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Alpha-tocopheryl phosphate is a novel apoptotic agent

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1. ABSTRACT

Alpha-tocopheryl succinate (TOS) is a well-known potent and selective apoptotic agent. This apoptotic activity has been ascribed to its detergent-like property which is also shared by the structurally related compound, alpha-tocopheryl phosphate (TOP). TOP meets the structural requirements that have been described for the apoptotic activity of TO esters, i.e. the combination of three structural, one functional, one signalling and one hydrophobic domain. In this study, we have investigated the effect of TOP on the osteosarcoma cell line MG-63 using TOS as a reference compound. As compared with TOS, TOP showed a higher proliferative and apoptosis inducing activity on the MG-63 cancer cell line. The cytotoxic effect of TOP and TOS seems to be due to the effect of the intact compounds, since only a minor conversion into alpha-tocopheryl (TO) could be detected. EPR experiments showed that TOS and TOP reduced membrane fluidity, whereas TO had no effect. In addition, induction of erythrocyte hemolysis by TOP depended on the pH. These results suggest that the detergent-like activity of these compounds might be involved in their biological effect. Due to the potent biological activities, TOP might be clinically useful.

2. INTRODUCTION

Recently, it has been discovered that alpha-tocopheryl phosphate (TOP) is endogenously present in plant and animal tissues, including human tissues. The concentrations of TOP are in general similar to those of alpha-tocopheryl (TO). In rat and pig liver, TOP levels even surpass those of TO by far (1,2). In foodstuffs such as chocolate and cheese the concentrations of TOP are 10 to 30 times higher than that of TO (1). It has been proposed that synthesis of TOP is catalyzed by kinases (3). We recently demonstrated that TOP interacts with biomembranes in a particular way, i.e. it acts as a potent antioxidant by its own, without conversion into TO (4). Moreover, TOP is not consumed during its protection against lipid peroxidation (4). It was suggested that the antioxidant effect of TOP is due to its detergent-like activity (4). Alpha-tocopheryl phosphate contains a charged phosphate group and a long aliphatic group and these characteristics are involved in the detergent activity.

In 1982, Prasad and Edwards-Prasad reported that alpha-tocopheryl succinate (TOS) induced inhibition of proliferation and cell death of murine melanoma B-16 cells in culture (5). Subsequently, it has been shown that TOS is a potent growth inhibitor of a variety of malignant cell

types in vitro (6-15) and in vivo (16-19). The antitumour activity of TOS seems to be selective for cancer cells, since TOS treatment is not toxic to normal cells (9,17). Although the precise mechanism for the growth inhibition activity by TOS remains unclear, it might be attributed to the detergent-like action of TOS (10,16). The detergent activity of TOS is due to the charged succinyl group and the long aliphatic hydrocarbon tail (10,16). Comparably, TOP contains a charged phosphate group and the same aliphatic chain as TOS.

Due to the structural similarity between TOP and TOS and the presumed comparable detergent-like activity, TOP may display a similar anti-proliferative activity as TOS. The present study has been designed to investigate the effect of TOP on the osteosarcoma cell line MG-63, using TOS as a reference compound.

3. MATERIALS AND METHODS

3.1. Chemicals

Alpha-tocopheryl, alpha-tocopheryl succinate, alpha-tocopheryl phosphate and 16-doxylstearic acid were obtained from Sigma (St. Louis, MO, USA). All other used chemicals were of analytical grade purity.

3.2. Cell Culture

Osteosarcoma cells (MG-63) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air.

3.3. Viability assay

MG-63 cells were seeded into 6-well plates at 0.5 x 10^6 cells per well and were cultured for 24 h in DMEM. Then the medium was replaced with 2.7 ml of balanced saline solution and 300 µl of medium, and in addition 24 µl of the tested compounds was added leading to the desired final concentration of 80 µM. Control cells were treated with 24 µM of both solvents, i.e. either deionised water (for TOP) or ethanol (for TO or TOS). After 6 h of incubation the cells were collected and tested for viability. Determination of cell number viability was assessed by the exclusion of trypan blue. Cells stained blue were counted as non-viable, whereas those excluding the dye were regarded as viable. Results were expressed as percentage cell death, which - because the controls mainly contained a negligible number of dead cells - was expressed as: number of dead cells/(number of dead cells + viable cells) * 100.

3.4. Proliferation assay (Qualitative and Quantitative)

MG-63 cells were cultured in flasks with a bottom of 25 cm^2 to 90 – 97% confluence. The medium was replaced with 4.5 ml of balanced saline solution and 500 µl of medium, and in addition 40 µl of TOP, TOS or TO was added resulting in a final concentration of 80 µM. In the control cells both solvents, i.e. either deionised water (for TOP) or ethanol (for TO or TOS) were added. After 4 h of incubation the balanced saline solution was replaced by medium and the cells were incubated further for 26 h. Photomicrographs were taken for a qualitative examination. For a quantitative determination, cells were harvested and then counted with a hemocytometer. The antiproliferative effect was calculated as: (number of control cells – number of treated cells)/number of control cells * 100.

3.5. DNA fragmentation assay for apoptosis

MG-63 cells were seeded into Lab-Tek Chamber Slide at 0.1 x 10^6 cells/ml per chamber and cultured in DMEM. After 24 h the medium was replaced with 900 µl of balanced saline solution and 100 µl of medium, and in addition 8 µl of the test compound was added to lead to a final concentration of 80 µM. Control cells were treated with both solvents, i.e. either deionised water (for TOP) or ethanol (for TO or TOS). After 4 h incubation the balanced saline solution was replaced by medium and further incubated for 26 h. Cells were washed with PBS and then fixed with 3.7% formaldehyde. After 30 min of incubation the cells were treated with 0.2 % triton X-100 and finally, stained with DAPI. Slides were evaluated microscopically. Cells with nuclei containing clearly condensed chromatin or cells with fragmented nuclei were counted and defined as apoptotic cells. Because the controls did not contain a noticeable number of apoptotic cells, the percentage of apoptotic cells was calculated as: number of apoptotic cells /(number of apoptotic cells + number of non-apoptotic cells) * 100.

3.6. Alpha-tocopheryl determination

MG-63 cells were seeded into 6-well plates at 0.5 x 10^6 cells per well and were cultured for 24 h in DMEM. Then the medium was replaced with 2.7 ml of balanced saline solution containing 300 µl of medium, and the cells were treated with 24 µl of the TOP, TOS or TO, leading to a final concentration of 80 µM. After 2 h of incubation the reaction was stopped by addition of 100 µl of 2.5 % SDS, and 2 ml ethanol containing 1 mg BHT to prevent further oxidation. Alpha-tocopheryl was extracted and determined as described in the following paragraph. Alpha-tocopheryl nicotinate (200 µg/ml) was added as an internal standard.

The mixture was shaken for 5 min, then 3 ml of hexane was added. Again the mixture was shaken for 15 min. After centrifugation the upper hexane layer was aspirated into a conical glass tube and evaporated under nitrogen. The residue was dissolved in 200 µl isopropylalcohol for HPLC analysis according to Burton *et al*. (20) using a Nucleosil C-18 column and absolute methanol as the mobile phase, with a flow rate of 2 ml/min. Alpha-tocopheryl was detected at 295 nm.

3.7. Membrane fluidity of human erythrocytes

The membrane fluidity of erythrocytes from healthy donors was measured by means of electron paramagnetic resonance (EPR) spectroscopy. Blood samples were obtained by venipuncture. Heparin was used as an anticoagulant. The plasma and buffy coat were carefully removed after centrifugation at 2000 g for 10 min at 4 ^oC. Erythrocytes were washed three times with phosphate-buffered saline (PBS) and finally resuspended in PBS to a hematocrit of 45%. PBS (0.5 ml) with the test

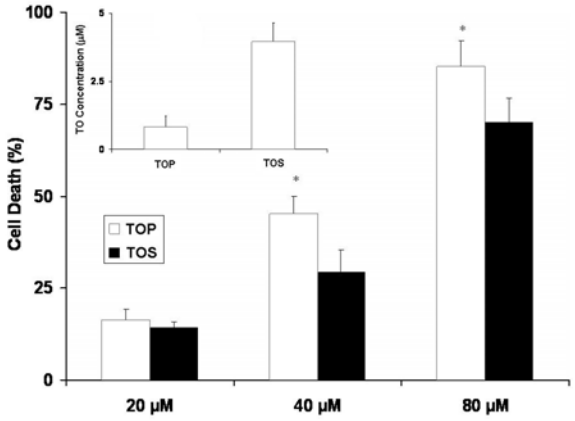

Figure 1. Cytotoxic effect of TOS and TOP in MG-63 cells. Cells were incubated with different concentrations of TOS and TOP. Percentages of cell death are shown. The insert shows the formation of TO from 80 µM TOS and TOP in MG-63 cells after 2 h of incubation. Values are presented as mean ± S.D. * Indicates a significant difference between TOP versus TOS ($p<0.001$).

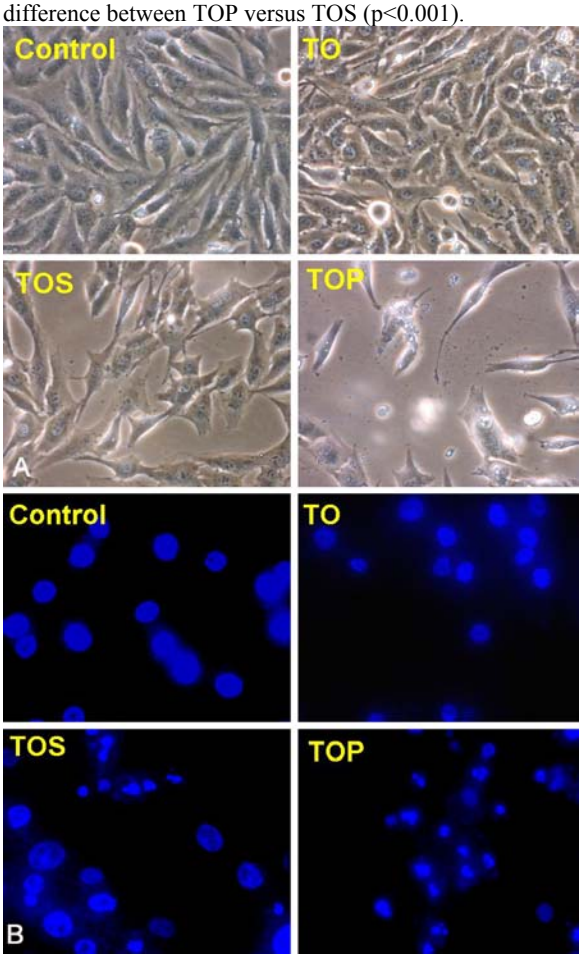

Figure 2. Qualitative antiproliferative (A) and apoptotic (B) effects of 80 µM of TO, TOS and TOP on MG-63 cells. Representative images for MG-63 cells from control and treated with TO, TOS and TOP were evaluated microscopically.

compound (final concentration 50 µM) was added to 0.5 ml of the erythrocyte suspension. Controls were treated with both solvents, i.e. deionised water (for TOP) and ethanol (for TO or TOS). After 90 min of incubation at 37 ^{o}C, spin labelled 16-doxylstearic acid was added and the samples were incubated for 90 min at 37 ^{o}C. Finally, EPR measurements were performed using an EPR spectrometer (Bruker EMX, GmbH, Freiburg, Germany) under the following conditions: microwave power 2 mW; modulation amplitude 1 G; scan width 50 G; modulation frequency 100 kHz and temperature 30 ^{o}C. Membrane fluidity was calculated from the peak height ratio (h_0/h_{-1}) as described previously (21). The higher the peak ratio, the lesser the freedom of the motion of the spin label in the biomembrane bilayer, indicating a lower membrane fluidity (21)

3.8. Erythrocyte Hemolysis

Erythrocyte hemolysis was assessed as described by Neuzil *et al*. (10) with minor modifications. Erythrocytes were prepared from freshly obtained heparin-treated human blood. One ml blood was diluted 80 times with PBS (pH 6.4 or pH 7.4) followed by centrifugation at 2000 *g* for 10 min at 4 °C. The pellet was resuspended with 45 ml PBS either of pH 6.4 or pH 7.4. One ml of the erythrocyte suspension was incubated with different concentrations of TOP at 37 °C. Controls were treated with both solvents, i.e. either deionised water (for TOP) or ethanol (for TO or TOS). After 2 h of incubation, the samples were centrifuged for 10 min at 2000 *g*. The supernatant was diluted 2 times, and the absorbance at 546 nm was determined.

3.9. Statistical analysis

All results are expressed as mean ± SD. Differences between the treatments were tested with Student's t-test.

4. RESULTS

In the viability assay with MG-63 cells, TOP and TOS induced cell death in a dose dependent way (Figure 1). At a concentration of 40 µM (n = 5) and 80 µM (n = 13) the effect of TOP (45 ± 5% and 85 ± 7% of cell death, respectively) was superior ($p < 0.001$ for both concentrations) to that of TOS (29 ± 6% and 70 ± 7% of cell death, respectively). The lowest concentration of 20 µM did not result in a significant difference in cytotoxicity between TOP and TOS (16 ± 3% *vs* 14 ± 2% of cell death, respectively (n = 3)). As shown in Figure 1 (insert), at a concentration of 80 µM both TOP and TOS hardly showed a conversion into TO (1 ± 0.4% and 4.6 ± 0.45%, respectively (n = 3)).

The cytotoxic effect of the TO esters was confirmed by their effect in the proliferation assay (Figures 2A and 3). The quantitative assay (Figure 3) confirmed the qualitative impression (Figure 2A) that TOP is a potent antiproliferative agent. The antiproliferative effects of TOP, TOS and TO were 75 ± 2% (n = 5), 41 ± 5% (n = 5) and 12 ± 3% (n = 5), respectively. The rank order of the potency of three compounds is TOP > TOS > TO. The difference

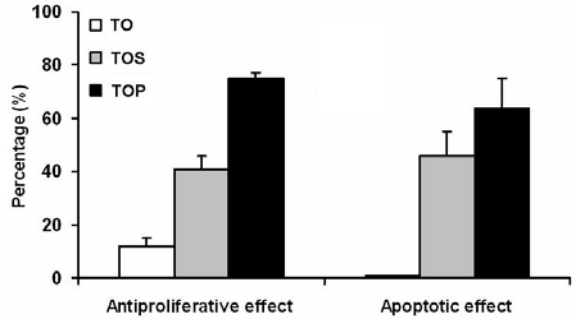

Figure 3. Quantitative antiproliferative and apoptotic effects of 80 μM of TO, TOS and TOP on MG-63 cells. The perecentage of antiproliferative effect was calculated as (number of control cells – number of treated cells)/number of control cells. The percentage of apoptotic cells was calculated as number of apoptotic cells /(number of apoptotic cells + number of non-apoptotic cells).

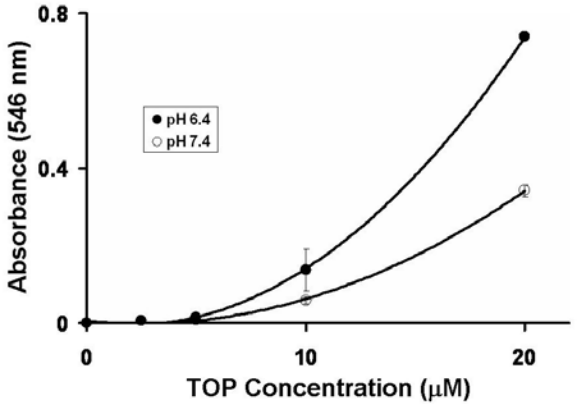

Figure 4. Induction of hemolysis by TOP. Isolated erythrocytes were incubated for 2 h with different concentrations of TOP at pH 6.4 or pH 7.4 at 37 ^{o}C. The data are presented as mean ± S.D. of three separate experiments.

between these effects were all highly significant, i.e. $p < 0.0003$.

The apoptotic effect of TOP and TOS was clearly observed in the MG-63 cells by DNA fragmentation and nuclear condensation. As shown in Figures 2B and 3, at a concentration of 80 μM the percentage of apoptotic cells was significantly higher after TOP (64 ± 11%, n = 21) than after TOS (46 ± 9%, n = 21) with a p-value of 10^{-6}. Alpha-tocopheryl had no apoptotic activity.

The membrane fluidity of erythrocytes, as measured by the ratio of the peak heights (h_0/h_{-1}), decreased significantly after treatment with 50 μM of TOP and TOS (h_0/h_{-1} = 8.1 ± 0.7 and 8.1 ± 0.3, respectively) in comparison to the controls (7.2 ± 0.2) with p-values of 0.03 and 0.001, respectively. Alpha-tocopheryl had no influence on the membrane fluidity (h_0/h_{-1} = 7.36 ± 0.13). The reduction in membrane fluidity by TOP and TOS was also significantly different compared to the effect of TO (p-values of 0.05 and 0.001, respectively).

Alpha-tocopheryl phosphate induced hemolysis of erythrocytes which was concentration dependent at pH 6.4 and 7.4 (Figure 4). Hemolysis increased when the concentration of TOP was increased and higher at pH 6.4 compared to 7.4

5. DISCUSSION

Several studies have shown that TOS induces growth inhibition and apoptotic activity on a variety of human and rodent cancer cell lines without affecting the proliferation of normal cells in vitro (6-15) as well as in vivo (16-19). Several authors have reported that TOS might be a highly selective anticancer agent. It was suggested that TOS is a promising anticancer drug or adjuvant, which is applicable in clinical practice. The goal of the present study is to determine whether TOP inhibits osteosarcoma cell proliferation and induces cell death and apoptosis. The effects of TOP are compared to those of TOS.

Alpha-tocopheryl phosphate induced cell death in a dose dependent manner and was more potent than TOS. The conversion of TOS or TOP into TO in MG-63 was very low, indicating that the induction of cell death by either TOS or TOP is due to a direct effect of the parent compounds. In contrast to the stability of TOP in the osteosarcoma cell line, Nakayama *et al*. (22) recently reported that in cultured mouse skin TOP showed a massive conversion into TO by alkaline phosphatase. Furthermore, daily supplementation of TOP (30 mg/kg) in rats for 32 days increased levels of TOP (62%) as well as TO (40%) in the liver, indicating a possible conversion of TOP into TO (1). The low conversion of TOP in the cancer cell line MG-63 and the substantial conversion of TOP in normal cells, cultured mouse skin (22), and liver tissue (1) indicate that differences in hydrolyzing enzymes, such as esterases and phosphatases exist between cancer and normal cells. This difference is used in the clinical application of amifostine as a protector of healthy tissues during chemo- and/or radiotherapy (23). Amifostine is a phosphorylated thiol and the removal of the phosphate group occurs at a higher rate in normal tissue compared to cancer tissue (23). In this way, more of the protective thiol is formed in normal tissue than in tumour tissue, leading to protection of healthy tissues without comprising the antitumour effect of chemo-and/or radiotherapy (23).

Of the tested compounds, TOP appeared to be the most potent inhibitor of cell proliferation. Alpha-tocopheryl has the lowest potency. Previously, it has been reported that alpha-tocopheryl inhibited cell proliferation while a chemically closely related form, beta-tocopheryl, is known to be ineffective (24). Esterification of the OH moiety of alpha-tocopheryl by succinate or phosphate boosts the inhibition of MG-63 cell proliferation. Again TOP was the most potent compound tested.

Alpha-tocopheryl phosphate also appeared to be most potent in inducing DNA fragmentation and nucleus

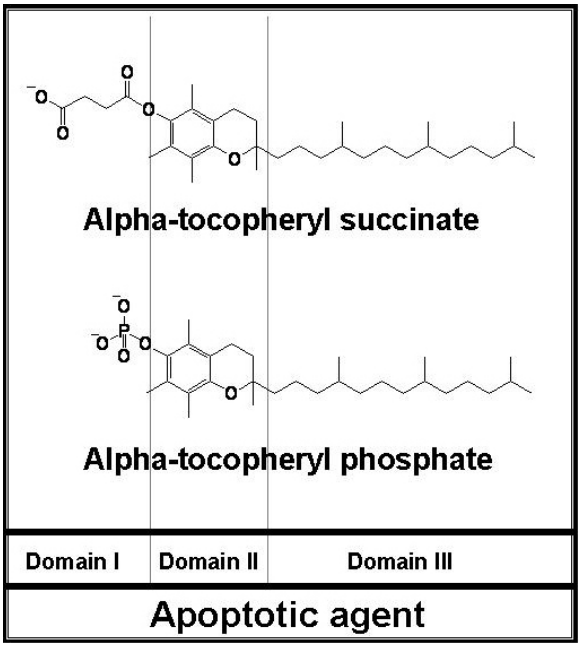

Figure 5. Structures of TOS and TOP showing 3 major domains (modified after Birringer *et al*. (9)). The domains are a functional domain (domain I), a signaling domain (domain II), and a hydrophobic domain (domain III). For apoptotic activity, the three domains are required. Alpha-tocopheryl phosphate is the TO derivative that has the highest activity of the TO esters tested.

condensation. Birringer *et al*. (11) found that neither trolox succinate nor phytyl succinate caused apoptosis. Methylation of the free succinyl carboxyl group (giving TO methyl succinate) completely abolished the apoptotic activity of TOS (11). This indicates that all parts of the molecule (i.e. the free succinate group, the chroman head and the phytyl tail) are essential for the induction of apoptosis by TOS. Alpha-tocopheryl glutarate has a very poor apoptotic activity compared to that of TOS, alpha-tocopheryl maleate and alpha-tocopheryl fumarate. Apoptotic activity also differs between alpha-tocopheryl oxalate, alpha-tocopheryl malonate, alpha-tocopheryl amide succinate and TOS (15, 25). The relative potency of these TO esters correlates with the flexibility of the terminal carboxyl containing moiety (15); the longer the terminal dicarboxylic moiety, the more flexible this moiety and the lower the apoptotic effect. The phosphate group is not flexible and, based on analogy with the TO esters, this might be an important feature of TOP in its apoptotic activity. Moreover, the TO esters, that are the less prone to enzymatic hydrolysis have a higher apoptotic activity (25). Results depicted in Figure 1 (insert) showed that conversion of TOP into TO was less than that of TOS, indicating a higher apoptotic activity of TOP compared to TOS.

Birringer *et al*. (11) reported that in all the apoptotic TO esters three structural domains can be recognized (Figure 5). Domain I is considered as functional domain which is the hydrophilic part of the detergent-like molecule of TOS and TOP. Domain II is regarded as signaling domain, which is accountable for cell signaling activity of TO, TOS and TOP. Domain III is a hydrophobic domain, which mediates embedding of TOS or TOP in the biomembranes.

The mechanism by which TOS induces apoptosis is not fully understood. A combination of membrane destabilizing activity and dysregulation of signaling pathways may be involved (10-17). Destabilization of the cell membrane probably occurs due to the detergent like action of TOS. Alpha-tocopheryl phosphate also has a detergent like activity (4).

The effect of TO, TOP and TOS on membrane fluidity has been tested. Both TOS and TOP decreased membrane fluidity of erythrocytes, whereas TO has no significant effect. Our recent study (4) showed that TOS and TOP induced erythrocyte hemolysis. Previously, Neuzil *et al*. (10) reported that both alpha-tocopheryl succinate and beta-tocopheryl succinate induced hemolysis to erythrocytes. The detergent-like activity of TOS increased with acidic pH (10). Alpha-tocopheryl succinate can exist in solution in two forms: the deprotonated charged species, and its protonated uncharged counterpart. The latter can enter cells by passive diffusion (10). Almost 99% of the total TOS will be charged at neutral pH. The protonated uncharged form will increase 25-fold at pH 6.2 (10). The apoptotic activity of TOS in Jurkat and MCF-7 cell lines was higher at acidic pH which may be due to the higher diffusion rate of protonated TOS over cell membranes at acidic pH (10). The pH dependent effect might contribute to the selective toxicity of TOS to cancer cells (9,17), since the pH of normal tissues is neutral compared to the acidic pH found in many tumours (26). Interestingly, we found that the toxicity of TOP on erythrocytes is also higher at acidic pH (Figure 4). Possibly, protonation of the phosphate group is involved, although the acidity (the pKa values) of TOP is not known. Thus, beside lower dephosphorylation in cancer versus healthy tissue, also the more acidic pH in tumour cells may contribute to a higher toxicity of TOP in tumour cells.

Alpha-tocopheryl phosphate is an important constituent in our diet which has been overlooked in the past, because HPLC techniques for the measurement of TO did not detect TOP (1, 2). The water solubility of TOP, that is presumably relatively low, and the charged phosphate group might be the impediments for straightforward detection. The dermal and oral LD_{50} values of mixture of TO and TOPs (mTOP) containing TOP (55.3%), di-TOP (30.6%), TO (5%), inorganic phosphate (2.6%), water (1.9%), oleic acid (1%) and impurities (3.57%), were determined to be more than 1130 mg/kg bw (27). Importantly, mTOP was not a dermal or eye irritant in rabbits and had no allergic activity in mice (27). However, the potency to induced erythrocyte hemolysis at a relative low concentration has to be considered in the application of TOP.

Various prominent health effects of TOP have been reported. TOP has been promoted as a radioprotector.

Felemovicius *et al*. (28) reported that a chronic oral supplementation of rats with TOP (5 mg/ml) significantly protected their intestine against acute irradiation. Pre-treatment of cultured mouse skin with a solution of 0.5% of TOP (9.4 mM) substantially protected against UV-B triggered skin damage (22). Also a significant beneficial effect of TOP in combination with semen plasma protein was observed on sperm survival (29). Furthermore, TOP improved acetylcholine-dependent relaxation in the aged aortic strips exposed to oxidative stress (30). In rabbits fed with a high cholesterol diet, mTOP induced a prominent atherosclerotic-preventing effect (31). These effects have been related to the ability of TOP to protect against lipid peroxidation, sunburn cell damage and DNA degradation (22). We have confirmed that TOP is indeed a potent inhibitor of lipid peroxidation (4).

Recently, Munteanu *et al*. (32) reported that a mixture of TOP modulated cell proliferation and gene expression in rat aortic smooth muscle cells (RASMC) and human THP-1 monocytic leukaemia cells at a relatively low concentration. At higher concentrations, the mixture of TOP induced apoptosis in human THP-1 monocytic leukaemia cells. Munteanu *et al*. (32) focused on a beneficial role of the mixture of TOP in atherosclerosis and inflammation. In our opinion, the apoptotic effect of TOP on monocytic leukaemia cells might be of interest too.

6. CONCLUSION

We conclude that TOP induces cell death, inhibits cell proliferation and provokes apoptosis in the MG-63 cell line, probably due to a membrane destabilising activity by acting as a detergent. TOP fits in the structural requirements that have been described for the apoptotic activity of TO esters, i.e. the combination of three structural domains, a functional, a signalling and an orientation domain. Due to its potent biological activities and the presence of relatively high amounts in the diet and our body, TOP might strengthen our therapeutic arsenal. Clinical studies are, however, needed to substantiate this hypothesis.

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