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Cytotoxicity of liposomal α -tocopheryl succinate towards hamster cheek pouch carcinoma (HCPC-1) cells in culture

Xinbin Gu^{a,b,*}, Joel L. Schwartz^c, Xiaowu Pang^a, Yanfei Zhou^{b,d}, David A. Sirois^e, and Rajagopalan Sridhar^{b,d}

a Department of Oral Diagnosis, College of Dentistry, Howard University, Washington, DC 20059, USA

b Cancer Center, Howard University, Washington, DC 20060, USA

c Institute for Cancer Prevention, One Dana Road, Valhalla, NY 10595, USA

d Department of Radiation Oncology and Howard University Hospital, Washington, DC 20060, USA

e Department Of Oral Medicine, College of Dentistry, New York University, NY 10010, USA

Abstract

There is compelling evidence for the cancer chemopreventive effects of vitamin E and related compounds. Of all the vitamin E derivatives that have been investigated to date, vitamin E acid succinate is the most effective anti-cancer agent. This report describes the preparation and testing of liposomal formulation of mono α -tocopheryl ester of succinic acid (α -TOS) for cytotoxicity against hamster cheek pouch carcinoma cell line (HCPC-1). Small unilamellar vesicles (SUV) of phosphatidylcholine incorporating 70 μ M α -TOS were superior to α -TOS alone or SUV without incorporated α -TOS, as inducers of apoptosis in HCPC-1 cells. Liposomal α -TOS perturbed the lipid structure in cells, promoted apoptosis, and decreased cell viability. The mechanism of action of α -TOS appears to involve membrane damage and induction of ceramide mediated apoptosis.

Keywords

Vitamin E succinate; Cancer therapy; Apoptosis; Ceramide; Ganglioside GD3

1. Introduction

The cancer chemopreventive action of α -tocopherol (vitamin E) has been studied for many years [1–8]. Chemopreventive potential of a combination of vitamin E acetate and selenium against prostate cancer is being evaluated in a multicenter selenium and vitamin E clinical trial designed to cover 400 study sites and enroll approximately 32,000 men from the United States, Puerto Rico and Canada [9,10]. Recent results indicate that α -tocopheryl succinate (vitamin E succinate, α -TOS) has the best anti-cancer profile of all vitamin E derivatives studied thus far in oral and other carcinogenesis studies [11–14]. This half ester of succinic acid has been reported to be more toxic to cancer cells in culture than normal cells [15,16]. The targeting of solid tumors by anti-cancer drugs can often be improved by incorporating the drugs into liposomes [17,18]. In the case of α -TOS, which is a hydrophobic compound, an injectable form of drug incorporated in small unilamellar vesicles (SUV) may be a desirable formulation. Therefore, it is important to compare the cytotoxicity of α -TOS and liposomal α -TOS towards tumor cells. Vitamin E has been suggested as a chemopreventive agent in head and neck cancer

* Corresponding author. Address: 600 W Street, NW. Dixon Building, Room 210, Washington, DC 20590, USA. Tel.: +1 202 8060345; fax: +1 202 8060354. E-mail address: xgu@howard.edu (X. Gu).

[7,19], and α -TOS may be considered as a prodrug that is converted into vitamin E through the action of carboxyesterases in tissues and tumor. Several investigators have reported preferential toxicity of α -TOS towards cancer cells [15,16,20–23]. The results presented in this article demonstrate that liposomal α -TOS is superior to α -TOS in inducing apoptosis in hamster cheek pouch carcinoma cell line (HCPC-1). Cell viability was much lower in cultures treated with liposomal α -TOS than in cultures treated with α -TOS alone or plain drug free liposomes. Sphingomyelinase activity, the levels of ceramide, ganglioside GD3 level and extent of apoptosis were measured in HCPC-1 cells treated with α -TOS-SUV, α -TOS, and SUV. The results show that liposomal α -TOS interacts with cell membrane to initiate sphingomyelinase activity and produce ceramide, which starts the signaling pathway towards apoptosis.

2. Materials and methods

2.1. Reagents

Chemicals were of the highest available grade. Dialysis tubing for unilamellar liposome preparation was obtained from Spectrum Company (Houston, TX). Ceramide (*N*-octadecanoyl-D-erythro-sphingosine) and α -tocopheryl succinate were obtained from Sigma Chemical Company (St Louis, MO). Silica gel pre-coated thin-layer glass plates (Kieselgel 60, TLC) were obtained from Merck (Darmstadt, Germany), and *Escherichia coli* sn-1,2-diacylglycerol kinase (specific activity >2 U/mg protein) was obtained from Calbiochem (La Jolla, CA). Octyl- β -D-glucopyranoside was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA) and [γ -³²P] ATP (3000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL).

2.2. Cell culture

7,2-dimethylbenz(a)anthracene induced golden Syrian hamster cheek pouch carcinoma cells (HCPC-1) [24] were grown in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY) and antibiotic-antimycotic mixture (50 U penicillin/ml, 50 μ g streptomycin/ml, and 2 μ g fungizone/ml; GIBCO). Cells were grown at 37 °C and subcultured to an initial density of 1×10^6 cells/ml every 5–6 days. Cell density was determined using a hemocytometer and a phase-contrast microscope. Trypan blue dye exclusion assay detected 0.1% dead cells in the untreated culture. All experiments were performed with cells in logarithmic phase of growth.

2.3. Liposome preparation

Two types of vesicle dispersions were prepared to obtain control-SUV and α -TOS encapsulated-SUV. The control-SUV was a suspension of phosphatidylcholine (PC) liposomes in phosphate buffered saline (PBS) buffer, and α -TOS-SUV consisted of PC liposomes encapsulating 70 μ M of α -TOS. A 1:1 mixture of chloroform: methanol was used to solubilize PC into a 0.2 g/ml lipid stock solution. In order to form pure PC liposomes, approximately 250 μ l of PC stock solution were dried under a blanket of dry nitrogen to form a lipid film. This film was then mixed with an isotonic solution of physiological saline (0.9% NaCl) along with the anionic detergent sodium cholate. The resulting mixed micellar solution was then dialyzed in a Mini Lipoprep dialyzer (5000 mw-cutoff) (Amika Corporation, Columbia, MD) for 4 h against an 8L reservoir of 0.9% NaCl solution. To encapsulate α -TOS into SUV, α -TOS was dissolved along with PC using the mixture of chloroform and methanol (1:1), and then processed as described above for the preparation of SUV.

2.4. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell viability

HCPC-1 cells were seeded in flat-bottom 96-well cell culture plate (Costar, Cambridge, MA) at a density of 30,000 cells/well and allowed to attach overnight and then treated with α -TOS-SUV (20 μ l) at a final concentration of 7 μ M for 3, 6 and 12 h. Twenty micro liters of MTT solution (Sigma, St Louis, MO) were added to each well and then the plate was incubated in a humidified CO₂ incubator at 37 °C for 5 h. After removing the media, 200 μ l of dimethyl sulfoxide were added to each well and mixed for 30 min at room temperature to dissolve crystals. The plate was placed inside a 37 °C incubator for 5 min. Finally, the plate was transferred to a microplate reader and absorbance at 550 nm was measured.

2.5. Flow cytometry assays

2.5.1. Cell cycle—Cultures were harvested by trypsinization and washed twice with ice-cold PBS buffer and cells (1×10^5) were incubated for 15 min at room temperature in the dark in a solution containing propidium iodide (5 μ g/ml) for fluorescence activated cell sorting (FACS) analysis using a FACStar flow cytometer equipped with a doublet discriminating module (Becton Dickinson & Co., San Jose, CA). Ten thousand cells were analyzed per sample.

2.5.2. GD3 expression—The cells were harvested by trypsinization and a single cell suspension of >95% viable cells ($\times 10^6$) was incubated with 100 μ l of (1:500) mouse anti-GD3 monoclonal antibody R24 recognizing ganglioside GD3 (the antibody was a gift from Dr Shunlin Ren, Medical College of Virginia). After that, the cells were incubated with a FITC-conjugated anti-Mouse IgG secondary antibody to detect R24 anti-GD3 antibody. Flow cytometry was then performed to quantify the level of GD3 expression.

2.6. Annexin V assays for apoptosis

Annexin V-EGFP (enhanced green fluorescent protein) *staining* kit was from BD Biosciences Clontech (Palo Alto, CA). HCPC-1 cells (1×10^3 /per well) were grown in 4-well Lab-Tek chamber slide (Nalge Nunc, Naperville, IL) with or without 10 μ l α -TOS-SUV (final concentration 7 μ M). The cells were rinsed with binding buffer and incubated with 5 μ l of fluorescently tagged Annexin V-EGFP at room temperature for 10 min in the dark. The cells with bound Annexin V-EGFP showed green staining in the plasma membrane under a fluorescence microscope.

2.7. TUNEL assay

TUNEL staining kit was from Biosource (Camarillo, CA). Exponentially growing monolayer cultures of HCPC-1 cells were treated with 100 μ l SUV or 100 μ l α -TOS-SUV (final concentration 7 μ M) in six well culture plate and then the attached cells were trypsinized to harvest cells. These cells were combined with the floating cells that were recovered by centrifugation from the culture medium before trypsinization. Cells were washed twice with PBS, resuspended in freshly prepared buffered formalin (10%) and fixed to glass slides [25]. The morphologic changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome bis-benzimide [26]. Five hundred cells per slide were scored for the incidence of apoptotic chromatin changes. The slides were viewed under a Leitz fluorescence microscope, which was attached to a digital imaging system (Scion, Frederick, MD). Apoptotic cells were stained dark brown and discernible under the microscope and in the captured images.

2.8. Photo microscopy of cytoskeleton and actin microfilaments

Monolayers of cells were grown on glass slides in 6-well chambers (Nalge Nunc International Corp., IL) and treated with 10 μ l SUV or 10 μ l α -TOS-SUV (final concentration 7 μ M) for 3–6

h and then washed twice with PBS. The washed monolayer cultures were then treated with phalloidin-FITC for 2 h in the dark. Confocal microscopy (Bio-Rad, CA) at 1500 $\times$ magnification was used to visualize changes in the microskeleton and actin.

2.9. Diacylglycerol kinase assay for determination of cellular levels of ceramide

Ceramide levels were determined on the basis of diacylglycerol kinase assay [27,28]. After incubation of monolayer cultures of HCPC-1 with 100 μ l α -TOS-SUV (final concentration 7 μ M) or 100 μ l SUV, the cells were harvested by trypsinization and the cells (10^7) were collected as pellets by centrifugation. Cellular lipids were extracted from the pellets using a mixture of chloroform: methanol: 1 N HCl in the volume to volume (v/v) ratio of 100:100:1, and then hydrolyzed with 0.1 N methanolic KOH for 1 h at 37 $^{\circ}$ C to remove glycerophospholipids. Ceramide containing samples were resuspended in 100 μ l of reaction mixture containing 150 μ g cardiolipin (Matreya, Inc.), 280 μ M diethylenetriaminepenta-acetic acid, 51 μ M octyl- β -D-glucopyranoside (Calbiochem, La Jolla, CA), 1 mM ATP, 10 μ Ci of [γ - 32 P]ATP (DuPont New England Nuclear, Boston, MA), and 35 μ g/ml *E. coli* diacylglycerol kinase, pH 6.5 (Calbiochem, La Jolla, CA). After 60 min at room temperature, the reaction was stopped by extraction of lipids with 1 ml of solvent mixture of chloroform:methanol:1 N HCl (100:100:1, v/v). Ceramide 1-phosphate was separated on TLC plates using a solvent system of chloroform:methanol:acetic acid (65:15:5, v/v) and detected by autoradiography, and the incorporated 32 P was quantified by liquid scintillation counting. Ceramide was determined by comparison with standard samples containing known amounts of ceramide.

2.10. Statistical analyses

Results are presented relative to untreated controls. Values represent mean $\pm$ standard deviation (SD) of three replicate tests. Data were analyzed by Duncan test following the ANOVA procedure when multiple comparisons were made. $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Cytotoxicity of α -TOS-SUV

Monolayer cultures of HCPC-1 cells were treated with α -TOS-SUV or SUV or α -TOS for up to 12 h and cell viability was assayed on the basis of MTT reduction by viable cells (Fig. 1). Treatment with α -TOS caused cell death as indicated by decrease of MTT reduction to the corresponding formazan in a time-dependent manner. For example, only 41 and 21% of the HCPC-1 cells survived 6 and 12 h exposure to 7 μ M α -TOS-SUV. However, 90 and 51% of the cells were viable after treatment with SUV or α -TOS alone for 12 h (Fig. 1). In addition, an accumulation of cells in G₁ phase and a reduction in numbers of cells in S phase also occurred following exposure to α -TOS-SUV with increasing exposure times from 1, 3, and 6 h in comparison to SUV treatment (Fig. 2). The experiments with free α -TOS gave erratic results because dilution of stock solutions of α -TOS in ethanol, by addition to the culture medium resulted in mixtures of unpredictable turbidity. This suggests that the dispersion of α -TOS into the medium gives emulsions, which vary in their micellar distributions. The cellular accessibility of α -TOS would also vary with differences in micellar composition. When α -TOS-SUV was used, the experiments were more reproducible compared to those with α -TOS alone in ethanol instead of SUV. The vitamin E derivative, α -TOS is a prodrug of the antioxidant, α -tocopherol (vitamin E). This prodrug has no antioxidant effect as such, but can be converted into the potent lipophilic antioxidant, vitamin E after enzymatic hydrolysis by the action of esterases present in tissues and tumor. In this context, liposomal formulations of α -TOS may differ from α -TOS itself with respect to the bioavailability and release kinetics of the active form of the drug (vitamin E). The liposomal formulation of α -TOS-SUV may also be better for delivery of α -TOS to cells, due to fusion of the small unilamellar vesicles with cell membrane and subsequent delivery of the incorporated or encapsulated α -TOS.

3.2. Induction of apoptosis by α -TOS-SUV

The MTT assay does not indicate whether the decrease in cell viability is due to apoptosis. Apoptosis is characterized by a variety of morphological features such as loss of membrane asymmetry and condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. In order to determine the contribution of apoptosis to cell killing in cultures treated with α -TOS-SUV, three different assays for apoptosis were used. One of the earliest indications of apoptosis is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Monolayer cell cultures were treated with α -TOS-SUV or SUV and then labeled with EGFP conjugated annexin V, which has high affinity for PS. The EGFP labeled PS was detected using a fluorescence microscope. The results indicated that α -TOS-SUV-treated HCPC-1 cells contained a higher level of PS that is accessible for binding to annexin V (annexin V index: $94.5 \pm 3.2\%$) compared to SUV-treated cells (annexin V index $14 \pm 2.2\%$, Fig. 3). The translocation of PS precedes other apoptotic processes such as loss of plasma membrane integrity, perinuclear membrane disruption, DNA fragmentation, and chromatin condensation. DNA degradation generates DNA strands with exposed 3'-hydroxyl ends. The TUNEL assay for detecting apoptosis-induced DNA fragmentation, clearly indicated a higher proportion of apoptotic cells in cultures treated with α -TOS-SUV (Apoptotic index: $47.7 \pm 3.9\%$) compared to cultures treated with SUV (Apoptotic index: $15.3 \pm 1.2\%$) (Fig. 4). These results were further confirmed by analysis of cytoskeleton and actin microfilaments using confocal microscopy (Fig. 5). Actin fragmentation and loss of stability were discernible with increased time of exposure of cells to α -TOS-SUV (Fig. 5). The cellular microfilaments and cytoskeleton also changed after HCPC-1 cells were exposed to α -TOS-SUV. The results indicated that the actin microfilaments were fewer and fragmented after α -TOS-SUV treatment for 3 h (Fig. 5(b)). The condensation of actin surrounding the nuclear membrane was also observed after 6-h treatment with α -TOS-SUV (Fig. 5(d)). The SUV control group did not exhibit any alteration in cytoskeleton features (Fig. 5(a) and (c)). There are reports indicating that actin fragmentation is a result of apoptosis, while others suggest that actin destabilization is a result of apoptosis. Caspases are proteolytic enzymes, which are involved in apoptosis. These proteases will degrade cellular proteins such as poly (ADP-ribose) polymerase and actin. Fragmentation of actin can be interpreted as evidence of caspase activity associated with apoptosis. Extensive morphological changes occur during apoptosis. Some of these changes are associated with actin fragmentation [29–31].

3.3. Effect of α -TOS-SUV on sphingolipids of cell membranes

Vitamin E succinate can be converted to vitamin E through the action of esterase activity in cells. As an antioxidant, vitamin E can be expected to inhibit apoptosis mediated by free radical-initiated events. But all the results so far indicate α -TOS-SUV causes an increase in apoptosis. In this context, it is important to consider the lipophilicity and surface-active properties of α -TOS-SUV, α -TOS, α -tocopherol. Since ceramide is known to feed into the signaling pathway towards apoptosis, we examined the level of ceramide in monolayer cultures treated with α -TOS-SUV or SUV from 30 min to 6 h. The cellular levels of ceramide increased with time of exposure to α -TOS-SUV (Fig. 6). For example, the ceramide level increased by 29 and 61% after treatment with α -TOS-SUV for 3 and 6 h, respectively, compared to cultures treated similarly with vehicular control SUV (Fig. 6). The modest but significant increase in sphingomyelinase activity was also seen with respect to ceramide levels in cultures treated with α -TOS-SUV [34]. In contrast, sphingomyelinase activity increased initially, but subsided with increasing duration of exposure to α -TOS-SUV.

Ganglioside GD3, which is an acidic sphingolipid located on the outside of cell membrane is a well-documented tumor associated antigen [32]. During α -TOS-SUV treatment, the level of GD3 on the outer membrane (which is accessible to the antibody used for FACS) was less compared to the levels detected for control cells treated with liposomes without α -TOS. The

GD3 levels decreased after a 1 h treatment with α -TOS-SUV, and this decrease was 13-fold higher in cells treated for 3 h with α -TOS-SUV (Fig. 7, Table 1). After 6 h of treatment with α -TOS-SUV the levels of GD3 in cells rebounded to nearly half the initial levels seen for untreated controls (Fig. 7, Table 1). The liposome control did not appear to alter normal levels of GD3. There is evidence in the literature to suggest that GD3 has several roles in the regulation of cell cycle and apoptosis. Recent literature suggests that increasing GD3 levels in cells is sufficient to initiate apoptosis through the mitochondrial signaling pathway [33,34]. Promotion of apoptosis by GD3 has been linked to the abolition of NF-kappa B dependent pathways [35].

Since vitamin E succinate is a prodrug of the potent antioxidant α -tocopherol, fluctuations in GD3 levels can also be anticipated in cells treated with vitamin E-succinate, depending on the kinetics of hydrolysis of the prodrug [32,36,37]. The MTT assay for cell viability measures the activity of the mitochondrial enzyme succinate dehydrogenase. GD3 levels may fluctuate during the cell cycle and during the apoptotic process. Therefore, it is possible that GD3 levels may not correlate with the time course of cell viability as measured by the MTT assay. GD3 triggers the mitochondrial-signaling pathway to apoptosis. GD3 may also affect the cell cycle progression. Therefore, it is not possible to draw conclusions about GD3 levels and the results of the MTT assay, which is a measure of cell viability and not necessarily a measure of apoptosis [38]. Mechanisms of GD3-induced apoptosis have been discussed as part of a review on glycosphingolipids and cell death [34].

These results indicate that α -TOS-SUV could have an effect on membrane-associated glycolipids in cells. In mammalian cells, ceramide promotes apoptosis. While sphingomyelin degradation produces ceramide, gangliosides also serve as a source of ceramide in cells. Sphingomyelin and gangliosides are located on the outer surface of cells. When sphingomyelin and gangliosides are translocated to the inside of the cell, enzymatic conversion to ceramide occurs. This initiates apoptosis. Our results suggest that liposomal α -TOS may translocate sphingomyelin and ganglioside GD3 from the outer surface of cells to intracellular regions and cause cellular damage.

Cellular response to vitamin E succinate may occur through a variety of mechanisms. A recent report suggests that this derivative can perturb mitochondria due to its detergent like behavior that may induce lysosomal and mitochondrial membrane fragility [39]. Bax translocation to mitochondria as well as roles for fas and c-Jun in vitamin E succinate induced apoptosis have been described [14,22,40].

The results with α -TOS-SUV and α -TOS suggest that interaction of α -TOS-SUV at the membrane level may be partly responsible for ceramide-mediated apoptosis, which is produced by the action of sphingomyelinase.

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Abbreviations

α -TOS

vitamin E succinate

SUV	Small unilamellar vesicles
HCPC-1	hamster cheek pouch carcinoma cell line
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling
PS	phosphatidylserine
PC	phosphatidylcholine
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
EGFP	enhanced green fluorescent protein
PBS	phosphate buffered saline
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Fig 1.

Effect of alpha (α)-TOS-SUV on the viability of HCPC-1 cells. The MTT assay was used for estimating the viability of HCPC-1 cells, before and after treatment of attached cells in 96-well culture plates with α -TOS-SUV (20 μ l, final concentration 7 μ M) or with an equal volume of SUV for 3, 6 and 12 h. One hundred percent survival was defined as the level of MTT metabolism in untreated control cultures. Data are expressed as the averages of triplicate determinations. *Error bars=SD* $P<0.001$.

Fig 2.

Effect of alpha (α)-TOS-SUV on cell cycle. HCPC-1 cells were treated with α -TOS-SUV (7 μ M) for 1, 3 to 6 h or with an equal volume SUV for 6 h. The cell cycle was analyzed using a

Fig 3. Annexin V-EGFP staining of cells for detection of α -TOS-SUV-mediated apoptosis of HCPC-1 cells. Monolayer cultures were treated for 6 h with an equal volume of SUV (Panel A) or α -TOS-SUV (7 μ M, Panel B) and then processed as described under Section 2 for binding of EGFP tagged Annexin V to phosphatidylserine moieties that become exposed and accessible in cellular membranes of apoptotic cells. Fluorescence due to EGFP-Annexin V bound to cell membrane is considerably higher in α -TOS-SUV treated cells (panel B; original magnification $\times 20$) than in SUV treated cells (Panel A; original magnification $\times 20$). (Arrow indicates EGFP-Annexin V bound cell).

Fig 4. α -TOS-SUV induced apoptosis detected using TUNEL assay. HCPC-1 cells were exposed to α -TOS-SUV or SUV for 6 h and then processed as described for the TUNEL assay in Section 2. Panel A: a typical photomicrograph of cells treated with an equal volume of SUV and subsequently stained for detection of apoptosis. Very few apoptotic cells are visible in this field. Panel B: a representative photomicrograph of cells treated with α -TOS-SUV (7 μ M) for 6 h and subjected to the procedure described for the TUNEL assay. A larger number of apoptotic cells, with characteristic brown stains in their nuclei, are seen in this field compared to Panel A. (Original magnification $\times 20$).

Fig 5.

Changes in the structure of actin microfilaments and cytoskeleton in α -TOS treated HCPC-1 cells were visualized using confocal microscopy. HCPC-1 cells were treated with α -TOS-SUV (7 μ M) or with an equal volume of SUV for 3 and 6 h. Magnification was 1500 $\times$ for all images, and in panel a, a micrometer scale is included for reference. The white horizontal strip above the scale represents 50 μ m. Arrows indicate actin microfilament (a), fragments of actin (b), a low level of actin around nuclear membrane (c), and a high level of actin around nuclear membrane (d).

Fig 6.

Measurement of the level of ceramide. HCPC-1 cells were treated with α -TOS-SUV ($7\mu\text{M}$) or SUV (an equal volume) for 0.5–6 h and the level of ceramide was determined using diacylglycerol kinase assay as described in the methods. Data are expressed as the averages of triplicate determinations. Error bars=SD $P<0.001$.

Fig 7.

Changes in the level of GD3 ganglioside in cells treated with α -TOS-SUV. HCPC-1 cells were treated with $7\mu\text{M}$ of α -TOS-SUV or with an equal volume of SUV for 1–6 h, and then harvested by trypsinization. The single cell suspensions were incubated with fluorescently labeled mouse anti-GD3 monoclonal antibody R-24. The level of GD3 was determined using a flow cytometer. Ten thousand cells were analyzed per sample.

Table 1
Effect of α -TOS-SUV on the level of ganglioside GD3 in HCPC-1 cells

Treatment	0 h	1 h	3 h	6 h
α -TOS-SUV	360.	115.7	8.86	150.9
SUV	N.A.	N.A.	N.A.	392.4

The data were collected from anti-GD3 monoclonal antibody (R-24) labeled flow cytometry assays as described in Fig. 7. *, mean counts for 5000 cells, $p \leq 0.001$.