

# Apoptosis Induction by the Photosensitizer Verteporfin: Identification of Mitochondrial Adenine Nucleotide Translocator as a Critical Target<sup>1</sup>

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## Abstract

We report that the photosensitizer verteporfin kills lymphoma cells by an apoptotic process involving a dissipation of the mitochondrial inner transmembrane potential ( $\Delta\Psi_m$ ). Light-activated verteporfin-induced apoptosis was abolished by transfection with Bcl-2, a procedure reported to inhibit the mitochondrial permeability transition pore complex (PTPC). Verteporfin triggered the  $\Delta\Psi_m$  loss in isolated mitochondria *in vitro*, and this effect was suppressed by bongrekic acid and cyclosporin A. Verteporfin plus light also permeabilized proteoliposomes containing the semipurified PTPC or the purified PTPC component adenine nucleotide translocator (ANT), yet had no effect on protein-free control liposomes. Verteporfin phototoxicity on ANT proteoliposomes was mediated by reactive oxygen species and was prevented by recombinant Bcl-2 or the adenine nucleotides ATP and ADP. In conclusion, verteporfin belongs to a class of clinically used chemotherapeutic agents acting on PTPC and ANT.

## Introduction

Recently, the mitochondrion has been proposed as a novel prospective target for chemotherapy-induced apoptosis (1–7). Indeed, four different anticancer agents, including the retinoic acid-derivative CD437, lonidamine, betulinic acid, and arsenite, have been shown to induce cancer cells apoptosis by a direct action on mitochondria. The interaction of these anticancer agents with mitochondria results in an increase of the permeability of the inner mitochondrial membrane attributable, at least in part, to the opening of the PTPC.<sup>4</sup> PTPC opening leads to swelling of the mitochondria matrix, the dissipation of the inner  $\Delta\Psi_m$ , enhanced generation of ROS, and the release of apoptogenic proteins from the intermembrane space to the cytoplasm. Such mitochondrial apoptogenic effectors include the caspase activator cytochrome *c*, AIF, and pro-caspases (2–6). All of the signs of apoptosis induced by CD437, lonidamine,

betulinic acid, and arsenite are prevented by two agents acting on specific PTPC proteins, namely CsA (a cyclophilin D ligand) and BA (a ligand of the ANT). It thus appears that PTPC opening is a critical event of apoptosis triggered by these agents.

The PTPC is a dynamic protein complex located at the contact site between the two mitochondrial membranes, its opening allowing the free diffusion of solutes  $M_r < 1500$  on the inner membrane. Formation of PTPC involves the association of proteins from different compartments, hexokinase (cytosol), porin, also called VDAC (outer membrane), PBR (outer membrane), ANT (inner membrane), and cyclophilin D (matrix). PTPC has been implicated in many examples of apoptosis because of its capacity to integrate multiple proapoptotic signal transduction pathways and because of its control by proteins from Bcl-2/Bax family (8, 9). The Bcl-2 family comprises death-inhibitory (Bcl-2-like) and death-inducing (Bax-like) members (10, 11), which, respectively, prevent or facilitate PTPC opening. Bax and Bcl-2 reportedly interact with VDAC (12) and ANT within PTPC (9, 13). In physiological conditions, ANT is a specific antiporter for ADP and ATP. However, ANT can also form a lethal pore on interaction with different proapoptotic agents including  $Ca^{2+}$ , Atr, the HIV-1 protein Vpr, and pro-oxidants (9, 13–15). Mitochondrial membrane permeabilization may also be regulated by the nonspecific VDAC pore modulated by Bcl-2/Bax-like proteins in the outer membrane (12, 16), and/or by changes in the metabolic ATP/ADP gradient between the mitochondrial matrix and the cytoplasm (17).

PDT is based on the combined use of light-absorbing compounds and light irradiation (18). PDT has been previously evaluated for treatment of tumors of bronchus, bladder, skin, head, neck, and cervix, and more recently for esophageal carcinoma or nonmalignant disorders such as age-related macular degeneration. As a consequence of their selective retention in cancer cells as compared with normal tissues, photosensitizers may kill cells by the local production of ROS on light irradiation. PDT has also been used as an *ex vivo* purging procedure for leukemia cells (19). Constant interest in producing new photosensitizers allowed production of a second generation of compounds including benzoporphyrin derivatives, which exhibit superior pharmacological and photochemical properties with minimal dark toxicity. Verteporfin or benzoporphyrin derivative monoacid ring A, is a photosensitizer that induces apoptosis in cancer cells and tumor cell lines (reviewed in Ref. 20). Cancer cells treated with verteporfin plus red light exhibit a rapid cytochrome *c* release into the cytoplasm, followed by caspase activation (21–24). Conflicting results have been reported on Bcl-2, which, depending on the concentration and the cell line studied, would inhibit all, some, or none of the signs of verteporfin-induced cell death (Ref. 25; for review, see Ref. 20). Although the efficacy of verteporfin to elicit proapoptotic mitochondrial photodamage has been clearly demonstrated in various cell lines, its primary molecular target in mitochondria has been elusive.

These observations prompted us to examine the functional interac-

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<sup>4</sup> The abbreviations used are: PTPC, permeability transition pore complex; ANT, adenine nucleotide translocator; Atr, atractyloside; BA, bongrekic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CsA, cyclosporin A; DiOC(6)<sub>3</sub>, 3-3'-dihexyloxacarbocyanine iodide;  $\Delta\Psi_m$ , mitochondrial transmembrane potential; PC, phosphatidylcholine; ROS, reactive oxygen species; 4-MUP, 4-methylumbelliferyl phosphate; 4-MU, 4-methylumbelliferone; PDT, photodynamic therapy; JCl1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; PBR, peripheral benzodiazepine receptor; SOD, superoxide dismutase; VDAC, voltage-dependent anion channel.

tion between verteporfin, PTPC components, and the oncoprotein Bcl-2. Using a variety of different experimental set-ups, studies of intact cells, purified mitochondria, proteoliposomes containing PTPC, or defined PTPC components, we show here that ANT is a critical target of verteporfin-dependent photochemotherapy. We also provide evidence that Bcl-2 regulates verteporfin-mediated membrane permeabilization via an effect on ANT.

## Materials and Methods

**Chemicals and Recombinant Protein.** When not specified, chemicals were purchased from SIGMA. Recombinant Bcl-2 was prepared as described previously (26).

**Photoactivation of Verteporfin.** Verteporfin was added to cells, mitochondria, or liposomes at the indicated dose. After 1-h, 5-min, or 10-min incubations, respectively, in the dark, photoactivation was performed with a tungsten-halogen lamp (100W) with a 650-nm-long pass filter in front of it at a dose of  $2\text{J}/\text{cm}^2$  as published previously (23).

**Cell Lines, Immunoblot, and Cytofluorometric Analysis of Apoptosis.** Jurkat cells (J-NEO) or Jurkat cells overexpressing Bcl-2 (J-Bcl-2) were kindly provided by N. Israel (Ref. 27; Pasteur Institute, Paris, France) and were cultured in DMEM supplemented with 10% heat-inactivated FCS and antibiotics at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$ . Bcl-2 and ANT expression levels in J-NEO and J-Bcl-2 were analyzed by immunoblot (12.5% SDS-PAGE; 500- $\mu\text{g}$  cell lysates/protein/lane) using a polyclonal rabbit antiserum recognizing ANT and a monoclonal antibody recognizing Bcl-2 (4C11; Santa-Cruz, CA). One  $\times 10^6$  cells/ml were treated with various doses of verteporfin for 1 h at  $37^\circ\text{C}$ . Cells were illuminated (or not) and then labeled with 40 nM DiOC(6)<sub>3</sub> (Molecular Probes, Eugene, OR) and 2  $\mu\text{M}$  dihydroethidine (hydroethidine; Molecular Probes, Eugene, OR) for 10 min at  $37^\circ\text{C}$ . Cells were then analyzed by cytofluorometry (FACSVantage, Becton Dickinson) as described previously (28).

**Flow Cytometric Analysis of Purified Mitochondria.** Mouse liver purified mitochondria were resuspended in a buffer containing 200 mM sucrose, 10 mM Tris-3-[N-morpholino]propanesulfonic acid (pH 7.4), 5 mM Tris-succinate, 1 mM Tris-phosphate, 2  $\mu\text{M}$  rotenone, and 10  $\mu\text{M}$  EGTA. Cytofluorometric (FACSVantage, Becton Dickinson) detection was restricted to mitochondria by gating on the FSC/sideward light scatters parameters and on the main peak of the FSC-W parameter. Confirmation *a posteriori* of the validity of the double gating was obtained by labeling of mitochondria with the  $\Delta\Psi\text{m}$ -insensitive mitochondrial dye MitoTracker Green (75 nM; Molecular Probes; green fluorescence). To determine the percentage of mitochondria having a low  $\Delta\Psi\text{m}$ , the  $\Delta\Psi\text{m}$ -sensitive fluorochrome JC 1 (200 nM; 570–595 nm) was added 10 min before CCCP or verteporfin. Percentage of mitochondria having a low  $\Delta\Psi\text{m}$  was determined in dot-plot FSC/FL-2 (red fluorescence) windows.

**PTPC Purification and Reconstitution in Liposomes.** PTPC was purified and reconstituted in liposomes after previous protocols (8). Briefly, four Wistar rat brains (3-month-old males) were homogenized in 40 ml of 1 mM  $\alpha$ -monothio-glycerol-10 mM glucose (pH 8.0) and were centrifuged twice (15 min,  $12,000 \times g$ ,  $4^\circ\text{C}$ ). The pellets were resuspended in the same buffer + 0.5% (v:v) Triton X-100 (Boehringer Mannheim, Mannheim, Germany) for 30 min at room temperature while stirring. The Triton-soluble protein fraction obtained by ultracentrifugation (40 min,  $50,000 \times g$ ,  $4^\circ\text{C}$ ) was mixed with 17 g of DE52 resin previously equilibrated with a buffer containing 1.5 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{K}_2\text{HPO}_4$ , 100 mM glucose, and 1 mM DTT (pH 8.0). The resin was packed into an FPLC column (XK16/20; Pharmacia) and eluted with previous buffer supplemented with 50 mM KCl or 400 mM KCl. After equilibration (0.8 ml/min, 6 ml), elution was performed on a linear gradient from 50 to 400 mM KCl, followed by determination of hexokinase activity. Lipid vesicles were prepared by mixing 100 mg of PC and 20 mg of cholesterol in 1 ml of chloroform, evaporation of the chloroform under nitrogen, and resuspension in 1 ml of liposomes buffer [125 mM sucrose + 10 mM HEPES (pH 7.4)] + 0.3% *n*-octyl- $\beta$ -D-pyranoside by vortexing (40 min, room temperature). One volume of liposomes was incubated with one volume of PTPC-containing fraction during 20 min at room temperature and dialyzed overnight against liposomes buffer at  $4^\circ\text{C}$ .

**ANT Purification and Reconstitution in Liposomes.** ANT was purified from rat heart mitochondria as described previously (9). After mechanical shearing, mitochondria were suspended in 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 200  $\mu\text{M}$  EDTA, 10 mM DTT, and 0.5 mg/ml subtilisin (pH 7.4), kept 8 min on ice, and sedimented twice by differential centrifugations (5 min,  $500 \times g$ ,

and 10 min,  $10,000 \times g$ ). Mitochondrial proteins were solubilized by 6% (v:v) Triton X-100 (Boehringer Mannheim) in 40 mM  $\text{K}_2\text{HPO}_4$ , 40 mM KCl, and 2 mM EDTA (pH 6.0), for 6 min at room temperature, and solubilized proteins were recovered by ultracentrifugation (30 min,  $24,000 \times g$ ,  $4^\circ\text{C}$ ). Then, 2 ml of this Triton X-100 extract were applied to a column filled with 1 g of hydroxyapatite (BioGel HTP; Bio-Rad), eluted with previous buffer, and diluted (v:v) with 20 mM MES, 200  $\mu\text{M}$  EDTA, and 0.5% Triton X-100 (pH 6.0). Subsequently, the sample was separated with a Hitrap SP column using a FPLC system (Pharmacia) and a linear NaCl gradient (0–1 M). Proteins concentration was determined using microBCA-assay (Pierce, Rockford, IL). Purified ANT and/or recombinant Bcl-2 were reconstituted in PC/cardioliipin liposomes. Briefly, to prepare liposomes, 45 mg of PC and 1 mg of cardioliipin were mixed in 1 ml of chloroform, and the solvent was evaporated under nitrogen. Dry lipids were resuspended in 1 ml of liposomes buffer containing 0.3% *n*-octyl- $\beta$ -D-glucopyranoside and mixed by continuous vortexing for 40 min at room temperature. ANT (0.1 mg/ml) and/or recombinant Bcl-2 (0.1 mg/ml) were then mixed with liposomes (v:v) and incubated for 20 min at room temperature. Proteoliposomes were finally dialyzed overnight at  $4^\circ\text{C}$ .

**ADP/ATP Translocase Assay.** ANT liposomes were sonicated in the presence of 5 mM ATP and 10 mM KCl (50 W, 22 s; Branson sonifier 250) on ice. Then, liposomes were separated on Sephadex G-25 columns (PD-10; Pharmacia) from unencapsulated products. 25- $\mu\text{l}$  aliquots of liposomes were mixed with various concentrations of ADP, verteporfin, or diamide, illuminated (or not), and incubated for 1 h at room temperature. Luciferase (25  $\mu\text{l}$ , 0.5 units/ml; Boehringer Mannheim) was added, and the emitted luminescence was determined using a Perkin-Elmer spectrophotometer. Results are expressed as the percentage of maximum translocase activity, as determined by the addition of 400  $\mu\text{M}$  ADP.

**Pore Opening Assay.** PTPC or ANT proteoliposomes were sonicated in the presence of 1 mM 4-MUP and 10 mM KCl (50W, 22 s; Branson sonifier 250) on ice as described previously (15). Then, liposomes were separated on Sephadex G-25 columns (PD-10; Pharmacia) from unencapsulated products. Aliquots (25- $\mu\text{l}$ ) of liposomes were diluted to 3 ml in 10 mM HEPES-125 mM saccharose (pH 7.4), mixed with various concentrations of the proapoptotic inducers Atr or verteporfin, illuminated (or not), and incubated for 1 h at room temperature. Potential inhibitors of mitochondrial membranes permeabilization such as histidine, SOD, catalase, DTT, ATP, and ADP, were added to the liposomes 30 min prior to verteporfin treatment. After addition of 10  $\mu\text{l}$  of alkaline phosphatase (5 units/ml; Boehringer Mannheim) diluted in liposomes buffer + 0.5 mM  $\text{MgCl}_2$ , samples were incubated for 15 min at  $37^\circ\text{C}$  under agitation, and the enzymatic conversion of 4-MUP in 4-MU was stopped by the addition of 150  $\mu\text{l}$  of Stop buffer [10 mM HEPES-NaOH and 200 mM EDTA (pH 10)]. Fluorescence was subsequently determined using a Perkin-Elmer spectrofluorometer. Atr, a proapoptotic permeability transition inducer, was used in each experiment as a standard to determine the 100% response. The percentage of 4-MUP release induced by verteporfin was calculated as follows:

$$\frac{\text{Fluorescence of liposomes treated by verteporfin} - \text{fluorescence of untreated liposomes}}{\text{Fluorescence of liposomes treated by Atr} - \text{fluorescence of untreated liposomes}} \times 100$$

## Results and Discussion

**Verteporfin Induces Apoptosis in Jurkat Cells.** The general principles of the apoptotic pathway appear to be similar in different cell types (29). We have used the widely studied human Jurkat T leukemia cell line to address the apoptosis-inducing mode of action of verteporfin. Jurkat cells, overexpressing (J-Bcl-2) or not (J-Neo) Bcl-2 in mitochondria (Fig. 1A), were cultured in the presence of verteporfin and irradiated by red light. Then mitochondrial and nuclear parameters of apoptosis were assessed by flow cytofluorometry as described in Material and Methods. The combination of verteporfin plus light, but not verteporfin alone, kill Jurkat cells in a dose-dependent manner (Fig. 1). When used at a dose of 140 nM, verteporfin induced a significant loss of the mitochondrial inner membrane potential ( $\Delta\Psi\text{m}$ ) 3 h after light irradiation, as shown by a decrease in the DiOC(6)<sub>3</sub> fluorescence (Fig. 1B). This event was accompanied by

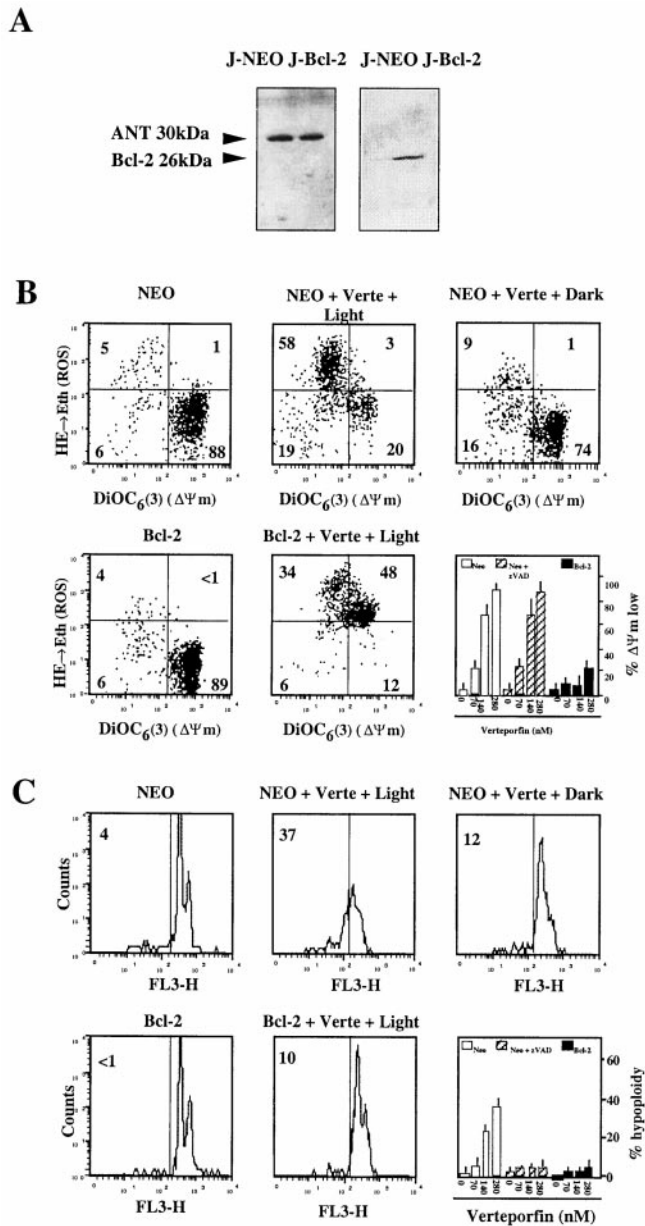


Fig. 1. Verteporfin induces a Bcl-2-dependent apoptosis in Jurkat cells. *A*, Bcl-2 and ANT expression levels in Jurkat cells (*J-NEO*) and Jurkat cells hyperexpressing Bcl-2 (*J-Bcl-2*). Cell lysate proteins (500  $\mu$ g) of *J-NEO* and *J-Bcl-2* were analyzed by immunoblot to detect ANT and Bcl-2. *Arrows*, the presence of ANT in *J-NEO* and *J-Bcl-2* as positive controls and the presence of Bcl-2 in *J-Bcl-2*. *B*, verteporfin induces the loss of  $\Delta\Psi_m$  and the generation of ROS. Jurkat cells (*J-NEO*) and Jurkat cells hyperexpressing Bcl-2 (*J-Bcl-2*) were treated with verteporfin (*Verte*) and irradiated with red light. Five  $\mu$ M CsA or 50  $\mu$ M z.VAD.fmk were added 15 min prior to verteporfin treatment. After 3-h culture, cells were labeled with 40 nM *DiOC<sub>6</sub>(3)* and 2  $\mu$ M hydroethidine and were analyzed by cytofluorometry. *C*, verteporfin induces nuclear signs of apoptosis. The frequency of hypoploid cells was determined by propidium iodide staining of ethanol-permeabilized cells.

Can enhanced generation of ROS capable of oxidizing hydroethidine oxidation to ethidium (Fig. 1*B*), as well as 8 h later by nuclear DNA loss, as shown by the staining with the DNA intercalating dye propidium iodide (Fig. 1*C*). No toxicity of verteporfin was observed in the absence of light (Fig. 1, *B* and *C*). As expected, preincubation of cells with zVAD, the caspase inhibitor, failed to inhibit the verteporfin-induced  $\Delta\Psi_m$  dissipation (Fig. 1*B*) but prevented the appearance of hypoploid cells (Fig. 1*C*).

Overexpression of Bcl-2, an inhibitory protein that stabilizes the barrier function of mitochondrial membranes (29–31), inhibited the  $\Delta\Psi_m$  dissipation (Fig. 1*B*). However, Bcl-2 did not reduce the

duction of ROS induced by verteporfin plus light. This latter observation suggests that ROS production may be stimulated independently of the  $\Delta\Psi_m$  loss, presumably by the photoactivation process of verteporfin itself, which leads to the local generation of ROS. Although Bcl-2 has been initially proposed to function as an antioxidant (32), this result is in agreement with several reports that indicate Bcl-2 protects from apoptosis via a mechanism that does not rely on the inhibition of ROS production or activity (33, 34).

Collectively, these data indicate that light-activated verteporfin elicits a mitochondrion-dependent apoptosis under the control of Bcl-2. Because Bcl-2 generally inhibits cell death induced by chemotherapeutic agents acting on PTPC, namely CD437, lonidamine, betulinic acid, and arsenite (3–6), a hypothesis was that verteporfin may act on mitochondria through the PTPC.

**Verteporfin Can Act Directly on Isolated Mitochondria.** To determine possible direct effects of verteporfin on mitochondria, these organelles were purified from healthy mouse liver and treated with verteporfin plus light; their  $\Delta\Psi_m$  was determined by flow cytofluorometry using the potential sensitive dye JC1. When mitochondria were incubated with the potentially sensitive fluorochrome JC1 (Fig. 2, *upper panels*), they exhibited a fluorescence significantly higher than unlabeled mitochondria (Fig. 2, *upper panel, Co.*). This JC1-dependent fluorescence was reduced by depolarization of the  $\Delta\Psi_m$  with the protonophore CCCP (Fig. 2, *upper panel, JC1+CCCP*). Verteporfin triggered a rapid loss of the  $\Delta\Psi_m$  measured with JC1, within less than 5 min (Fig. 2, *lower panel, JC1+verteporfin*). This effect was complete, as compared with that of the maximum  $\Delta\Psi_m$  depolarizing agent CCCP (Fig. 2, *upper panel, JC1+CCCP*). The  $\Delta\Psi_m$  loss triggered by verteporfin was inhibited by pretreatment of mitochondria with BA and CsA, two PT inhibitors (Fig. 2, *lower panel*), which suggests again that verteporfin mediates its proapoptotic activity by acting on PTPC. In addition, this indicated that verteporfin toxicity did not require any pre-mitochondrial activation of an intermediate cytoplasmic compound which, in turn, would act on mitochondria. We conclude that verteporfin can exert its toxicity directly on isolated mitochondria.

**Verteporfin Effect on PTPC Reconstituted into Liposomes.** We have previously shown that rat brain PTPC can be purified and

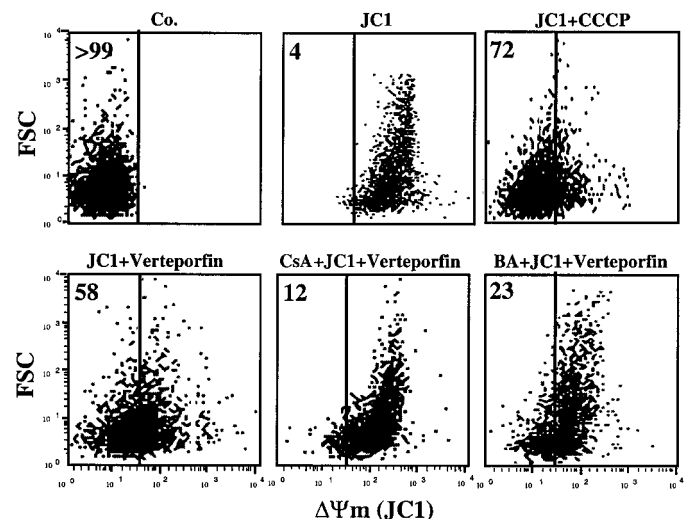


Fig. 2. Verteporfin triggers dissipation of  $\Delta\Psi_m$  in isolated mitochondria. Purified mouse liver mitochondria (40  $\mu$ g of proteins) were incubated or not (*Co.*) with the  $\Delta\Psi_m$ -sensitive dye JC1 (200 nM; 10 min), followed by the addition of CCCP (100  $\mu$ M; 5 min.) and/or verteporfin (100 ng/ml; 10 min.). Thereafter, mitochondria were subjected to flow cytometric determination of the fluorescence (570–595 nm) and the particle size (FSC). *Numbers*, the percentage of JC1-<sup>10w</sup> mitochondria among  $\sim 10^4$  events. Pretreatment of mitochondria with the PTPC inhibitors CsA (5  $\mu$ M; 5 min.) or BA (50  $\mu$ M; 5 min.) strongly reduce the  $\Delta\Psi_m$  dissipation induced by verteporfin. One experiment representative of three is shown.

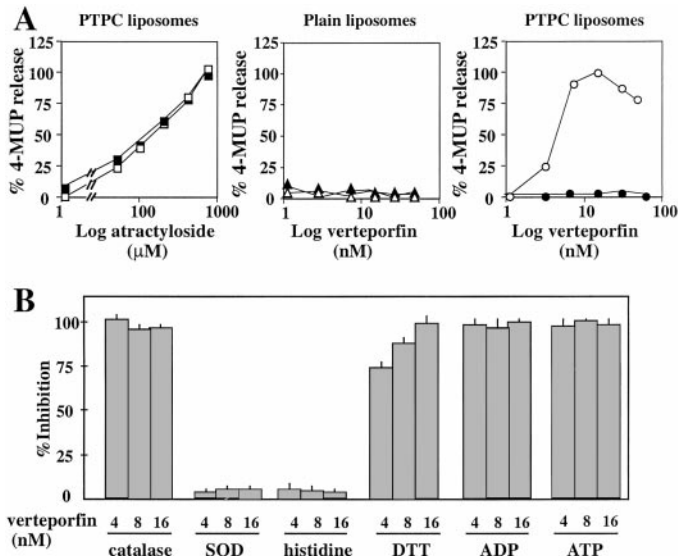


Fig. 3. Verteporfin induces a liposomal membrane permeabilization via the opening of PTPC pore. *A*, verteporfin induces the release of encapsulated 4-MUP from PTPC liposomes. PTPC liposomes were treated with Atr or verteporfin and illuminated (*open symbols*) or not (*full symbols*) with red light. In addition, protein-free vesicles (*Plain liposomes*; phosphatidylcholine:cholesterol, 5:1, w:w) were treated with verteporfin. After 1 h of incubation, the released 4-MUP was converted to 4-MU and determined spectrofluorometrically, as described in "Materials and Methods." *B*, inhibitory profile of verteporfin-induced PTPC-dependent membrane permeabilization. Before verteporfin plus light treatment, PTPC-liposomes were incubated for 30 min with 500 units/ml catalase, 500 units/ml SOD, 1 mM histidine, 1 mM DTT, 1 mM ADP or 1 mM ATP. The release of 4-MUP was determined as above and the inhibition of 4-MUP release ( $X \pm \text{SD}$ ;  $n = 3$ ) was calculated.

reconstituted into liposomes to mimic the function of mitochondrial PTPC *in vitro* (8, 9, 13, 15). On treatment with inducers of mitochondrial permeability transition, the PTPC response consists in an increase in liposomal permeability that can be measured as the release of a liposome-encapsulated substrate, 4-MUP, which becomes accessible to externally added alkaline phosphatase to yield the fluorescent product 4-MU (15). Verteporfin plus light, but not verteporfin alone, induced the release of 4-MUP from PTPC liposomes, yet had no effect on protein-free control liposomes (Fig. 3A). The verteporfin-induced 4-MUP release was dose-dependent and attained the same magnitude as did the 4-MUP release induced by the Atr, a proapoptotic ligand of ANT (Fig. 3A). To further investigate the mechanism of action of verteporfin, we evaluated a series of ROS-detoxifying agents that were added to PTPC containing liposomes prior to treatment with verteporfin plus light. Catalase, an  $\text{H}_2\text{O}_2$ -scavenging enzyme, and DTT, a thiol reducing agent, fully inhibited the membrane permeabilization induced by verteporfin plus light. In contrast, SOD and histidine, a singlet oxygen quencher, failed to inhibit the permeabilization of liposomal membranes under the same conditions (Fig. 3B). These results suggest that the PTPC opening effect of light-activated verteporfin are mediated, at least in part, by  $\text{H}_2\text{O}_2$  and/or thiol oxidation of PTPC components. ATP and ADP, the two physiological ligands of ANT, also inhibited the effect of verteporfin plus light on PTPC, thus confirming that ANT constitutes (one) of the physical target(s) of the photosensitizer within PTPC (Fig. 3B).

**Verteporfin Effects on ANT Reconstituted in Liposomes.** In an attempt to identify the functional target of verteporfin within PTPC, we purified rat heart ANT to homogeneity and reconstituted it into phosphatidylcholine/cardiophilip liposomes. In a similar experimental set-up as for PTPC, we showed that verteporfin plus light induced the permeabilization of ANT proteoliposomes (Fig. 4A). As expected, verteporfin did not trigger the opening of the ANT pore in the absence of light irradiation, nor did it induce permeabilization of plain liposomes, underscoring the

specificity of the verteporfin effect for ANT and its absence of effects on lipids and notably cardiolipin, a phospholipid that is reputed for its sensitivity to ROS (35). In contrast to diamide, a proapoptotic thiol-oxidating agent (15), verteporfin had no inhibitory effect on the antiporter function of ANT (Fig. 4B). Again, the two ligands of ANT, ATP and ADP, prevented the pore opening effect of verteporfin plus light (Fig. 4C). Pretreatment of ANT liposomes with DTT and catalase, confirmed that the light-activated verteporfin effect is mediated by an oxidative modification of ANT by  $\text{H}_2\text{O}_2$  and/or by thiol oxidation. In contrast, SOD did not inhibit the light-activated verteporfin-induced permeabilization of ANT liposomes, indicating an oxidative mechanism independent of the anion superoxide generation or, alternatively, an incapacity of SOD to intercept the formation of anion superoxide as an intermediate in the cascade of reactive oxygen intermediates triggered by the photoactivation of verteporfin (Fig. 4D).

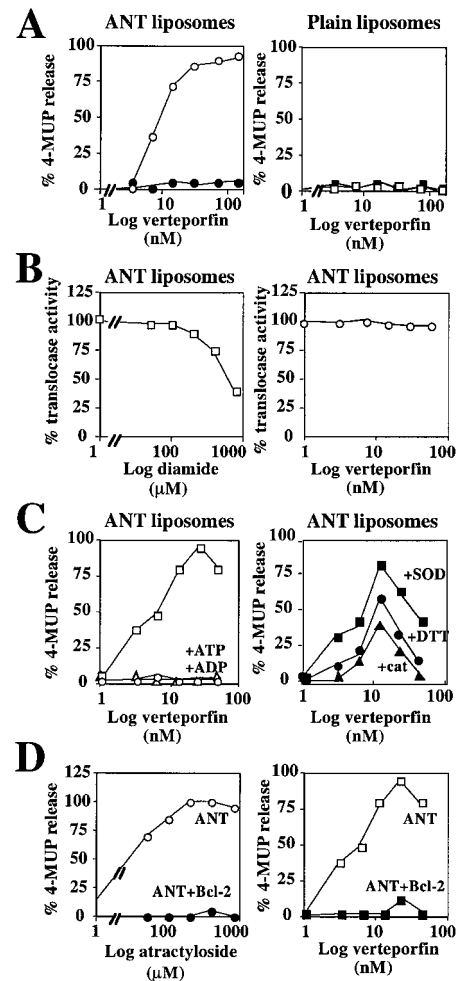


Fig. 4. Verteporfin effects on liposomes containing ANT. ANT-, ANT-Bcl-2- and plain phosphatidylcholine/cardiophilip liposomes (45:1, w:w) were generated and either 4-MUP or ATP were encapsulated. Liposomes were treated with various agents, illuminated or not with red light, and incubated for 1 h. The percentage of 4-MUP release (indicative of membrane permeabilization) or ATP translocated (indicative of enzymatic translocase activity) were determined as described in "Materials and Methods." *A*, verteporfin plus light opens the ANT pore. The effect of verteporfin was determined on ANT-liposomes or plain liposomes after light irradiation (*open symbols*) or not (*full symbols*). *B*, verteporfin does not alter the translocator function of ANT. ANT liposomes containing ATP were pretreated with diamide or verteporfin, illuminated with red light, and then treated with 400  $\mu\text{M}$  ADP. The ADP-induced ATP release was then quantitated by means of the luciferase chemoluminescence assay. *C*, the ANT pore opening is mediated by ROS production and can be prevented by ANT ligands. ANT liposomes were left untreated ( $\square$ ) or were preincubated with 1 mM ATP ( $\circ$ ), 1 mM ADP ( $\triangle$ ), 1 mM DTT, 500 units/ml catalase ( $\blacktriangle$ ), and 500 units/ml SOD before verteporfin plus light was applied. *D*, Bcl-2 inhibits the verteporfin pore-opening effect on ANT. ANT liposomes (*open symbols*) and ANT-Bcl-2 liposomes (*full symbols*) were incubated with Atr (*circles*) or verteporfin (*squares*) and illuminated with red light. Experiments are representative of three independent determinations.

As shown above, Bcl-2 prevents the mitochondrial membrane permeabilization induced by verteporfin plus light in intact cells (Fig. 1). Because ANT appears (one of) the verteporfin targets in mitochondria, we wondered whether Bcl-2 exerts its protective effect via ANT. To clarify this issue, verteporfin was added to proteoliposomes containing either ANT alone or ANT combined with recombinant Bcl-2. In this chemically defined, synthetic set-up, Bcl-2 totally inhibited the ANT-dependent permeabilization of membranes induced by verteporfin plus light (Fig. 4D). These data constitute the first demonstration, to our knowledge, that a clinically relevant chemotherapeutic agent may act on ANT to exert its cytotoxic function. Moreover, they establish for the first time that Bcl-2 may intercept proapoptotic photochemotherapy at the level of ANT. In conclusion, based on the present results, ANT becomes a plausible target of the photosensitizer verteporfin. Of note, the PBR has been proposed in the past to be the mitochondrial receptor for porphyrin derivatives (25, 36, 37), and PBR has been shown to physically interact with ANT (38). However, PBR may not be the only target of verteporfin, given that Jurkat cells lacking PBR expression (39) are efficiently killed by this porphyrin derivative. It remains possible, however, that interaction of porphyrins with PBR ameliorates their proapoptotic potential, by increasing the local concentration of verteporfin, near to its true physiological target, ANT. Irrespective of these theoretical considerations, the present data suggest ANT to be a target of at least one photosensitizing agent. Future studies will determine whether the active search for agents acting on ANT will yield a new class of anticancer agents.

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