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Only one carbon difference determines the pro-apoptotic activity of α -tocopheryl esters

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Abbreviations: TS, α -Tocopheryl succinate; Tglu, α -Tocopheryl glutarate; ROS, reactive oxygen species; ER, endoplasmic reticulum; IP₃R, 1,4,5-triphosphate receptor; 2-APB, 2-aminoethyl diphenylborinate; DAPI, 4',6-diamidino-2-phenylindole; GRP78, glucose regulated protein 78; PQC, protein quality control; UPR, unfolded protein response; PKC, protein kinase C; TMRE, tetramethyl rhodamine ethyl ester; HSP5A, heat shock protein family A member 5; HPLC, high performance liquid chromatography; AcOEt, ethanol ethylacetate.

Keywords:

α-Tocopheryl ester; Structure-activity relationship; Endoplasmic reticulum stress; GRP78; Apoptosis

Conflict of interest:

The authors declare no conflicts of interest.

Abstract

 α -Tocopheryl succinate (TS), a redox-silent succinyl ester of natural α -Tocopherol, has emerged as a novel anticancer agent. However, the underlying mechanism is unclear. We found that the terminal dicarboxylic moiety of tocopheryl esters contributes to apoptosis induction and thus cytotoxicity. To further examine this relationship, we compared the pro-apoptotic activity of TS, which has four carbon atoms in the terminal dicarboxylic moiety, to that of a newly synthesized, tocopheryl glutarate (Tglu), which has five. Cytotoxicity assays in vitro confirmed that TS stimulated apoptosis, while Tglu was non-cytotoxic. In investigating biological mechanisms leading to these opposing effects, we found that TS caused an elevation of intracellular superoxide, but Tglu did not. TS increased intracellular Ca²⁺ in cultured cells, suggesting induction of endoplasmic reticulum (ER) stress; however, Tglu did not affect Ca²⁺ homeostasis. 1,4,5-trisphosphate (IP₃) receptor antagonist 2-Aminoethyl diphenylborinate (2-APB) decreased TS-induced intracellular Ca²⁺, restored mitochondrial activity and cell viability in TS-treated cells, establishing the ER-mitochondria relationship in apoptosis induction. Moreover, real-time PCR, immunostaining and Western blotting assays revealed that TS downregulated glucose-regulated protein 78 (GRP78), which maintains ER homeostasis and promotes cell survival. Conversely, Tglu upregulates GRP78. Taken together, our results suggest a model in which TS-mediated superoxide production and GRP78 inhibition induce ER stress, which elevates intracellular Ca^{2+} and depolarizes mitochondria, leading to apoptosis. Because Tglu does not affect superoxide generation and increases GRP78 expression, it inhibits ER stress and is thereby non-cytotoxic. Our research provides insight into the structure-activity relationship of tocopheryl esters regarding the induction of apoptosis.

Introduction

The apoptosis pathway is a promising target for cancer therapies and may provide a foundation for developing novel selectively targeted anticancer drugs [1,2]. Recently, α -tocopheryl succinate (TS) was identified as a novel anticancer agent, potentially via its interaction with apoptotic machinery. TS is a fat-soluble succinyl ester of naturally occurring α -tocopherol (Fig. 1). Multiple researchers have considered it the most effective form of vitamin E in inducing apoptosis in cancer cells [3]. TS induces apoptosis preferentially in the cancer cell with limited toxicity toward normal cells [4].

The mechanisms by which apoptosis is induced by TS are complex, and they have not yet been completely elucidated. The understanding of the cytotoxic mechanisms of TS and related tocopheryl esters may provide information about new molecular targets for killing cancer cells and thereby improving cancer therapy.

TS has been shown to induce the production of reactive oxygen species (ROS), and the resulting oxidative stress activates apoptotic pathways in various types of cancer cells [5–9]. In addition, the ROS-generating capability of TS has also been shown to correlate with endoplasmic reticulum (ER) stress and apoptosis in human gastric cancer cells [10,11]. However, the type of ROS generated by TS in these studies is not explicitly mentioned. Previously, we have shown that TS induces apoptosis in vascular smooth muscle cells via superoxide generation [5].

Recently, ER stress and related apoptotic pathways have emerged as an attractive field of study for the development of effective cancer pharmacotherapies [12]. Oncogene activation, loss of tumour suppressors, hypoxia, nutrient deprivation and acidosis cause the generation of misfolded proteins in cancer cells [13]. The protein quality control (PQC) system encompassing the chaperone system and protein degradation system controls the number of misfolded proteins within the cells. Depending on the functionality of these two systems, the amount of misfolded protein may vary in the cancer cells. Usually, these systems are defective in the cancer cells due to gene mutation, hostile microenvironment, inflammation, and rapid ROS production. The lack of clearance of misfolded proteins due to the dysfunctional PQC of the cancer cells causes misfolded proteins accumulate within the cells. However, the type and quantity of the accumulated misfolded proteins in the cancer cells are unknown. These defective proteins subject the ER to stress, and the organelle consequently activates a highly conserved adaptive survival mechanism, the unfolded protein response (UPR), to support cancer cell survival [14,15]. UPR, however, fails to manage misfolded proteins and prevent apoptosis when ER stress is prolonged [16–18].

Glucose-regulated protein (GRP78) is a key ER-resident molecular chaperone and is also known as the master regulator of UPR signal transduction. Accordingly, GRP78 has been found to be overexpressed in various cancer cells [19]. GRP78 keeps unfolded protein concentrations to a minimum and thus protects the cancer cells from UPR-mediated apoptosis [20]. Several recent studies have suggested that downregulation of GRP78 inhibits the proliferation and migration of various cancer cells, enhances their radio- and chemo-sensitivity, and ultimately facilitates the killing of cancer cells by other therapeutics [21–28]. In these regards, then, GRP78 represents a potential target for the induction of ER stress by TS, but experimental evidence to support this hypothesis is not yet available.

While direct targets of α -tocopherol derivatives have not yet been identified, structure-activity relationship studies have suggested that the pro-apoptotic activity of these molecules depends on the length and charge of the terminal dicarboxylic moiety [29]. Previously, we showed that other derivatives of α -tocopherol, α -tocopheryl oxalate and α -tocopheryl malonate also have pro-apoptotic activity [30]. Interestingly, the strength of the pro-apoptotic activity differs among these derivatives, with α -tocopheryl oxalate having the highest activity among the derivatives tested. Further investigation allowed us to conclude that the terminal dicarboxylate moiety must contain four or less than four carbon atoms in order for a derivative of α -tocopherol to display pro-apoptotic activity [31].

Despite this correlative understanding of the influence of structure of tocopheryl ester derivatives on their activity, the mechanisms by which the side chain length of α -Tocopherol derivatives affect pro-apoptotic activity are unknown.

Previously, we have reported that TS directly interacts with and activates protein kinase C (PKC) [31]. Considering the interaction between TS and PKC, we considered PKC a possible molecular target of the tocopheryl esters. The PKC enzyme plays a crucial role in different cellular signal transduction cascades. PKC activation may induce superoxide production, causing oxidative stress and downregulation of GRP78 leading to ER stress. We hypothesized that the conformational flexibility of tocopheryl ester derivatives or their ability to interact with molecular targets results in variation in some biological activities of different α -tocopheryl esters, including intracellular superoxide production and ER stress induction. It should also be noted that the effects of TS and other analogues of α -tocopherol on ER stress have not been studied extensively.

To test our hypothesis, we compared the pro-apoptotic activities of TS and a newly synthesized tocopheryl ester, α -tocopheryl glutarate (Tglu). These molecules are identical chemically, except that TS contains four carbon atoms in the terminal dicarboxylic moiety, while Tglu contains five (Fig. 1). By examining the individual effects of TS and Tglu on intracellular superoxide production, ER stress, mitochondrial integrity, and apoptosis *in vitro*, we were able to gain insight into structural requirements for the cytotoxic biological activities of α -tocopherol derivatives.

Results

Cytotoxic effects of TS and Tglu in vitro

To investigate the cytotoxic effects of TS and Tglu in mouse melanoma B16F1, human hepatic carcinoma HepG2 cells, and mouse 3T3-Swiss albino, we incubated the cells with different concentrations of TS and Tglu liposome for 24 and 48 h. TS was found to decrease cell viability, as measured by trypan blue exclusion assays, in all the cell lines in a dose- and time-dependent manner (Fig. 2A, 2B and 2C). Even a relatively low concentration (50 μ M) of TS decreased cell viability to around 75% in B16F1 cells, 77% in HepG2 cells and around 91% in mouse 3T3-Swiss albino cells after 48 h. As the dose increased to 75 μ M and 100 μ M, the viability of B16F1 cells at 48 h decreased further, to around 68% and 65%, respectively. In addition, TS decreased the viability of HepG2 cells by around 65% and 56% at 75 μ M and 100 μ M concentrations, respectively. On the other hand, 75 μ M and 100 μ M TS treatment decreased cell viability by about 83% and 77%, respectively, in mouse 3T3-Swiss albino cells after 48 h. 75 μ M and 100 μ M TS treatment for 24 h also caused the formation of apoptotic bodies when we stained the nucleus of the TS-treated B16F1 cells with 4',6-diamidino-2-phenylindole (DAPI) after 24 h of TS treatment (Fig. 2D). Interestingly, Tglu treatment did not show cytotoxicity in both cancer cells, even at a higher dose of 125 μ M (Fig. 2E, 2F).

Effect of TS on mitochondrial integrity

Mitochondrial cytochrome c release due to rupture of the outer mitochondrial membrane is the early stage of apoptosis [37]. To identify the effect of TS on mitochondrial integrity, considering cytochrome c release in the cytoplasm as an indicator, we treated the cells with 75 μ M TS containing liposomes for 12 h and stained the cells with a mitochondria-specific fluorescent dye, MitoTracker and fluorescent tag antibody (secondary) that binds with cytochrome c antibody (primary). The yellow fluorescent in merged images (Fig. 3A) represents the localization of cytochrome c in the mitochondria. As shown in Fig. 3A, the yellow fluorescence is higher in control cells, while notable lower yellow fluorescence was observed in TS-treated cells. The cytofluorograms (Fig. 3B, C) obtained by the 'colocalization finder' function of ImageJ represent the correlation between the fluorescence of MitoTracker and cytochrome c. The value of the Pearson coefficient obtained from control was 0.99 and 0.45 in TS-treated cells.

Effects of TS and Tglu on mitochondrial membrane potential

Mitochondrial membrane potential is a well-known indicator of mitochondrial functionality. To identify the influence of structural changes of tocopheryl esters and the involvement of the tocopheryl ester-induced altered intracellular calcium level on mitochondrial membrane potential, we treated B16F1 and HepG2 cells with TS, Tglu or 2-APB or both with TS and 2-APB for 48 h and stained the cells with tetramethylrhodamine ethyl ester (TMRE), a mitochondria-specific fluorescent dye, which accumulates in mitochondria in a membrane potential-dependent manner (Fig. 4). Therefore, we have considered the loss of TMRE fluorescence as a readout for mitochondrial depolarization.

TS treatment was found to correlate with decreased mitochondrial fluorescence, while Tglu and 2-APB treatment led to increased fluorescence in both cells. The quantification of fluorescence intensity by image analysis revealed that TS treatment resulted in a decrease of mitochondrial fluorescence to around 38% relative to control (Fig. 4C) in the B16F1 cells. In the case of HepG2 cells, TS treatment reduced mitochondrial fluorescence to around 53% relative to control. Conversely, mitochondrial fluorescence increased to around 152% and 169% of control in Tglu-treated B16F1 and HepG2 cells, respectively (Fig 4C, D). In addition, the IP₃R antagonist, 2-APB treated cells exhibited increased mitochondrial fluorescence of about 134% and 115% that of control in B16F1 and HepG2 cells, respectively. Notably, in the case of cells treated with both 2-APB and TS, mitochondrial fluorescence was around 89% in B16F1cells and 84% in the HepG2 cells relative to control.

Measurement of the amount of TS and Tglu transferred to the cell

To confirm the transfer of TS and Tglu to the cell, we quantified the amounts of TS and Tglu transferred to the cells and remaining in the culture medium by HPLC. We found that amount of TS increased intracellularly while decreasing in the cell culture medium in a time-dependent manner. Figure 5A shows that around 20% of the applied TS transferred to the cell immediately after adding TS to the cell culture medium. After 24 h and 48 h of addition of TS, no TS remained in the cell culture medium. However, the amount of TS found in the cells after 24 h, and 48 h was 90% and 95% of the applied TS, respectively.

In the case of Tglu, we found around 80% of applied Tglu in the cell culture medium and 20% of Tglu inside the cell immediately after adding Tglu to the cell culture medium (Fig. 5B). After 24 h of Tglu-addition, around 23% of applied Tglu was transferred to the cell, while about 20% of Tglu recovered from the cell culture medium. However, around 22% Tglu was found inside the cell, while no Tglu remained in the medium after 48 h.

Effects of TS and Tglu on intracellular superoxide production

The induction of superoxide production is a key mechanism leading to the cytotoxicity of TS [5,6,32]. To investigate how length of the ester moiety of the tocopheryl esters influence intracellular superoxide production, we examined the production of superoxide by B16F1 and HepG2 cells in the presence of TS and Tglu. The known superoxide-inducing bacterial metabolite pyocyanin was used as a positive control. α -Tocopherol and NAC were used as antioxidants. Superoxide was detected with a superoxide-activated fluorophore. As shown in Fig. 6A and 6C, the fluorescence intensity was notably higher in cells treated with pyocyanin or TS than in untreated cells. On the other hand, cells treated with Tglu exhibited fluorescence that was similar to that exhibited by untreated cells.

According to the quantification of fluorescence by image analysis (Fig. 6B), pyocyanin increased intracellular superoxide production in B16F1 cells by around 186%, and TS increased intracellular superoxide by around 165%, as compared to untreated cells. On the other hand, in HepG2 cells, pyocyanin increased intracellular superoxide by around 383%, while TS increased intracellular superoxide by around 273% (Fig. 6D). Conversely, Tglutreated cells exhibited similar superoxide production as did untreated cells. Notably, cells treated with both TS and α -Tocopherol exhibited around 109% and 95% of the superoxide production of untreated cells in B16F1 and HepG2 cells, respectively. NAC treatment showed about 115% superoxide production in both TS-treated B16F1 and HepG2 cells.

Effects of TS and Tglu on intracellular Ca²⁺

A key mechanism that has been proposed to explain the pro-apoptotic activity of TS is the induction of ER stress [10,11]. Therefore, we compared the effects of TS and Tglu on ER stress by evaluating changes in cytoplasmic Ca²⁺ levels, as increased cytoplasmic calcium is a well-known consequence of ER stress [33–35]. Using an assay based on a calcium-activated cell-permeable dye, intracellular calcium was detected in the cytoplasm of B16F1 after 48 h of TS and Tglu treatment (Fig. 7). Treatment with TS, however, resulted in increased fluorescence. In contrast, treatment with Tglu correlated with decreased calcium-stimulated fluorescence relative to control and TS-treated cells. Upon quantifying the fluorescence intensity by image analysis, we found that the fluorescence in TS-treated cells was around 202% of that of control in B16F1 cells (Fig. 7B). Conversely, the fluorescence intensity of Tglu-treated B16F1 cells was around 52% that of control cells.

IP₃R is the main Ca²⁺ release channel among the calcium releasing channels on ER [36]. To examine the involvement of IP₃R in TS-induced calcium release, we treated the cells with both IP₃R antagonist 2-APB (2-Aminoethyl diphenyl borinate) and TS. We found that cells treated with both 2-APB and TS exhibited fluorescence of around 126% (Fig. 7B). Again, cells treated with 2-APB alone showed reduced fluorescence of about 77% of that of control. However, the reduction was not statistically significant when compared to the control.

Effect of TS on intracellular Ca²⁺ in Ca²⁺ free cell culture medium

To investigate the source of Ca^{2+} in TS-induced elevation of intracellular Ca^{2+} level, we treated B16F1 cells with 75 µM TS containing liposomes in Ca^{2+} free cell culture medium to exclude the possibility of Ca^{2+} influx from outside the cell. After 6 h of TS treatment, intracellular calcium was detected using calcium-activated cell-permeable dye (Fig. 8). Treatment with TS resulted in increased Ca^{2+} stimulated fluorescence relative to control, while combined treatment of TS and 2-APB reduced fluorescence to control level. The quantification of fluorescence intensity by image analysis, we found that the fluorescence in TS-treated cells was approximately 148% of that of control (Fig. 8B). Notably, cells treated with both TS and 2-APB exhibited fluorescence that was similar to that exhibited by control cells.

Effect of 2-APB on TS-induced cytotoxicity in vitro

Based on our result of TMRE fluorescence restoration by 2-APB in TS-treated cells, we investigated the effect of 2-APB on TS-induced cytotoxicity by treating the cells for 48 h with either different concentrations of 2-APB or a combination of 75 μ M TS and different concentrations of 2-APB. We found that 2-APB is non-cytotoxic as measured by trypan blue exclusion assays (Fig. 9A). However, pre-treatment with 2-APB for 1 h was found to improve cell viability in both TS-treated B16F1 and HepG2 cells in a dose-dependent manner (Fig. 9B, C). TS treatment decreased cell viability to around 72% in B16F1 cells, while 25 μ M and 50 μ M 2-APB increased cell viability to about 78% and 89% relative to control, respectively, in TS-treated B16F1 cells. In addition, TS treatment decreased cell viability in HepG2 cells treated with both 25 μ M 2-APB and 75 μ M TS, while cell viability was 85% in cells treated with both 50 μ M 2-APB and 75 μ M TS.

Effects of TS and Tglu treatment on total GRP78 activity

GRP78 initiates adaptive responses to protect cells from ER stress-induced apoptosis [19,21,28,38], providing a potential mechanism whereby TS and Tglu may modulate ER stress. To analyze the activity of GRP78 on normal cells (mouse 3T3-Swiss albino) and untreated cancer cells, we used real-time PCR to quantify the mRNA expressed from the *HSP5A* gene, which encodes for GRP78. The RT-PCR signal from GRP78 mRNA was normalized to that of the housekeeping gene *GAPDH* and β -actin so as to control for cytotoxic effects. We found that the activity of GRP78 is higher in B16F1 cells compared to mouse 3T3-Swiss albino cells (Fig. 10A, B, C). The GRP78 mRNA in B16F1 cells was around 190% of that of mouse 3T3-Swiss albino cells. We also assessed GRP78 protein expression by western blotting. We found higher expression of GRP78 protein in B16F1 cells compared to 3T3-Swiss albino cells (Fig. 10A). Quantification of western blot band intensities by image analysis showed that GRP78 band intensity was about 250% that of mouse 3T3-Swiss albino cells.

We examined the GRP78 protein activity in TS-treated B16F1 and HepG2 cells. We found that GRP78 protein decreased in a time-dependent manner in TS-treated cells (Fig. 10D, 10G). Quantification by image analysis revealed that the intensity of the GRP78 band from TS-treated B16F1 cells was around 71% that of control after 12 h, 63% that of control after 24 h, and 47% that of control after 48 h (Fig. 10E). In addition, GRP78 band intensity was around 90% that of control after 12 h, 45% that of control after 24 h, and 66% that of control after 48 h (Fig. 10H) in TS-treated HepG2 cells.

To investigate the underlying mechanism of TS-induced GRP78 downregulation in the cancer cells, we quantified the GRP78 mRNA level by real-time PCR. As shown in Fig. 10F, treatment with TS correlated with a decrease of GRP78 mRNA in B16F1 cells relative to that in untreated control cells in a time-dependent manner. Specifically, after 24 h of treatment, TS suppressed GRP78 mRNA to around 64% of that of control. After 48 h, GRP78 mRNA was suppressed to around 37% of the control level in TS-treated B16F1 cells. The degree of suppression of GRP78 mRNA by TS was significantly higher after 48 h as compared to the effect at 24 h. However, in the case of HepG2 cells, 24 h of TS treatment increased GRP78 mRNA to around 198% that of control, while after 48 h, GRP78 mRNA decreased to the control level (Fig. 10I). We also examined the effect of Tglu treatment on GRP78 activity. Results of Western blotting assays demonstrated that GRP78 protein increased over time (Fig. 11A, D). Specifically, quantification of band intensities by image analysis showed that GRP78 increased significantly in Tglu-treated B16F1 cells to around 172% and 137% relative to control after 24 h and 48 h,

respectively (Fig. 11B). In contrast, Tglu treatment initially decreased GRP78 protein after 12 h of treatment, but after 24 h, it increased GRP78 band intensity to around 147% that of control and 117% that of control after 48 h in HepG2 cells (Fig. 11E).

As shown in Fig. 11C and 11F, treatment with Tglu induced time-dependent and significant increases in GRP78 mRNA relative to control in both B16F1 and HepG2 cells. 24 h of Tglu treatment induced significant increases in GRP78 mRNA to around 119% in B16F1 cells and around 149% in HepG2 cells compared to control. In addition, GRP78 mRNA level increased to around 146% that of control in Tglu-treated B16F1 cells, while 48 h of Tglu treatment induced around 126% increase in GRP78 mRNA level compared to control in HepG2 cells.

Localization of GRP78 after TS and Tglu treatment

Generally, GRP78 is thought to reside in the lumen of ER; however, it also shows a diffuse distribution throughout the cytoplasm [39]. To investigate the changes in GRP78 localization between ER and cytoplasm upon TS and Tglu treatment, we performed immunostaining using anti-calreticulin antibody as the marker of ER lumen. GRP78 was detected as green fluorescence and calreticulin as red fluorescence in B16F1 after 48 h of TS and Tglu treatment (Fig. 12). Here, the yellow signal in merged images represents the localization of GRP78 in the lumen of ER. However, TS-treated B16F1 cells showed decreased yellow fluorescence compared to control, while yellow fluorescence calculated by the colour thresholding method of image analysis revealed that in control cells, the ratio was about 48% (Fig. 12B). On the other hand, the ratio was 17% in TS-treated cells. In accord with previous results, the opposite effect was observed upon treatment with Tglu: Tglu treatment increased the ratio of yellow to total green signal to about 62%.

Discussion

We previously reported that the terminal dicarboxylate moiety must contain four or less than four carbon atoms in order for a derivative of α -tocopherol to display pro-apoptotic activity [31]. However, the relation between structural changes in the terminal dicarboxylate moiety and biological activities of the tocopheryl ester derivatives leading to pro-apoptotic activity has not been elucidated yet. In the present study, we studied the effect of changes in the side chain length at the terminal ester moiety of the tocopheryl esters on their pro-apoptotic activity.

TS caused cytotoxicity in B16F1 cells (Fig. 2A), human hepatic carcinoma HepG2 cells (Fig. 2B) and mouse 3T3-Swiss albino cells (Fig. 2C). However, the higher cytotoxicity of TS in B16F1 and HepG2 cells compared to mouse 3T3-Swiss albino cells indicates that the cancer cells are more sensitive to TS treatment than normal cells. Previously we have shown the higher sensitivity of cancer cells toward TS treatment is due to the failure of their antioxidative defence system [32]. As shown in Fig. 2D, TS-induced morphological changes of the nucleus characterized by the formation of the apoptotic bodies confirm cellular apoptosis upon TS treatment.

In contrast, Tglu, with a single additional carbon in the terminal ester (Fig. 1), was not cytotoxic to B16F1 and HepG2 cells (Fig. 2E, F). These results suggest that an increase in carbon number from four to five in the terminal ester group of tocopheryl esters diminishes the cytotoxic effect. We investigated this interesting phenomenon to identify the underlying mechanisms.

TS has been reported to preferentially accumulate in the mitochondria of the hepatocytes [40]. However, the possibility of accumulation of TS to other organelle is not excluded. Again, it is well established that TS interferes with the activities of mitochondrial respiratory chain components and generates superoxide [6,9,40–42]. Taken together, we hypothesized that accumulation of the generated superoxide by TS may cause oxidative stress leading to ER stress. In our current study, we found that TS downregulated ER residing chaperone protein GRP78 (Fig. 10), suggesting the possibility of ER as another target organelle of TS. We think the hydrophobic nature of TS may allow its distribution between lipid membranes of other organelles. Therefore, the primary target organelle of TS is unknown.

Our current study confirmed superoxide generation by TS using superoxide specific fluorescence probe, which is insensitive to other peroxide, hydroxyl radical, peroxynitrite, chlorine and bromine species. The quantification of fluorescence intensity showed that TS significantly increases superoxide production in B16F1 and HepG2 cells (Fig. 6) as compared to untreated cells. At the same time, α -Tocopherol and NAC-treatment significantly reduced

TS-induced superoxide production (Fig. 6B, D). Importantly, though, the newly synthesized tocopheryl ester, Tglu, did not generate superoxide like TS does, lending support to the conclusion that Tglu is less cytotoxic than TS. These findings further suggest that the structural change in the dicarboxylic moiety drastically affects the superoxide generation by the tocopheryl esters.

ROS generation has also been demonstrated to cause ER stress, which leads to apoptosis in human gastric cancer cells [10,11]. The ER lumen has a highly regulated microenvironment consisting of molecular chaperones and folding enzymes and high concentrations of ATP and Ca²⁺ [43]. Changes in these components may alter ER homeostasis, leading to ER stress [44], which is characterized by increased cytoplasmic $Ca^{2+}[45]$. In addition, superoxide may induce Ca^{2+} release from ER by stimulating IP₃R, which is the main Ca^{2+} releasing channel on ER [36,46]. Hence, taking the superoxide generation ability by TS into account, we hypothesized TS might induce Ca^{2+} release by indirectly stimulating IP₃R. To test our hypothesis, we examined intracellular Ca²⁺ level upon TS, Tglu and 2-APB (IP₃R antagonist) treatment in B16F1 cells. We found that TS-treated B16F1 exhibited increased cytoplasmic Ca²⁺ (Fig. 7), suggesting induction of ER stress in B16F1 cells. In addition, a significant reduction in cvtoplasmic Ca²⁺ in cells treated with both TS and IP₃R antagonist 2-APB compared to TStreated cells support TS-induced Ca²⁺ release through the IP₃R channel. Conversely, Tglu treatment actually decreased Ca²⁺-specific fluorescence, suggesting that Tglu may compensate for, rather than cause, ER stress. Furthermore, TS-treated cells exhibited higher Ca²⁺-specific fluorescence relative to control in Ca²⁺-free cell culture medium, while 2-APB treatment restores TS-induced elevation of Ca^{2+} to the control level (Fig. 8). This finding confirms ER as the source of Ca^{2+} in TS-induced Ca^{2+} inflation.

In either event, the increased cytoplasmic Ca^{2+} may cause mitochondrial Ca^{2+} overload, with accompanying mitochondrial swelling, perturbation or rupture of the outer membrane, and release of apoptotic factors [47]. Our immunoassay of cytochrome c showed that cytochrome c and MitoTracker signal overlapped in control cells. The cytofluorogram obtained from the control cells (Fig 3B) by using "co-localization finder" of ImageJ represents linear co-relation between cytochrome c and MitoTracker signal. The Pearson coefficient of 0.99 suggests perfect localisation of cytochrome c in the mitochondria of the control cells. On the other hand, the cytofluorogram obtained from TS-treated cells did not represent a linear co-relation between cytochrome c and MitoTracker signal (3C). The Pearson coefficient value of 0.45 represents a lack of overlapping between cytochrome c (green) and MitoTracker (red) signal

indicating that cytochrome c is no longer in the mitochondria. This finding suggests cytochrome c release due to mitochondrial membrane rupture upon TS treatment.

Again, the loss of mitochondrial integrity is associated with mitochondrial depolarization.

We confirmed mitochondrial depolarization in TS-treated B16F1 and HepG2 cells (Fig. 4). Notably, 2-APB treatment restored mitochondrial membrane potential loss induced by TS in both cells suggesting the involvement of Ca^{2+} leakage from ER in mitochondrial depolarization (Fig. 4C, D). In addition, we found that blocking of Ca^{2+} leakage by pre-treatment with 25 μ M and 50 μ M 2-APB dose-dependently improves cell viability in TS-treated B16F1 (Fig. 9B) and HepG2 cells (Fig. 9C). These findings suggest that Ca^{2+} overload on mitochondria due to TS induced Ca^{2+} leakage from ER is responsible for TS-induced apoptosis and demonstrates a crucial relationship between ER and mitochondria in apoptosis induction.

Oxidative stress induction by TS may damage intracellular proteins structurally, resulting in the accumulation of misfolded proteins and, consequently, ER stress. Chaperone proteins play a vital role in managing these misfolded proteins and reestablishing ER homeostasis. GRP78 is an essential chaperone that acts as a Ca^{2+} buffer in the lumen of the ER [48]. GRP78 also maintains mitochondrial functionality by reducing Ca²⁺ efflux from the ER to mitochondria and by managing misfolded mitochondrial proteins and mitochondrial steroidogenesis [49–51]. Recently, it has been proposed that GRP78 overexpression aids neuroprotection by triggering the mitophagy process [52]. Our real-time PCR and western blotting results confirm that GRP78 activity is higher in B16F1 cells than mouse 3T3-Swiss albino cells (Fig. 10A-C), providing an important link between GRP78 expression and cancer cell survival under hostile conditions. Furthermore, downregulation of GRP78 has been proposed to cause apoptosis in several types of cancer cells [21-28]. Thus, the role of GRP78 in maintaining ER stress homeostasis and mitochondrial functionality suggests important relationships among downregulation of GRP78, ER stress, mitochondrial dysfunction, and apoptosis in cancer cells. We found that TS downregulates GRP78 expression in both B16F1 and HepG2 cells (Fig. 10D, E and Fig. 10G, H). Not only that decreased ratio of luminal GRP78 to total GRP78 in TStreated cells indicates a loss of GRP78 in the ER lumen (Fig. 12). Hence, TS may negatively affect protein folding and, consequently, Ca^{2+} homeostasis in the lumen of the ER, contributing to Ca²⁺ leakage from the ER into the cytoplasm. Taken together, GRP78 downregulation by TS might be one of the key events in the induction of ER stress and mitochondrial depolarization that lead to apoptosis (Schema). However, it is still inconclusive whether TS indirectly causes ER stress via superoxide generation or directly induces ER stress by

downregulating GRP78. Further investigation is required to find out the trigger of ER stressinduced by TS.

On the other hand, Tglu treatment was found to upregulate GRP78 expression in B16F1 and HepG2 cells (Fig. 11). Tglu-treated cells were found with an increased amount of GRP78 in the ER lumen (Fig. 12A, B). Thus, upregulation of GRP78 by Tglu may support ER homeostasis by decreasing the amount of intracellular misfolded proteins and preventing Ca²⁺ leakage from the ER. The reduced cytoplasmic Ca²⁺ in Tglu-treated B16F1 (Fig. 7A, B) is consistent with this model. These results further support the inhibition of ER stress by Tglu. Through this mechanism, Tglu would protect mitochondria from intracellular Ca²⁺ overload, thereby leading to a non-cytotoxic character. The increase of mitochondrial fluorescence in TMRE assay upon Tglu-treatment (Fig. 4) and cytotoxicity results (Figs. 2E and 2F) support this statement.

Furthermore, we investigated the mechanism of GRP78 protein downregulation by TS and upregulation by Tglu. Our GRP78 mRNA quantification in the TS-treated cancer cells suggests that TS treatment decreases GRP78 mRNA (Fig. 10F and 10I). Conversely, Tglu-treated cells have shown an increased level of GRP78 mRNA (Fig. 11C and 10F). These findings suggest that TS may downregulate GRP78 protein by interfering with the transcription or stability of GRP78 mRNA, while Tglu does the opposite and upregulate GRP78 protein. However, protein synthesis involves a complex interplay of macromolecules like mRNA, tRNA, rRNA and non-ribosomal protein factors. Therefore, GRP78 mRNA reduction by TS does not confirm the target organelle of TS in GRP78 downregulation.

Again, it was observed that GRP78 protein continued to decrease even after 12 h and 24 h of treatment (Figs. 10D, E and 10G, H). Considering that the half-life of GRP78 is longer than 36 h [53], this observation suggests that intracellular degradation systems, such as the ubiquitinproteasome or autophagy systems, may be activated by TS. Therefore, the mechanism of GRP78 downregulation by TS is not clearly understood. Previously, we have found that TS directly interacts with and activates protein kinase C (PKC) [31]. Hence, there is a possibility that activation of PKC by TS is correlated with the suppression of GRP78 expression. In addition, some other natural compounds like docosahexaenoic acid, curcumin, and lanatoside C have also been reported to suppress GRP78 expression [54–56]. Interestingly, these compounds also activate PKC [57–59], lending further support to the correlation between the PKC activation and GRP78 downregulation. However, another research group found that the inhibition of PKC suppresses GRP78 mRNA level under hypoxic condition [60]. Therefore, the regulation of GRP78 by PKC is controversial. Further investigation is required to confirm the correlation between activation of PKC by the tocopheryl esters and GRP78 expression.

We have confirmed the transfer of TS and Tglu from the cell culture medium to the cell. As shown in Fig. 5A, almost all the applied TS transferred to cells. On the other hand, the intracellular presence of 23% and 20% of applied Tglu remaining in the cell culture medium after 24 h indicates around 57% of applied Tglu disappeared from the cell culture medium and cell. It suggests that the possibility of the hydrolysis of Tglu in the cell in our experimental condition (Fig. 5B). These findings suggests that TS is less prone to hydrolysis than Tglu. However, our HPLC analysis confirms that TS and Tglu reached the intracellular region and initiated opposite biological activities.

In conclusion, a single carbon difference between TS and Tglu resulted in opposing effects on cellular apoptosis, superoxide generation, ER stress induction, and mitochondrial depolarization due to loss of mitochondrial integrity. We also demonstrated the involvement of GRP78 protein in the induction of ER stress by TS and the interplay between ER and mitochondria in TS-induced apoptosis. The structural difference may affect the stability, conformation, or structural flexibility of the tocopheryl esters, affecting their interactions with different molecular targets. Importantly, this report demonstrates the flexibility of tocopheryl esters in terms of biological activities. These results may indicate a broader potential for the application of tocopheryl esters to the treatment of different diseases, including cancer.

Materials and methods

Materials

TS and egg phosphatidylcholine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glutaric anhydride, 4-dimethylaminopyridine, and D- α -tocopherol were purchased from Tokyo Chemical Industry (Tokyo, Japan). Tetrahydrofuran was purchased from Kanto Chemical (Tokyo, Japan). Other reagents were of the highest grade commercially available.

Synthesis of α-tocopheryl glutarate

Tglu was synthesized by the condensation of D-α-tocopherol and glutaric anhydride in the presence of a catalytic amount of 4-dimethylaminopyridine. To a solution of D-α-tocopherol (201 mg, 0.467 mmol) in tetrahydrofuran (3 mL) were added glutaric anhydride (160 mg, 1.40 mmol) and 4-dimethylaminopyridine (5.7 mg, 0.0467 mmol) at room temperature. After stirring for 40 h, saturated NH₄Cl (10 mL) was added, and the mixture was then extracted twice with 20 mL of ethanol ethylacetate (AcOEt). The organic layer was washed twice with brine (15 mL), dried over anhydrous MgSO₄, filtered, and concentrated *in vacuo*. The residue was purified by two-column chromatography steps using an IsolaraTM automated purification system (Biotage, Sweden). The first step employed a linear gradient of *n*-hexane: AcOEt from 10:1 to 70:30 to afford Tglu (66.4 mg, 26%) as white solid.

Liposome preparation

A chloroform solution (0.1 mL) containing 0.1 M TS or Tglu was mixed with 0.64 mL of a chloroform solution containing egg phosphatidylcholine (0.1 g/mL) in a glass tube. The mixture was evaporated to dryness under a nitrogen stream to form a lipid film. The lipid film was hydrated with 192 μ L PBS and 8 μ L 1 N NaOH. The hydrated lipid film was incubated at 55 °C for 10 min in a water bath. After the incubation, the glass test tube was sonicated in an ultrasonic bath for 10 to 15 minutes to form liposomes. The size and surface charge of the liposomes was measured with a Zetasizer Nano (Malvern Panalytical Ltd., UK).

Cell culture

Mouse melanoma B16F1 cells, human hepatic carcinoma HepG2 cells, and mouse 3T3-Swiss albino cells were purchased from American Type Culture Collection and were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a 5% CO₂ humidified environment. When the cells

reached 80% confluency, they were treated with TS or Tglu for 12 h, 24 h and 48 h time intervals.

Cell viability assay

Cells were seeded at a density of 1.5×10^5 cells/well in 6-well plates and were cultured for 24 h. The cells were treated with different amounts of TS and Tglu-containing liposomes and incubated for 24 h and 48 h at 37 °C. The cells were collected by trypsin treatment, and cell viability was measured with the trypan blue dye exclusion method. Cell viability was calculated as the percentage of stained cells relative to the total number of cells.

Apoptosis analysis via DAPI staining

Cells were seeded at a density of 1.5×10^5 cells/well in a 35mm glass-bottom dish. The cells were treated with 75 µM and 100 µM TS-containing liposomes and incubated for 24 h. After 24 h, cells were fixed with 4% formaldehyde-phosphate buffer saline (PBS) at 37 °C for 15 min. Then, cells were washed with PBS and permeabilized with 0.1% Triton-bovine serum albumin (BSA) at 37 °C for 15 min. Afterwards, 1 ml DAPI (1 µg/ ml) was added to the dish and incubated the cells for another 15 min at 37 °C. After 15 min of incubation, cells were washed with PBS 3 times and observed under LSM700 confocal laser scanning microscope.

Corrected total cell fluorescence (CTCF) calculation

ROI was drawn around a certain number of cells using polygon selection and measured the area, mean and integrated density by ImageJ. We also measured the area and mean fluorescence of the background by selecting a non-fluorescence area of the image. After that, we calculated the corrected total cell fluorescence (CTCF) using the following formula-

CTCF = Integrated Density – (Area of selected cells X Mean fluorescence of background)

Analysis of cytochrome c release

Mitochondria were stained using MitoTracker Deep Red FM (Cell Signaling Technology, Massachusetts, USA). Mitochondria possess a membrane potential (around -150 mV), an indicator of mitochondrial activity. On the other hand, MitoTracker dyes are cell-permeable positively charged probes that contain a mildly thiol-reactive chloromethyl moiety. When cells are incubated with MitoTracker, these probes passively diffuse across the plasma membrane and accumulate in mitochondria in a membrane potential-dependent manner. After entering mitochondria, MitoTracker probes bind to the thiol group of mitochondrial proteins and show fluorescence under the confocal microscope. After treatment with TS for 12 h, cells were washed with PBS and loaded with 500 nM MitoTracker Deep Red FM that was diluted in

culture media and incubated for 30 min at 37 °C. After incubation, cells were fixed in ice-cold 100% methanol for 15 min at -20 °C. Cells were washed with PBS and permeabilized with 0.1% Triton-BSA at 37 °C for 15 min. Afterwards, cells were washed with 5% BSA-PBS three times and incubated in anti-cytochrome c antibody (ab90529; Abcam) at a dilution of 1:1000 for 1 h at 37 °C. Next, cells were washed with 5% BSA-PBS three times and incubated the cells another 1 h at 37 °C with Alexa Fluor 488-conjugated secondary antibody (secondary, dilution 1:500). Then, cells were rinsed with PBS three times and observed with an LSM700 confocal laser scanning microscope. Images were processed for background subtraction with a rolling ball radius of 50 pixels (default). Afterwards, images were converted to 16-bit images and ROI was drawn around the boundaries of 4-5 cells/image. Next, cytofluorograms and colocalization ratio (Pearson coefficient) of MitoTracker and cytochrome c were obtained using the "colocalization finder" function of the ImageJ. At least, 30 images were analyzed during each observation.

TMRE assay for mitochondrial membrane potential

Tetramethylrhodamine, ethyl ester (TMRE, ab113852; Abcam) is a cell-permeant, positively charged mitochondria-specific fluorescence dye that diffuses through the plasma membrane of the cells and accumulates in active mitochondria due to the relative negative charge of mitochondria. Depolarized mitochondria fail to sequester TMRE and show weak fluorescence under a confocal laser scanning microscope. After treatment, TMRE solution was added directly to the cell culture dishes to a final concentration of 200 nM and incubated the cells for 30 min at 37 °C. Next, cells were washed with PBS three times and observed under LSM700 confocal laser scanning microscope. 6-15 cells were analyzed in each image for quantification of fluorescence intensity and corrected total cell fluorescence (CTCF) was calculated. At least 30 images were analyzed during each observation.

Analysis of TS and Tglu amount

We quantified the amount of TS and Tglu transferred to the cells and remained in the cell culture medium by high performance liquid chromatography (HPLC). We collected the culture medium and cells after different incubation periods, and lipids were extracted using the Folch method [61]. Briefly, cells were resuspended in 250 μ l water, and 1.25 ml of chloroform/methanol (2:1) was added. The following vortex for 1 min cells was incubated on ice for 5 min and again vortexed for 1 min. The organic and aqueous phases were separated by centrifugation at 13400 g for 10 min at 4°C. The organic phase was dried under N₂ and dissolved in methanol. The extracted samples were subjected to HPLC using a column of

TSKgel ODS-80T_M (150 X 4.6 mm, Tosoh Bioscience, Tokyo) and monitored TS samples at λ_{max} 286 nm, while Tglu at λ_{max} 284 nm. The solvent system used was methanol/water (99:1) for TS and methanol/water (90:10) for Tglu.

Superoxide detection

Cells were seeded at densities of 1.5×10^5 cells per 35mm glass-bottom dish. Intracellular superoxide anion was detected using the ROS-ID Superoxide Detection Kit (Enzo Life Sci., New York, USA) according to the manufacturer's instructions. At 60% confluency, some cells were treated with 75 µM liposome containing TS or Tglu. Other cells were pretreated with or 50 µM α -tocopherol or 5 mM NAC for 30 min prior to the addition of TS. After incubation for 3 h, the media was removed, and the cells were washed with PBS. Next, the cells were loaded with 1 mL superoxide staining solution and incubated for 30 min at 37 °C, and pyocyanin solution was added to a final concentration of 200 µM only to the culture dish representing the positive control. The cells were incubated for an additional 30 min. The cells were washed twice with wash buffer and observed with an LSM700 confocal laser scanning microscope (Carl Zeiss, Germany). To quantify fluorescence (CTCF) was calculated based on the method described above.

Cytoplasmic Ca²⁺ detection

Cells were seeded at densities of 1.5×10^5 cells per 35mm glass-bottom dish. Some cells were treated with 75 µM liposomes containing TS or Tglu or 2-APB (Selleck Chemicals LLC, Houston, USA) for 48 h. Other cells were pre-treated with 50 µM 2-APB for 1 h, then TS-containing liposomes were added to the cells. After 48 h cells were washed with PBS and intracellular Ca²⁺ was detected using the Fluo 4 Calcium Assay Kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, cells were loaded with 1 mL Fluo 4-AM reagent containing 0.04% Pluronic F-127 and 1.25 mmol/L probenecid and incubated for 1 h at 37 °C. After incubation, cells were observed with an LSM700 confocal laser scanning microscope. 6-15 cells were analyzed in each image for quantification of fluorescence intensity and corrected total cell fluorescence (CTCF) was calculated.

Real-time PCR

Total RNA was extracted and purified from cells with RNeasy Plus Mini kit (QIAGEN) according to the manufacturer's instructions. The total RNA concentration was determined with

a Nanodrop 8000 spectrometer (Thermo Fisher Scientific). Then, cDNA was synthesized from 200 ng of total RNA with PrimeScript RT Master Mix (Perfect Real Time, Takara Bio, Otsu, Japan) using an MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA, USA). The reverse transcription reaction was performed at 37 °C for 15 min and 85 °C for 5 s. Real-time PCR analysis was performed using TB GreenTM Premix Ex TaqTM II (Tli RNaseH Plus, Takara Bio) and a Thermal Cycler Dice Real-Time System III (Takara Bio). To analyze the mRNA levels of GRP78 and GAPDH or β actin, the cDNA was denatured at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s for amplification. The sequences of the primers used for the real-time PCR are shown in Table 1. The mRNA level of GRP78 was calculated using the 2^{- $\Delta\Delta$ Ct} method by normalization relative to GAPDH or β actin.

Western blotting

The following antibodies were used: anti-GRP78 rabbit polyclonal antibody (ab21685; Abcam, Cambridge, UK), anti-GAPDH rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, U.S.A), anti- β actin rabbit polyclonal (ab8227, Abcam), and anti-horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG polyclonal antibody (A24531; Thermo Fisher Scientific, Waltham, MA, USA). To observe the expression of GRP78 in TS- and Tglutreated cells, total protein (7 µg) was separated with 10% SDS-PAGE, and the proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was incubated with 5% skim milk dissolved in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 for 1h at 37 °C, then with anti-GRP78 antibody (1:3000) for 24 h at 4 °C. The membrane was washed and incubated with the HRP-conjugated secondary antibody at a dilution of 1:4000 for 1 h at 37 °C. After incubation of the membrane with a chemiluminescent substrate reagent (ECL Prime; GE Healthcare, Little Chalfont, UK), bands were detected with an LAS-4000 mini system (Fuji Film, Tokyo, Japan). After GRP78 was detected, the membrane was washed three times for 45 minutes with Tri-buffered saline (pH 7.4) containing 0.1% Tween 20, incubated with an anti-GAPDH antibody (1:3000) for 24 h at 4 °C, and developed as above. Band intensities were quantified using ImageJ.

Immunostaining for GRP78 localization

After treatment with TS- and Tglu-containing liposomes, cells were washed twice with 0.5 mL PBS. Then, cells were fixed with 4% paraformaldehyde in PBS for 15 min at 37 °C. Following three washes with PBS containing 1% bovine serum albumin (BSA), cells were permeabilized for 15 min at 37 °C with a PBS solution containing 1% BSA and 0.1% Triton X-100. The cells were washed and incubated with the primary antibody (anti-GRP78 BiP) for 1 h at 37 °C.

Following several washes, goat anti-rabbit IgG labeled with Alexa Fluor 488 (ab150077, Abcam) in 1%BSA/PBS (1:500) was added, and the cells were incubated for 1 h at 37 °C. After incubation, the cells were washed three times with 1% BSA-PBS and incubated with primary antibody (anti-calreticulin, ab92516, Abcam) for 1 h at 37 °C. Following several washes with 1% BSA-PBS, goat anti-rabbit IgG labeled with Alexa Fluor 647 (ab150079, Abcam) in 1%BSA/PBS (1:500) was added, and the cells were incubated for another 1 h at 37 °C. Afterwards, cells were rewashed, and nuclei were counterstained for 15 min with DAPI solution (1 µg/mL). The cells were washed with PBS and observed with an LSM700 confocal laser scanning microscope. We performed quantitative analysis by drawing the ROI around boundaries of 4-5 cells/image and measuring the area of the yellow signal area by the "color threshold" function of the ImageJ. Then we measured the total green signal area obtained from the green channel in the same cells. An equal number of cells was selected during the analysis of different images. At least 30 images were analyzed during each observation. The ratio was calculated by dividing the yellow signal area by the total green signal area. The ratio represents the percentage of total GRP78 located in the ER lumen.

Statistical analysis

Statistical significance was determined using one-way ANOVA to compare multiple groups, followed by Tukey's honestly significant difference test using JASP software. The unpaired Student's t-test was performed to calculate statistical significance between the two groups. *P* values less than 0.05 were considered to indicate statistical significance.

Author contributions

KK conceptualized the study; MNR performed the study; MNR, KK and MO were involved in the analysis and interpretation of the experimental data; MN and SS synthesized tocopheryl glutarate; MNR and KK wrote the manuscript; KK and MO supervised the study.

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Primer	Sequence (5' to 3')
Mouse GRP78 forward	AGAAACTCCGGCGTGAGGTAGA
Mouse GRP78 reverse	TTCCTGGACAGGCTTCATGGTAG
Mouse GAPDH forward	GAGGACCAGGTTGTCTCCTG
Mouse GAPDH reverse	ATGTAGGCCATGAGGTCCAC
Human GRP78 forward	TGGGTCGACTCGAATTCCAAAG
Human GRP78 reverse	GTCAGGCGATTCTGGTCATTGG
Human β -actin forward	CACTCTTCCAGCCTTCCTTCC
Human β -actin reverse	CGTACAGGTCTTTGCGGATGTC

Table 1. Sequences of primers used for real-time PCR

Figure legends

Fig. 1. Structures of α -tocopherol, TS and Tglu

Fig. 2. Cytotoxicity of TS and Tglu

(A) Viability of B16F1 cells after TS treatment. (B) Viability of HepG2 cells after TS treatment. (C) Viability of mouse 3T3-Swiss albino cells after TS treatment (D) DAPI staining assay showing apoptotic bodies (arrowheads) (E) Viability of B16F1 cells after Tglu treatment. (F) Viability of HepG2 cells after Tglu treatment. Data are shown as mean \pm SD (n = 6). * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 relative to control (one way ANOVA; Tukey's Post hoc test); # *P* < 0.05, ## *P* < 0.01(unpaired Student's t-test). Scale bar = 20 µm.

Fig. 3. Detection of cytochrome c release upon TS treatment

B16F1 cells were treated with 75 μ M TS-containing liposomes for 12 h. Cells were incubated with MitoTracker for 30 min. After that, cells were fixed and permeabilized prior to immunostaining with an anti-cytochrome c antibody and an Alexa Fluor 647-conjugated secondary antibody. Nuclei were counterstained with DAPI (blue). Cytofluorograms were obtained using the "colocalization finder" function of ImageJ. (A) Mitochondria (Red) and cytochrome c (Green) were detected. The yellow signal in the merged images represents the localization of cytochrome c in the mitochondria. (B) Cytofluorogram of the control cells. (C) Cytofluorogram of the TS-treated cells. Individual experiments were repeated five times. Scale bar = 20 μ m.

Fig. 4. Effects of TS and Tglu on mitochondrial membrane potential

B16F1 and HepG2 cells were treated with 75 μ M TS- or Tglu-containing liposomes and inositol 1,4,5-triphosphate receptor (IP₃R) antagonist 2-aminoethyl diphenyl borinate (2-APB). Cells were pre-treated for 1 h with 50 μ M 2-APB prior to the addition of TS. Mitochondria were stained with Tetramethylrhodamine, ethyl ester (TMRE). Mitochondria are stained red under confocal microscopy 48 h after treatment in B16F1 cells (A) and HepG2 cells (B). The fluorescence intensities were quantified with ImageJ (C) Quantification of fluorescence intensity in B16F1 cells. (D) Quantification of fluorescence intensity in HepG2 cells. Data are shown as mean \pm SD (n = 5). * *P* < 0.05, *** *P* < 0.001 relative to control; # *P* < 0.05, ### *P* < 0.001(one way ANOVA; Tukey's Post hoc test). Scale bar = 20 μ m.

Fig. 5. Measurement of TS and Tglu inside the cell

B16F1 cells were separately treated with 75 μ M TS- and Tglu-containing liposomes in serumfree medium. Cells and culture medium was collected after 0 h, 24 h and 48 h. The amount of TS and Tglu in the collected samples was quantified by HPLC. Data are shown as mean \pm SD (n = 6). *** *P* < 0.001vs 0 h (one way ANOVA; Tukey's Post hoc test).

Fig. 6. Effects of TS and Tglu on intracellular superoxide production

(A) Intracellular superoxide production in B16F1 cells was detected with a superoxideactivated fluorophore (orange). (B) The fluorescence intensities in B16F1 cells were quantified with ImageJ. (C) Superoxide production in HepG2 cells (D) The fluorescence intensities in HepG2 cells were quantified with ImageJ. Data are shown as mean \pm SD (n = 6). ** *P* < 0.01, *** *P* < 0.001 relative to untreated; # *P* < 0.05, ### *P* < 0.001 (one way ANOVA; Tukey's Post hoc test). Scale bar = 20 µm.

Fig. 7. Effects of TS and Tglu on intracellular Ca²⁺

(A) A fluorescent indicator (Fluo4) was used to detect intracellular Ca²⁺ in B16F1 cells 48 h of treatments. (B) The fluorescence intensity was quantified by ImageJ. Data are shown as mean \pm SD (n = 5). * *P* < 0.05, *** *P* < 0.001 relative to control; # *P* < 0.05, ## *P* < 0.01, ### *P* < 0.001 (one way ANOVA; Tukey's Post hoc test). Scale bar = 20 µm.

Fig. 8. Effect of TS on intracellular Ca²⁺ in Ca²⁺ free cell culture medium

(A) A fluorescent indicator (Fluo4) was used to detect intracellular Ca²⁺ in B16F1 cells after 6 h of 75 μ M TS treatment in Ca²⁺ free culture medium (B) The fluorescence intensity was quantified by ImageJ. Data are shown as mean \pm SD (n = 5). ** *P* < 0.01, *** *P* < 0.001 (one way ANOVA; Tukey's Post hoc test). Scale bar = 20 μ m.

Fig. 9. Effect of 2-APB on TS-induced cytotoxicity in vitro

B16F1 cells were treated with different concentrations of 2-APB in dimethyl sulfoxide (DMSO) for 48 hr. Cell viability was measured by trypan blue exclusion assay. (A) Viability of B16F1 cells after 2-APB treatment for 48 h. B16F1 and HepG2 cells were treated with 25 μ M and 50 μ M 2-APB for 1 h prior to addition of 75 μ M TS-containing liposome. (B) Viability of B16F1 cells treated with both 2-APB and TS (C) Viability of HepG2 cells treated with both 2-APB and TS. Data are shown as mean \pm SD (n = 6). ** *P* < 0.01, *** *P* < 0.001 vs control, #*P* < 0.05; ## *P* < 0.01; ### *P* < 0.001 (one way ANOVA; Tukey's Post hoc test).

Fig. 10. Effect of TS on GRP78 expression

B16F1 and HepG2 cells were treated with 75 μ M TS-containing liposomes. The whole-cell lysate was collected from control cells and cells treated with 75 μ M TS-containing liposomes after three-time points (12, 24, and 48 h), and Western blotting was performed, with GAPDH and β -actin serving as an internal control. The intensities of protein bands were quantified by ImageJ. (A) GRP78 protein bands in untreated mouse 3T3-Swiss albino and B16F1 cells (B) Quantification of western blot band intensities. (D, E) GRP78 protein bands and quantification in TS treated B16F1 cells. (G, H) GRP78 protein bands and quantification in TS treated HepG2 cells. Total RNA was isolated 24 and 48 h after treatment, and quantitative real time-PCR was performed (n = 5). Shown are levels of GRP78 mRNA relative to that of the housekeeping gene *GAPDH or* β -actin. Quantification of GRP78 mRNA in (C) untreated mouse 3T3-Swiss albino and B16F1 cells, (F) TS treated B16F1 cells, (I) TS treated HepG2 cells. Data are shown as mean \pm SD (n = 5). * *P* < 0.05, ** *P* < 0.01 (unpaired Student's t-test; one way ANOVA; Tukey's Post hoc test).

Fig. 11. Effect of Tglu on GRP78 expression

B16F1 and HepG2 cells were treated with 75 μ M Tglu-containing liposomes. The whole-cell lysate was collected from control cells and cells treated with 75 μ M Tglu-containing liposomes and Western blotting was performed, with GAPDH and β -actin serving as an internal control. The intensities of protein bands were quantified by ImageJ. (A, B) GRP78 protein bands and quantification in Tglu treated B16F1 cells. (D, E) GRP78 protein bands and quantification in Tglu treated B16F1 cells. (D, E) GRP78 protein bands and quantification in Tglu treated HepG2 cells. Total RNA was isolated 24 and 48 h after treatment, and quantitative real time-PCR was performed (n = 5). Shown are levels of GRP78 mRNA relative to that of the housekeeping gene *GAPDH or* β -actin. Quantification of GRP78 mRNA in (C) Tglu treated B16F1 cells, (F) Tglu treated HepG2 cells. Data are shown as mean \pm SD (n = 5). * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 (one way ANOVA; Tukey's Post hoc test).

Fig. 12. Effects of TS and Tglu on GRP78 localization

B16F1 cells were separately treated with 75 μ M TS- and Tglu-containing liposomes for 48 h. Cells were fixed and permeabilized prior to immunostaining with an anti-GRP78 antibody and an Alexa Fluor 488-conjugated secondary antibody. Next, cells were immunostained with an anti-calreticulin antibody and an Alexa Fluor 647-conjugated secondary antibody. Nuclei were counterstained with DAPI (blue). (A) GRP78 (green) and calreticulin (Red) were observed after the noted treatments. Yellow fluorescence in the merged images represents colocalization of GRP78 and calreticulin (B) Percent ratio of yellow signal to total green signal. Data are shown as mean \pm SD (n = 5). * *P* < 0.05, *** *P* < 0.001 relative to control; ### *P* < 0.001 (one way ANOVA; Tukey's Post hoc test). Scale bar = 20 µm.



 α -Tocopheryl succinate (TS)



 α -Tocopheryl glutarate (Tglu)



Figure	7
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Figure 9

