated (see the legend to Fig. 2), and then incubated for an additional 5 min at 37°C. Levels of DEVDase were then measured. Data represent mean \pm SD for four determinations.

UDCA(μM)	DEVDase (nmol/min/mg protein)		
	No sensitizer	CPO	SnET2
0	0.62 \pm 0.011	1.69 \pm 0.15	1.84 \pm 0.28
10	0.074 \pm 0.019	4.54 \pm 0.72 ^a	3.75 \pm 0.96 ^a
30	0.075 \pm 0.013	6.2 \pm 1.03 ^a	6.99 \pm 1.83 ^a
100	0.128 \pm 0.017	12.9 \pm 2.7 ^a	14.68 \pm 3.58 ^a

^a Significantly greater than the control value, $P < 0.05$.

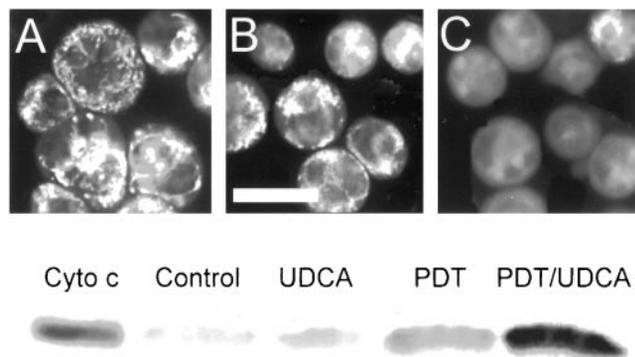


Fig. 3. UDCA potentiation of PDT-mediated disruption of $\Delta\Psi_m$ and cytochrome *c* release by L1210 cells. Mitochondrial membrane potential ($\Delta\Psi_m$) detected with MTO in: A, control L1210 cells; B, cells treated with 2 μM SnET2; C, cells treated with the same PDT dose but with 100 μM UDCA present during the loading incubation. Cells were labeled with MTO 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).

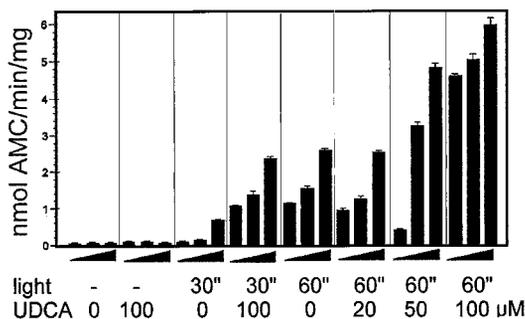


Fig. 4. Effects of UDCA on PDT-induced activation of caspase-3 in Hepa 1c1c7 cells. Cultures were incubated with 2 μM SnET2 and/or specified levels of UDCA for 25 min, washed, and then irradiated for 0, 30, or 60 s (0, 45, or 90 mJ/cm²). After irradiation, UDCA was added back, and the cultures were harvested 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 quadruplicate analyses; bars, SD.

(19). Neither GUDCA nor TUDCA was cytostatic or cytotoxic to L1210 cells, but both conjugates were as potent as UDCA in potentiating the cytotoxic effects of SnET2 when used in a PDT protocol (Table 1).

Caspase-3 Activation in Hepa 1c1c7 Cells. Treatment of Hepa 1c1c7 cells with 100 μM UDCA for 25 min did not result in the activation of caspase-3 (Fig. 4) nor the development of apoptotic nuclei (data not shown). A 30 s irradiation of Hepa 1c1c7 cultures treated with 2 μM SnET2 stimulated the activation of caspase-3. This activation was detected 90 min after irradiation. A concentration of 100 μM UDCA dramatically accelerated the kinetics of appearance and the magnitude of caspase-3 activation after a 30-min irradiation.

Similar effects were 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.

Clonogenic Studies with Hepa 1c1c7 Cells. Exposure of Hepa 1c1c7 cultures to 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 LD₉₉ dose. Significant potentiation of cell killing was detected even with a 10 μM concentration of UDCA (Table 3).

Effects of UDCA on Accumulation of SnET2. Cotreatment of either L1210 or Hepa 1c1c7 cells with 100 μM UDCA during the sensitizer-loading incubation did not promote accumulation of SnET2 (Table 4). Similarly, UDCA did not promote the accumulation of CPO in L1210 cells (Table 4). In other studies, we could not detect any effects of UDCA on subcellular sites of sensitizer localization (not shown).

DISCUSSION

Several earlier reports indicated that UDCA protected cells in culture from the apoptotic effects of a variety of stimuli (6–12). This protective

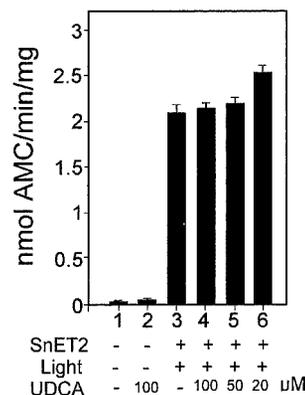


Fig. 5. Effects of postirradiation addition of UDCA on PDT-induced activation of caspase-3. Hepa 1c1c7 cultures were incubated with 2 μM SnET2 for 25 min, washed, and then irradiated for 60 s (90 mJ/cm²). After irradiation, varied concentrations of UDCA were added back, and the cultures were harvested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

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

Hepa 1c1c7 cultures were incubated with SnET2 (2 μM) and specified concentrations of UDCA for 25 min at 37°C, then transferred to fresh medium and irradiated at 660 nm (45 mJ/cm²). Colonies were counted 7–8 days later. Numbers represent the mean \pm SD for four culture dishes.

Sensitizer and UDCA (μM)	Colonies	% control
None		
None	75 \pm 5	100
100	74 \pm 14	100 \pm 18
SnET2		
None	39 \pm 16 ^a	52 \pm 21
10	12 \pm 5 ^b	20 \pm 7
20	9 \pm 5 ^b	12 \pm 6.5
50	2.6 \pm 1.5 ^b	3.5 \pm 2
100	1.0 \pm 0.7 ^b	1.3 \pm 1

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

^b Significantly less than survival in photosensitized and irradiated cells with no UDCA present, $P < 0.05$.

Table 4 Effect of UDCA on sensitizer accumulation

L1210 and Hepa 1c1c7 cells were incubated with 2 μM concentrations of specified sensitizer \pm 100 μM UDCA for 15 and 25 min, respectively, at 37°C prior to being washed and lysed for subsequent determination of intracellular content of sensitizer. Data represent the mean \pm SD for four to five determinations. The addition of UDCA did not significantly affect the accumulation of sensitizer by either cell line, NA, not assayed.

Photosensitizer	Accumulation (nmol/10 ⁶ cells)	
	L1210	Hepa 1c1c7
CPO	116.4 \pm 3.87	NA
CPO + UDCA	119.8 \pm 5.47	NA
SnET2	74.2 \pm 4.33	33.8 \pm 5.7
SnET2 + UDCA	76.1 \pm 6.26	27.9 \pm 1.2

mechanism was attributed to stabilization of the mitochondrial structure, thereby preventing the loss of cytochrome *c* (9–12). The latter event is known to trigger an apoptotic response via the Apaf-1/caspase-9 pathway (3). In this report, we describe a different effect of UDCA: promotion of the apoptotic response to mitochondrial photodamage.

UDCA at a 100 μM concentration, in the absence of any other treatment, neither induced caspase-3 activation in L1210 or 1c1c7 cells nor was cytotoxic. Similar effects have been noted by several groups in a variety of cultured cell types (6–12). However, coexposure of L1210 and 1c1c7 cells to mitochondrial sensitizers and UDCA prior to irradiation markedly potentiated PDT-induced apoptosis. This potentiation reflected enhanced loss of mitochondrial membrane potential, release of cytochrome *c*, and activation of caspase-3. The basis for the potentiating effects of UDCA is unclear. However, data shown in Table 4 clearly demonstrate that this potentiation does not reflect an enhanced accumulation of the sensitizer in UDCA-treated cells.

The very hydrophobic UDCA analogues, lithocholic acid and DCA, can elicit a very rapid apoptotic response (12), as can other agents with amphipathic properties, *e.g.*, Triton X-100 (20). These effects are likely the consequence of a chaotropic interaction with the mitochondrial membrane, leading to the spontaneous release of cytochrome *c*. UDCA has been shown to protect against the apoptogenic effects of lithocholic acid and DCA by competing with the hydrophobic bile acids for mitochondrial binding sites. We propose that UDCA alone does not cause mitochondrial membrane damage. Rather, it lowers the threshold at which photodamage destabilizes the mitochondrial membrane. This idea is supported by the finding that potentiation of PDT with mitochondrial sensitizers occurred when cells were exposed to UDCA prior to, but not after, irradiation (Figs. 4 and 5).

PDT is currently being investigated as a means for selective tumor eradication (1). The results described here indicate an enhanced phototoxic response when UDCA is used in conjunction with two photosensitizing agents that catalyze mitochondrial photodamage. A variety of other sensitizers fall into this class including Photofrin (21), benzoporphyrin derivative (22), and protoporphyrin derived from administration of 5-aminolevulinic acid (21). All of these agents have received Food and Drug Administration approval for photodynamic therapy or are in clinical trials. Several procedures have been suggested for enhancing the efficacy of PDT, including fractionated light dose (23) and hyperoxygenation of tissues (24). We propose that the use of UDCA may be a simpler approach to this same end.

Because UDCA has a long history of clinical safety (4, 5), addition of this agent to a clinical protocol might present a minimal challenge with regard to potential adverse reactions. In addition, UDCA has two features that facilitate its use in PDT protocols. Enhancement of the PDT response by UDCA only requires it to be present at the time of loading with the sensitizer. Moreover, the taurine and glycine conjugates of UDCA retain proapoptotic activity in PDT protocols with mitochondrial sensitizers. The latter is an important factor because

metabolism of UDCA to these conjugates occurs readily in humans (19). The potential for UDCA as a means for enhancing PDT efficacy *in vivo* remains to be established, especially with regard to selectivity for normal *versus* neoplastic tissues. Because many of the photosensitizing agents in current clinical use have mitochondrial targets, UDCA may be a valuable addition to current protocols.

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