γ-Tocopheryl quinone induces apoptosis in cancer cells via caspase-9 activation and cytochrome c release

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Recently, it was suggested the potential role of γ-tocopheryl quinone (γ-TQ), an oxidative metabolite of γ-tocopherol, as a powerful chemotherapeutic agent, since it was shown that this molecule exerts powerful cytotoxic effects, induces apoptosis and escapes drug resistance in human acute lymphoblastic leukemia and promyelocytic leukemia cells. We have studied the apoptogenic potential of γ-TQ in cultured human leukemia HL-60 and colon adenocarcinoma WiDr cells, and in murine thymoma cells growing in ascites form. The cells were treated with γ-TQ and apoptosis was evaluated morphologically by acridine-orange staining and cytometrically by Annexin V binding assay. γ-TQ-induced apoptosis in a dose- and time-dependent manner in all the cell types tested, although HL-60 and thymoma cells were much more sensitive than WiDr cells. In HL-60 cells apoptosis was mediated by the activation of the caspase-3 cascade. In particular, we observed a time- and dose-dependent increase in the activities of the upstream caspase-9 and caspase-8 and of the downstream caspase-3. The activation of caspase-9 preceded that of caspase-8 and its specific inhibition completely prevented apoptosis. These findings and data showing the precocious release of cytochrome c from mitochondria, a decrease in Bcl-2, and a change in mitochondrial transmembrane potential (ΔΨm), all suggest that the intrinsic mitochondrial pathway is primarily involved in the development of γ-TQ-induced apoptosis. The late activation of caspase-8 and data showing the partial cleavage of pro-apoptotic protein BID suggest that the initial activation of caspase-9 may be potentiated by a feedback amplification loop involving the caspase-8/BID pathway.

Introduction

In recent years, different components and derivatives of the vitamin E group of compounds including α-tocopherol (α-T) (1,2), α-tocopherol succinate (3), δ-tocopherol (4), γ-tocopherol (γ-T) (5,6) and γ-, α-, and δ-isoforms of tocotrienols (4,7) have received particular attention as chemotherapeutic agents. An anticancer therapeutic role for the oxidative derivatives γ- and δ-tocopheryl quinone (γ-TQ and δ-TQ) was put forward recently by Cornwell et al. (8–10). Quinones represent a clinically important category of chemotherapeutic agents with a wide range of applications in both antitumor and anti-microbial therapy (11–13). Some peculiar features of tocopherol quinones make them particularly interesting. In acute lymphoblastic and promyelocytic leukemia cell lines, γ-TQ and δ-TQ were more cytotoxic than doxorubicin, a xenobiotic quinone currently used in chemotherapy (8–10). The cytotoxicity of γ-TQ appeared related to its property as an arylating electrophile, as the non-arylation compounds, α-tocopherylquinone (α-TQ) and the glutathion-S-yl derivative of γ-TQ, are not cytotoxic (8–10). γ-TQ retained its cytotoxicity when tested in multidrug resistance (MDR) leukemic cell lines (8–10), differing in this behavior from many xenobiotics widely used in cancer chemotherapy and known to stimulate MDR (14–16).

Unlike α-TQ, significant amounts of γ-TQ are not identified in animal tissues for several reasons. The concentration of precursor γ-T is low in most tissues because the liver synthesizes a specific transport protein for α-T (17). Furthermore, large amounts of γ-T are selectively metabolized to γ-[2,7,8-trimethyl-2-(beta-carboxyethyl)-6-hydroxy chroman] (γ-CEHC) and excreted in the urine (18). On the other hand, γ-T is widely distributed and abundant in vegetable oils (19). It is plausible that the inability of the tissues to retain γ-T represents an evolutionary advantage by allowing the production of only small amounts of its mutagenic metabolite γ-TQ (20). Finally, γ-TQ is an arylating electrophile rapidly converted to the glutathion-S-yl and other nucleophile derivatives (8) that, unlike α-TQ, would not be identified by conventional assay methods. It has been suggested that, even though scarce, the possible tissue production of γ-TQ may induce a state of tolerance toward it, that would explain the ability of this quinone to escape MDR (8).

Recently it has been shown that the effect of γ-TQ, similarly to different cytotoxic quinones used in chemotherapy, including doxorubicin (21), mitomycin C (22,23) and menadione (24), is mainly related to its ability to induce apoptosis in leukemia cells and in breast cancer cells (10). In the present investigation, we have evaluated the apoptogenic potential of γ-TQ in WiDr colonic adenocarcinoma, HL-60 leukemia and murine thymocyte cells, and studied pathways through which γ-TQ mediates the expression of different proteins involved in the apoptotic cascade. We found the activation of the mitochondrial death pathway characterized by the disruption of mitochondrial transmembrane potential, release of cytochrome c and activation of caspase-9.

Materials and methods

Cells

Two human cell lines were used: HL-60 cells, a promyelocytic leukemia and WiDr cells, a colon adenocarcinoma (American Type Culture Collection,
Manassas, VA). We also used a murine ascites thymoma grown in Balb/c mice originally provided by the Institute of Pathology, University of Perugia, Italy (25). HL-60, and WiDr cells were maintained in RPMI 1640 (Sigma, St Louis, MO) supplemented with 10% (v/v) fetal calf serum (PCS, Gibco-Invitrogen Corporation, San Diego, CA), 2 mM L-glutamine, 1% penicillin-streptomycin, and in a humidified atmosphere containing 5% CO2 at 37°C. HL-60 cells were seeded at $3 \times 10^5$ cells/ml twice a week to maintain log phase growth. WiDr cells were split by trypsinization and plated at $3 \times 10^5$ cells/ml. Experiments were performed 1 day after trypsinization. Thymoma cells were grown by weekly intraperitoneal (i.p.) transplantation in Balb/c mice. Cells were harvested after 7–8 days, washed twice and resuspended in Ringer-HEPES, (130 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1.3 mM CaCl2, 50 mM HEPES, pH 7.4). For morphological experiments, thymoma cells were incubated at $37^\circ$C under O2 for variable length of time at a cell density of $2 \times 10^5$ cells/ml.

γ-TQ was synthesized and purified as described previously (8,9). γ-TQ, which was dissolved in ethanol, was diluted to the final concentration in the appropriate culture medium.

### Evaluation of apoptosis

#### Morphological evaluation.
Cell suspensions were stained with a mixture of chromotomod, the membrane permanent dye acridine-orange (100 µg/ml) and membrane impermeant ethidium bromide (100 µg/ml). Stained cells were examined by fluorescence microscopy at $\times 400$ magnification. Necrotic cells (damaged plasma membrane and non-condensed nuclei) and apoptotic cells (condensed or fragmented nuclei) were scored manually. At least 200 cells/tissue point were scored (26). When indicated, 200 µM AC-LEHD-CHO or Ac-IETD-AMC (Alexis Biochemical Italia, FI, Italy), specific inhibitors of caspase-9 and caspase-8, respectively, were added to the incubation medium.

#### Cytofluorimetric evaluation.
Apoptotic cells were also identified using the Apo-alert Annexin V-FITC apoptosis detection kit (Calbiochem, La Jolla, CA) according to the manufacturer’s instructions. This allows the detection of phosphatidyl-serine on the external cell membrane early in apoptotic cell death. Increased permeability to propidium iodide (PI) is noted in the late phase of the apoptosis beyond apoptotic chromatin condensation.

Cytofluorimetric analysis. Apoptotic cells were also identified using the Apo-alert Annexin V-FITC apoptosis detection kit (Calbiochem, La Jolla, CA) according to the manufacturer’s instructions. This allows the detection of phosphatidyl-serine on the external cell membrane early in apoptotic cell death. Increased permeability to propidium iodide (PI) is noted in the late phase of the apoptosis beyond apoptotic chromatin condensation. The results are presented as the mean ± SEM. The data were analyzed using analysis of variance (ANOVA). Post hoc comparisons of means were made using Fisher’s test (significance $P < 0.05$).

### Results

#### Morphological evaluation of apoptosis
The morphological examination of cells stained with the fluorescent dye acridine-orange revealed that γ-TQ caused a concentration- and time-dependent increase in the percentage of apoptotic cells in all the strains of cells evaluated (Figure 1). WiDr cells were less sensitive to the pro-apoptotic effect of γ-TQ than either HL-60 or thymoma cells after 4 h exposure. Whereas 50 µM γ-TQ induced apoptosis in ~60% of HL-60 cells and neoplastic thymocytes (Figure 1A and B), the maximal percentage of apoptotic WiDr cells, obtained with 200 µM γ-TQ, was only 25% (Figure 1C). Neoplastic thymocytes were the most responsive to the pro-apoptotic effect of γ-TQ, showing a significant percentage of apoptotic cells already at 2 h of γ-TQ treatment (Figure 1B). The other two cell strains had an initial lag phase of 2 h before showing the morphological changes of apoptosis.

#### Translocation of cell membrane phosphatidyl serine
The apoptosis-inducing effect of γ-TQ was confirmed in HL-60 cells (Figure 2) by flow cytometric evaluation of Annexin V binding, which measures phosphatidyl serine on the external leaflet of the plasma membrane, an event characteristic of early apoptosis. Four hour treatment with 50 µM γ-TQ increased the

![Fig. 1. Effect of γ-TQ on the percentage of apoptotic cells. Apoptosis was measured by morphological examination of HL-60 (A), thymoma (B) and WiDr (C) cells stained with acridine-orange. Cells were exposed to indicated amounts of γ-TQ for 4 h. Values were the mean ± SEM of six to eight experiments. *Significantly different from control ($P < 0.05$).](https://academic.oup.com/carcin/article-abstract/24/3/427/2608415)
Pathways of γ-TQ-induced apoptosis

Fig. 2. Representative dot plot showing the effect of γ-TQ on apoptosis on HL-60 cells. Proportion of apoptotic cells in a population was measured by the bivariate Annexin V/PI flow cytometry in control cells and cells treated with 50 µM γ-TQ for 4 h. One representative plot of three similar experiments is shown.

Fig. 3. Time-dependent and dose-dependent activation of caspase-9, -8 and -3 by γ-TQ in HL-60 cells. (A) Cells were treated with 50 µM γ-TQ for the indicated times. The activation was measured as the increase in activity (%) with respect to control cells (100%). The control basal value was 640 ± 31, 635 ± 32 and 57 ± 4 arbitrary fluorescence units for caspase-3, -8 and -9, respectively. In (B–D), cells were treated with increasing concentration of γ-TQ for the indicated times. Values were the mean ± SEM of five experiments.

percentage of apoptotic cells from 5.4 ± 0.5 (control condition) to 56.9 ± 5.4%.

Involvement of caspase-3, -8 and -9

As a family of aspartate-specific cysteiny1 proteases (caspases) plays a pivotal role in the execution of programmed cell death (31–34), we tested whether treatment of HL-60 cells with γ-TQ resulted in activation of two upstream caspases-8 and -9 and of the downstream caspase-3 (Figure 3). In particular, we evaluated caspase-8 activity because it represents the apical caspase in the death receptor (extrinsic) pathway (32) and caspase-9, as it serves as the apical caspase of the mitochondrial (intrinsic) pathway (33). Moreover, we analyzed caspase-3 because it has been shown to be one of the most important cell executioners for apoptosis (34). To determine whether activation of caspase-3, -8 and -9 plays a role in γ-TQ-induced apoptosis, HL-60 cells were incubated with γ-TQ and the cleavage of the fluorochrome AMC from the specific fluorogenic peptide substrates (Ac-DEVD-AMC, Ac-IETD-AMC and Ac-LEHD-AMC for caspase-3, -8 and -9, respectively) was analyzed fluorimetrically (Figure 3). A marked time-dependent increase in the activities of caspase-3, -8 and -9 was observed in HL-60 cells treated with 50 µM γ-TQ (Figure 3A). However, the temporal pattern of activation of the three caspases was markedly different. There was significant activation of caspase-9 as early as 2 h of treatment, and the activity continued to increase at 3 and 4 h of the assay. On the other hand, significant activation of caspase-8 was not found until after 3 h. Caspase-3 activity was very low at 2 h and then increased abruptly. The early activation of caspase-9 suggests that the γ-TQ pro-
apoiesis may be elicited mainly via the mitochondrial pathway. Figure 3(B–D) shows that γ-TQ-induced activation of caspases is both dose-dependent and time-dependent.

To further evaluate whether caspase-9 mitochondrial pathway was primarily induced by γ-TQ, we investigated to which extent its activation may be responsible for apoptosis induced by the quinone. Figure 4 shows the percentage of apoptotic HL-60 cells after treatment with 50 μM γ-TQ for 4 h in the absence or in the presence of 200 μM AC-LEHD-CHO or Ac-IETD-CHO, specific caspase-9 and -8 inhibitors, respectively. Apoptosis was evaluated morphologically using acridine-orange to stain the cells. The specific inhibition of caspase-9 led to the complete suppression of γ-TQ-induced apoptotosis. Lower concentrations of the inhibitor (10–20 μM AC-LEHD-CHO) had less of an effect (data not shown). In contrast, there was a much smaller decrease in the percentage of apoptotic cells when cells were incubated with the caspase-8 inhibitor. A similar limited inhibition of γ-TQ-induced apoptosis was observed in cells treated with a higher concentration (300 μM) of caspase-8 inhibitor (data not shown). These findings provide evidence that γ-TQ induces apoptosis through the mitochondrial pathway.

Cytochrome c release from mitochondria

To confirm the involvement of the mitochondrial pathway of apoptosis, we measured the induction of cytochrome c release from the mitochondria by γ-TQ. It is known that cytochrome c released from mitochondria into the cytosol binds to the apoptotic protease activating factor (Apaf) complex and triggers the activation of pro-caspase-9 to the active caspase-9 (35). As shown in Figure 5, a marked fraction of the cytochrome c was released from the mitochondria of γ-TQ-treated cells at 2 h, and the release was more pronounced at 3 h. This increase in the release of cytochrome c was in agreement with the data showing the continued increase in caspase-9 activity after 2 and 3 h of treatment (Figure 3A). The close association of the release of cytochrome c from mitochondria with the concurrent increase in caspase-9 provide evidence that γ-TQ induces apoptosis in HL-60 cells through the mitochondrial pathway.

Δψm loss

Cytochrome c may be released from mitochondria into the cytosol by opening a pore during membrane permeability transition, and changes in the opening of this pore have been postulated to play a role in cellular events leading to apoptosis of certain types of cells (36). Figure 6 presents the data of FACS analysis of the incorporation of fluorochrome DiOC6(3) used to test for changes in the membrane potential of intracellular mitochondria in intact cells treated with γ-TQ. HL-60 cells were incubated with 0, 20 and 50 μM γ-TQ for 2 and 3 h. A time- and dose-dependent loss of mitochondrial membrane potential (Δψm) was observed. Δψm decreased...
after 2 h of γ-TQ treatment by 24.7 ± 3.0% at the highest concentration tested (50 µM). After 3 h, DiOC₆(3) incorporation decreased by 20.6 ± 3.2 and 55.0 ± 5.1% in HL-60 cells treated with 20 and 50 µM γ-TQ, respectively. The data suggest that the early ∆Ψₘ decrease may trigger cytochrome c release and its subsequent activation of caspase-9.

Alterations in the expression of cellular Bcl-2 and BID
Caspase-8 activation, when triggered downstream of mitochondrial pathway of apoptosis, may further amplify caspase-9 and -3 activation through cleavage of the pro-apoptotic protein BID (37,38). Cleaved BID binds to mitochondria, antagonizes anti-apoptotic proteins of the Bcl-2 family and causes a further efflux of cytochrome c into the cytosol (39). Therefore, we investigated whether γ-TQ elicits the cleavage of BID, thus activating an amplifying loop for caspase-9 and -3. Figure 7 shows that there was a significant decrease of uncleaved BID in cells treated with 20 or 50 µM γ-TQ for 3 h (25.4 and 42.0% decrease, respectively, as compared with control). Interestingly, the level of uncleaved BID was similar to controls at 2 h when caspase-8 had not yet been activated (data not shown). Thus, it is possible to speculate that caspase-8 activation and BID cleavage could trigger a feedback amplification pathway for caspase-9 activation.

We also studied the effect of γ-TQ on Bcl-2. The anti-apoptotic protein Bcl-2 is an integral membrane protein located mainly on the outer membrane of mitochondria (40). As some observations suggest that Bcl-2 can be involved in the release of cytochrome c from mitochondria (41), we measured the expression of Bcl-2 in HL-60 cells treated with 20 and 50 µM γ-TQ. A small but significant decrease in Bcl-2 was observed after 3 h of treatment with γ-TQ, which indicated that Bcl-2 was involved in γ-TQ-induced release of cytochrome c from mitochondria.

Discussion
A number of studies have implicated apoptosis as an important mechanism by which chemotherapeutic agents kill susceptible cells (42,43). The results shown in this report demonstrate that γ-TQ, an oxidative metabolite of γ-tocopherol (8–10), is able to induce apoptosis in different strains of tumor cells of human (leukemia HL-60 and colonic adenocarcinoma WiDr cells) and murin origin (Balb/c neoplastic thymocytes). Moreover, they provide some insights into the signaling mechanisms that underlie γ-TQ-induced apoptosis. Recently, a potential chemotherapeutic role for γ-TQ has been hypothesized, which was shown to exert cytotoxic effects more powerful than the widely studied chemotherapeutic drugs doxorubicin and vinblastine in human leukemia cell lines (8). Moreover, it was recently reported that this compound is able to induce apoptosis in breast cancer and leukemia cells (10). γ-TQ was also shown to be able to escape drug resistance, exerting its cytotoxic and pro-apoptotic effects also in MDR leukemia cells (8–10). This property suggests a high tolerance of the organism toward this
compound, which has been related to the molecular structure of γ-TQ (8). γ-TQ induces apoptosis in a concentration- and time-dependent manner in cells of different origin and the various cell types show different sensitivities with a more powerful pro-apoptogenic action toward HL-60 and thymoma cells, than toward the colonic cell line. Our previous studies (8–10) and the present finding suggest that cells of leukocytic origin may represent the preferential target of the possible chemotherapeutic action of γ-TQ. Furthermore, the morphological features of apoptosis were detectable very early during the treatment of all the types of cells (2–3 h), and the pro-apoptotic effect of γ-TQ was confirmed by the cytofluorimetric analysis of Annexin V binding in HL-60 cells.

Some of the molecular and biochemical pathways involved in γ-TQ-induced apoptosis (44) were investigated in HL-60 cells. We found that γ-TQ induced apoptosis by the activation of the downstream caspase-3, which has been shown to play an important role in apoptosis induced by several conditions (45–47), and to be necessary in determining the nuclear alteration of apoptosis (34). In our model, caspase-3 activation was preceded by the activation of caspase-9, the apical caspase of the intrinsic mitochondrial pathway of apoptosis (occurring at the second hour of treatment). On the other hand, the activation of caspase-8, the apical caspase of the extrinsic pathway, became evident only later (at the third hour), concomitantly to the activity of caspase-3. This finding suggests that caspase-9 may play the main role in the initial triggering of the cleavage and activation of pro-caspase-3 and that caspase-8 activation may represent a downstream event after the activation of caspase-9. This hypothesis is in agreement with recent evidences (37,38) suggesting that activation of caspase-8 may also occur as a consequence of the activation of caspase-9, even though traditionally it was associated with Fas receptor-induced apoptosis. It is conceivable that the lipophilic quinone permeates the membrane and activates the intrinsic mitochondrial pathway, leading to the activation of caspase-9, thus supporting the hypothesis that caspase-9 represents the most apical caspase in chemical induced apoptosis (48). The dose- and time-dependent decrease in ΔΨm occurring after only 2 h of γ-TQ treatment, further indicates the early activation of the intrinsic mitochondrial pathway (33). Similarly, γ-TQ induced the release of cytochrome c into the cytosol after 2 h, and this release markedly increased after 3 h. The primary involvement of the mitochondrial pathway in γ-TQ-induced apoptosis was confirmed by the observation that a specific inhibitor of caspase-9 (Ac-LEHD-CHO) completely inhibited γ-TQ-induced apoptosis in HL-60 cells, whereas specific inhibitor of caspase-8 (Ac-IETD-CHO) caused only a small, but significant decrease in apoptosis. The data support the hypothesis that caspase-8 activation is secondary to the activation of caspase-9 and functions to amplify caspase-9 activation in γ-TQ-induced apoptosis. Recent reports (37,38) suggest that caspase-8 activation, when triggered downstream of the mitochondrial pathway of apoptosis, may amplify caspase-9 activation through the cleavage of the pro-apoptotic protein BID, which, in turn, elicits a further efflux of cytochrome c from mitochondria (39). We hypothesize that this amplification loop for caspase-9, involving the caspase-8/BID pathway, may take place in our model. In agreement, we found that, after 3 h of treatment, when caspase-8 became activated, a significant caspase-8-dependent BID cleavage occurred in HL-60 cells treated with γ-TQ.

Our data show a slight but significant reduction in the expression of the anti-apoptogenic protein Bcl-2 following γ-TQ treatment, which suggests that this protein is involved in the release of cytochrome c from mitochondria.

The biochemical and molecular mechanisms underlying the pro-apoptotic and cytotoxic effects of γ-TQ were recently clarified by Jones et al. (10). They put forward an ‘arylation hypothesis’ based on the notion that γ-TQ, as other alkylating and arylating quinones, is detoxified through the formation of glutathione (GSH) adducts (10). They hypothesized that the consequent depletion of intracellular GSH, known to be a common event in damage-induced apoptosis (49), may be essential in triggering γ-TQ-induced apoptosis.

In conclusion, γ-TQ-induced apoptosis appears to be primarily associated with the early activation of caspase-9 through the mitochondrial pathway, as demonstrated by the early ΔΨm loss and cytochrome c release from mitochondria. The activation of the mitochondrial pathway may in turn activate caspase-3, -8 and BID cleavage, triggering a feedback amplification loop for caspase-9. These results, together with previous findings concerning the high cytotoxic and pro-apoptogenic effects of the quinone toward tumor cells suggest a potential role of γ-TQ as a powerful chemotherapeutic agent.

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