



Article

Antiproliferative Role of Natural and Semi-Synthetic Tocopherols on Colorectal Cancer Cells Overexpressing the Estrogen Receptor β

Irene Falsetti ¹, Gaia Palmi ² , Roberto Zonefrati ², Kristian Vasa ³ , Simone Donati ¹, Cinzia Aurilia ¹, Allegra Baroncelli ³, Caterina Viglianisi ³ , Francesco Ranaldi ¹ , Teresa Iantomasi ^{1,*} , Piero Procacci ³, Stefano Menichetti ³ and Maria Luisa Brandi ²

¹ Department of Experimental and Clinical Biomedical Sciences “Mario Serio”, University of Florence, Viale Pieraccini 6, 50139 Firenze, Italy; irene.falsetti@unifi.it (I.F.); simone.donati@unifi.it (S.D.); cinzia.aurilia@unifi.it (C.A.); francesco.ranaldi@unifi.it (F.R.)

² Italian Foundation for Research on Bone Disease (F.I.R.M.O.), Via San Gallo 123, 50129 Firenze, Italy; gaia@fondazionefirmo.com (G.P.); marialuisa@marialuisabrandi.it (M.L.B.)

³ Department of Chemistry “Ugo Schiff”, University of Florence, Via Della Lastruccia, 3–13, 50019 Sesto Fiorentino, Italy; kristian.vasa@unifi.it (K.V.); allegra.baroncelli@edu.unifi.it (A.B.); caterina.viglianisi@unifi.it (C.V.); piero.procacci@unifi.it (P.P.); stefano.menichetti@unifi.it (S.M.)

* Correspondence: teresa.iantomasi@unifi.it

Abstract: Estrogen receptor β (ER β) is the most highly expressed subtype in the colon epithelium and mediates the protective effect of estrogen against the development of colon cancer. Indeed, the expression of this receptor is inversely related to colorectal cancer progression. Structurally estrogen-like compounds, including vitamin E components, affect cell growth by binding to ERs. In the present study, cell proliferation was measured by cell counting in a Bürker hemocytometer, and ER β expression was measured by Real-Time qPCR and immunoenzymatic methods. The results obtained show that natural δ -tocopherol (δ -Toc) and two of its semi-synthetic derivatives, bis- δ -tocopheryl sulfide (δ -Toc)₂S and bis- δ -tocopheryl disulfide (δ -Toc)₂S₂, play an antiproliferative role and upregulate ER β expression, similar to 17- β -estradiol (17 β -E2), in human colon adenocarcinoma HCT8 cells engineered to overexpress ER β protein (HCT8- β 8). These events are not present in HCT8-pSV2neo and in HCT8- β 8 pretreated with ICI 182,780, suggesting that they are mediated by the binding of compounds to ER β , as also boosted by an in silico assay. The antiproliferative effect is independent of the intracellular redox state and (δ -Toc)₂S and (δ -Toc)₂S₂ reduce cell proliferation at concentrations lower than that of δ -Toc and all tested compounds are also able to upregulate ER β expression. Taken together, the data indicate that, through the involvement of ER β activity and expression, δ -Toc, (δ -Toc)₂S, and (δ -Toc)₂S₂ may provide potential therapeutic support against colorectal cancer.

Keywords: estrogen receptor β ; colorectal cancer; δ -tocopherol; δ -tocopherol sulfide/disulfide; cell proliferation; reactive oxygen species



Academic Editor: Elena Zocchi

Received: 13 November 2024

Revised: 26 February 2025

Accepted: 4 March 2025

Published: 5 March 2025

Citation: Falsetti, I.; Palmi, G.; Zonefrati, R.; Vasa, K.; Donati, S.; Aurilia, C.; Baroncelli, A.; Viglianisi, C.; Ranaldi, F.; Iantomasi, T.; et al. Antiproliferative Role of Natural and Semi-Synthetic Tocopherols on

Colorectal Cancer Cells Overexpressing the Estrogen Receptor β . *Int. J. Mol. Sci.* **2025**, *26*, 2305. <https://doi.org/10.3390/ijms26052305>

<https://doi.org/10.3390/ijms26052305>

Copyright: © 2025 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Estrogen receptors (ERs), including ER α and ER β , belong to the nuclear receptor family and have a similar structure; both receptor subtypes, after binding to estrogen, induce the transcription of estrogen-responsive genes [1]. However, the biological functions of ER α and ER β are different as well as their tissue targets [2]. Generally, ER α shows proliferative and anti-apoptotic effects, whereas ER β exerts antiproliferative and pro-apoptotic roles [3–6]. ER α is very poorly expressed in the colon, where ER β is highly

expressed in both normal and malignant epithelium, although ER β expression is inversely associated with colorectal cancer (CRC) progression [7,8]. The advanced stage and grade of colon cancer is associated with a decreased ER β expression, which is responsible for the increased proliferation and decreased differentiation and apoptosis in colon cancer cells [9,10]. Given that CRC affects more men than women and the incidence is reduced by hormone replacement therapy in ER β -positive postmenopausal women [11], a protective role in the prevention and/or progression of CRC has been attributed to estrogen and ER β [10,12,13]. Estrogen exerts its protective role in CRC through the involvement of ER β [14–17], and the activation and/or increased expression of ER β reduce intestinal tumorigenesis in animal models [18–20]. Reduced cell growth occurs in ER β -transfected colon cancer cells and their stimulation with phenolic compounds; having a structure similar to that of 17- β -estradiol (17 β -E2) induces an upregulation of ER β expression and downregulation of cell proliferation and viability [21–24].

Vitamin E is the fat-soluble, most antioxidant compound found in humans. It consists of eight different molecules, specifically, α -, β -, γ -, and δ -tocopherol (α -, β -, γ -, and δ -Toc) and α -, β -, γ -, and δ -tocotrienol [25]. Due to its antioxidant capacity, vitamin E may have a beneficial effect on diseases associated with oxidative stress, such as inflammatory, neurodegenerative, and cardiovascular diseases [26–28]. However, in addition to its antioxidant activity, vitamin E is also able to regulate the cell cycle, apoptosis, and cell proliferation by influencing the activity of a number of enzymes, the activation of transcription factors, and signal transduction pathways [25,29]. It has been shown that natural forms and synthetic derivatives of vitamin E have an antiproliferative effect in cancer cells but not in normal cells [30–34].

Due to the presence of a phenolic group in the structure, vitamin E compounds can influence cell proliferation by binding to ERs. In fact, both tocopherols and tocotrienols interact with ERs, even if δ isoforms appear to have a higher affinity than α and γ . Then, they can modulate the ER-dependent gene expression [35,36]. Tocotrienols have been shown to have a high affinity for ER β , but not for ER α , and to exert antiproliferative and pro-apoptotic effects through the nuclear translocation of ER β and the expression of pro-apoptotic estrogen-responsive genes in ER β -expressing breast cancer MDA-MB-231 cells and in human breast cancer MCF-7 cells expressing both ER α and ER β [37,38]. Tocopherols inhibit cell invasion in ER-positive breast cancer, while a derivative of α -Toc, unlike α -Toc, can reduce MCF-7 cell proliferation [39,40]. Although it has been shown that γ - and δ -Toc, but not α -Toc, inhibit colon carcinogenesis [41,42], to our knowledge, no data have been reported in the literature on the role of vitamin E components on ER-mediated cell proliferation in colon cancer. Therefore, the aim of this study was to evaluate the role of natural δ -Toc and two of its semi-synthetic derivatives, bis- δ -tocopheryl sulfide (δ -Toc) $_2$ S and bis- δ -tocopheryl disulfide (δ -Toc) $_2$ S $_2$, on ER β -mediated cell proliferation and the regulation of ER β expression in human colon adenocarcinoma HCT8 cells transfected with the plasmid vector pCXN2-hER β for the overexpression of ER β (HCT8- β 8). Semi-synthetic derivatives of δ -Toc containing sulfide and disulfide bonds were tested, as the intrinsic ability of a ligand to activate a receptor to induce the receptor-mediated cellular response is also related to its chemical structure [43]. In addition, they have previously been shown to exhibit differences in chain-breaking antioxidant activity in vitro [44], and may be differently affected by the intracellular redox state and, thus, differentially influence potential redox-mediated biological processes. The use of HCT8- β 8 allowed the in vitro evaluation of the role of ER β in colon tumorigenesis and how this can be regulated by molecules, structural analogues of 17 β -E2, through their binding to the estrogen receptor. To clarify the actual involvement of ER β in the role of the tested compounds, experiments

were also performed in the presence of 17β -E2 and in HCT8 cells transfected with the control pSV2neo vector (HCT8-pSV2neo).

2. Results

The experiments were performed in medium containing 0.5% charcoal-stripped fetal bovine serum (FBS) (starved medium) to deplete steroids, and the range of concentrations used for δ -Toc, $(\delta$ -Toc) $_2$ S, and $(\delta$ -Toc) $_2$ S $_2$ also includes those that have been reported in the literature for vitamin E compounds [36,39]. However, in our experiments, the normal concentration (μ N) was used for the tocopherol-containing compounds because $(\delta$ -Toc) $_2$ S and $(\delta$ -Toc) $_2$ S $_2$ have two δ -tocopherol units in their structures compared to δ -Toc (Figure 1).

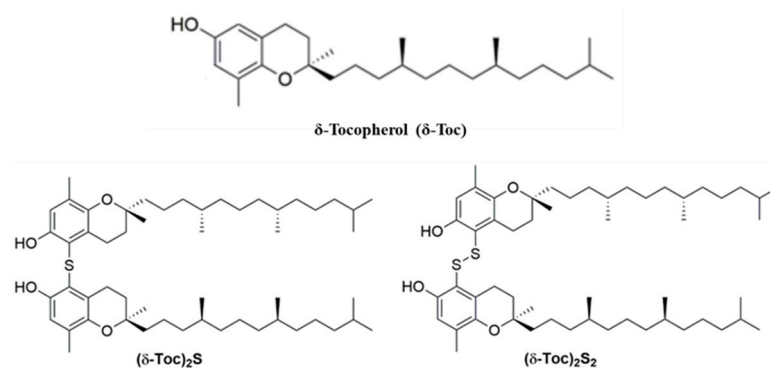


Figure 1. Structure of δ -tocopherol, δ -Toc, bis- δ -tocopheryl sulfide, $(\delta$ -Toc) $_2$ S, and bis- δ -tocopheryl disulfide, $(\delta$ -Toc) $_2$ S $_2$.

In this way, we can compare the effects of the natural δ -Toc and the semi-synthetic derivatives, considering the same concentration of tocopherol units. Consequently, the molarity of the $(\delta$ -Toc) $_2$ S and $(\delta$ -Toc) $_2$ S $_2$ is half that of the δ -Toc. Then, for δ -Toc, the concentrations 12.5, 25, 50, 100, and 250 μ N are equivalent to the same concentrations expressed in μ M. On the contrary, for semi-synthetic $(\delta$ -Toc) $_2$ S and $(\delta$ -Toc) $_2$ S $_2$, the concentrations 12.5, 25, 50, 100, and 250 μ N correspond to 6.25, 12.5, 25, 50, and 125 μ M. In order to have uniformity in the concentrations, we preferred to use the normal concentration.

2.1. Effect of 17β -E2, Natural δ -Toc, and Semi-Synthetic $(\delta$ -Toc) $_2$ S and $(\delta$ -Toc) $_2$ S $_2$ on Proliferation of HCT8- β 8 Cells, Treated or Not with ICI 182,780, and in HCT8-pSV2neo Cells

Figure 2a shows that 17β -E2, used as a positive control for an antiproliferative effect, significantly reduced the proliferation of HCT8- β 8 (94 ± 20 h for the population-doubling time) compared to untreated cells (51 ± 10 h for the population-doubling time) with an inhibitory effect of 76%. δ -Toc, $(\delta$ -Toc) $_2$ S, and $(\delta$ -Toc) $_2$ S $_2$ were shown to have an antiproliferative effect similar to that of estrogen. In particular, the antiproliferative effect of sulfur derivatives appeared to be superior to that of δ -Toc, as only the highest concentration (250 μ N) of δ -Toc was able to significantly reduce HCT8- β 8 proliferation (100 ± 23 h for the population-doubling time with a 96% inhibition) (Figure 2b). All concentrations of $(\delta$ -Toc) $_2$ S used significantly inhibited proliferation with 12.5 μ N as the minimum responsive concentration (93 ± 16 h for the population-doubling time with an inhibition of 82%) (Figure 2c), while the antiproliferative effect of $(\delta$ -Toc) $_2$ S $_2$ was evident from the concentration of 50 μ N with a population-doubling time of 96 ± 21 h with an inhibition of 88% (Figure 2d). However, it should be noted that the sulfur derivatives did not show a concentration-dependent effect in the responsive concentration range. In fact, the antiproliferative effect was not significantly different among the different effective concentrations (Figure 2c,d).

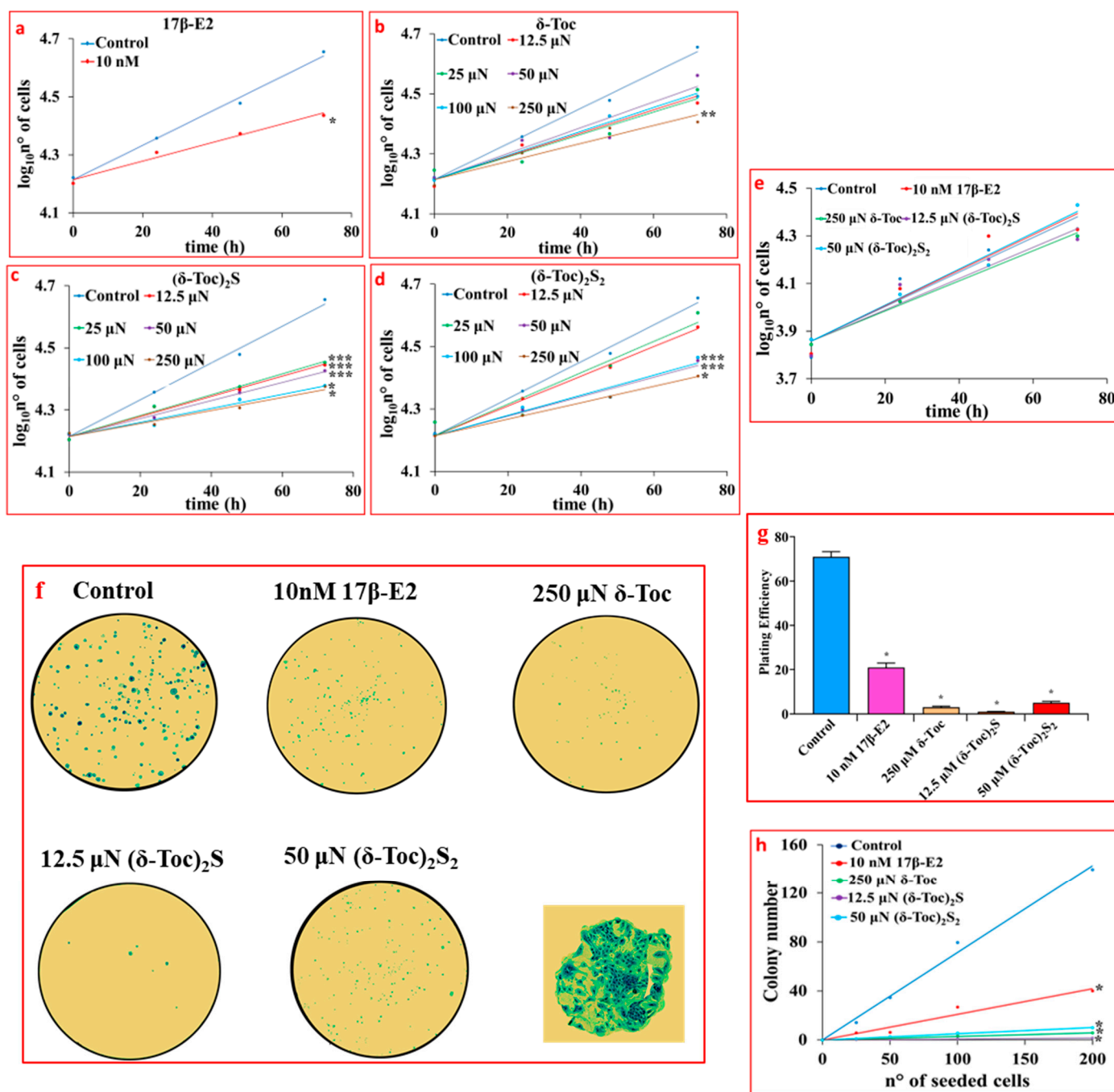


Figure 2. Proliferation (a–e) and colony formation (f–h) assays in cells treated with 17β-E2, natural δ-Toc, or semi-synthetic (δ-Toc)₂S and (δ-Toc)₂S₂. HCT8-β8 and HCT8-pSV2neo cells were cultured in charcoal-stripped 0.5% FBS and untreated (control) or treated with 10 nM 17β-E2 or concentrations in the range (12.5–250 μN) of δ-Toc, (δ-Toc)₂S, or (δ-Toc)₂S₂ for 0, 24, 48, and 72 h to detect proliferation, as described in Section 4. The same treatments were performed for 10 days to detect colony formation in HCT8-β8. (a–e) Proliferation values are the mean ± SE of three experiments repeated in tetraplicate and are expressed as the log₁₀ of cell number. (f) Representative colony formation images in 200 cells/well; amplification of a representative colony is shown in the square. (g) Plating efficiency values in 200 cell/well are the ratio between number of colonies formed and number of cells seeded × 100, (h) Colony number relative to number of cells seeded (25–200 cells). Plating efficiency and colony number values are the mean ± SE of three independent experiments. * $p \leq 0.001$; ** $p \leq 0.01$; *** $p \leq 0.05$ compared to control.

Taking all this into account, in the following experiments, δ-Toc was used at 250 μN, which is the only effective concentration, while the semi-synthetic compounds, (δ-Toc)₂S

and $(\delta\text{-Toc})_2\text{S}_2$, were tested at the lowest concentrations at which they were effective (12.5 and 50 μN , respectively).

Figure 2e shows that the proliferation of HCT8-pSV2neo cells was not affected by 10 nM $17\beta\text{-E}_2$, 250 μN $\delta\text{-Toc}$, 12.5 μN $(\delta\text{-Toc})_2\text{S}$, and 50 μN $(\delta\text{-Toc})_2\text{S}_2$. These treatments were carried out in HCT8- β 8 in order to confirm the antiproliferative role of the tocopherol-containing compounds by means of the colony formation assay. The results obtained show that, when 200 cells were seeded in 6-well plates and treated with $17\beta\text{-E}_2$, $\delta\text{-Toc}$, $(\delta\text{-Toc})_2\text{S}$, and $(\delta\text{-Toc})_2\text{S}_2$ for 10 days, the number of colonies was very, very low compared to the control (Figure 2f,g). In addition, the number of HCT8- β 8 colonies was dependent on the different number of cells seeded in the range of 25–200 cells/well and was reduced by treatments compared to control (Figure 2h). In the presence of treatments, the number of colonies in HCT8-pSV2neo was not different from that in controls.

To assess the involvement of ER β in the antiproliferative role of tocopherol-containing compounds, the proliferation and colony formation were investigated in HCT8- β 8 pre-treated for 1 h with 10 μM ICI 182,780 (fulvestrant) to which were added 10 nM $17\beta\text{-E}_2$, 250 μN $\delta\text{-Toc}$, 12.5 μN $(\delta\text{-Toc})_2\text{S}$, or 50 μN $(\delta\text{-Toc})_2\text{S}_2$. ICI 182,780, an estrogen-receptor-signaling antagonist capable of binding to ERs, causes the alteration of receptor dimerization and increases receptor degradation [45,46]. The results show that ICI 182,780 prevented the antiproliferative effect of $17\beta\text{-E}_2$ (Figure 3a) and tocopherol-containing compounds (Figure 3b–d), suggesting the involvement of ER β in their effect on proliferation and a possible binding of these compounds to the receptor. In the presence of ICI 182,780, the number of colonies was similar in all conditions (Figure 3e–g).

2.2. Effect of $17\beta\text{-E}_2$, Natural $\delta\text{-Toc}$, and Semi-Synthetic $(\delta\text{-Toc})_2\text{S}$ and $(\delta\text{-Toc})_2\text{S}_2$ on Viability of HCT8- β 8 and HCT8-pSV2neo Cells

Cell viability was determined using acridine orange (AO) staining to detect the lysosome formation, an indicator of cell death, and trypan blue dye which is only taken up by dead cells. In fact, an increased lysosome formation and activation is an early sign of cell distress leading to subsequent apoptosis and cell death [47], whereas the trypan blue dye exclusion test is based on the staining of dead or damaged cells with compromised cell membranes [48]. The lysosomal fluorescence staining at 72 h (Figure 4a,b) and the relative lysosomal activity (Figure 4c) detected in HCT8- β 8 and HCT8-pSV2neo are shown. In particular, HCT8- β 8 cells treated with 10 nM $17\beta\text{-E}_2$ showed a significant increase in lysosomal activity, resulting in a decrease in viability at all times tested, with no differences between the different times; on the contrary, $\delta\text{-Toc}$, $(\delta\text{-Toc})_2\text{S}$, and $(\delta\text{-Toc})_2\text{S}_2$, tested at the lowest responsive concentrations for proliferative activity (250, 12.5, and 50 μN for $\delta\text{-Toc}$, $(\delta\text{-Toc})_2\text{S}$, and $(\delta\text{-Toc})_2\text{S}_2$, respectively), did not cause a significant change in viability compared to the control (Figure 4a–c). Results at 24 h and 48 h were similar to those at 72 h. The trypan blue dye exclusion assay performed at 72 h confirmed these results and showed that only $17\beta\text{-E}_2$ significantly reduced HCT8- β 8 viability by approximately 25% compared to control cells (Figure 4d). The lysosomal activity and trypan blue assay showed that the viability of HCT8-pSV2neo cells was not affected by either $17\beta\text{-E}_2$ or tocopherol-containing compounds (Figure 4b–d).

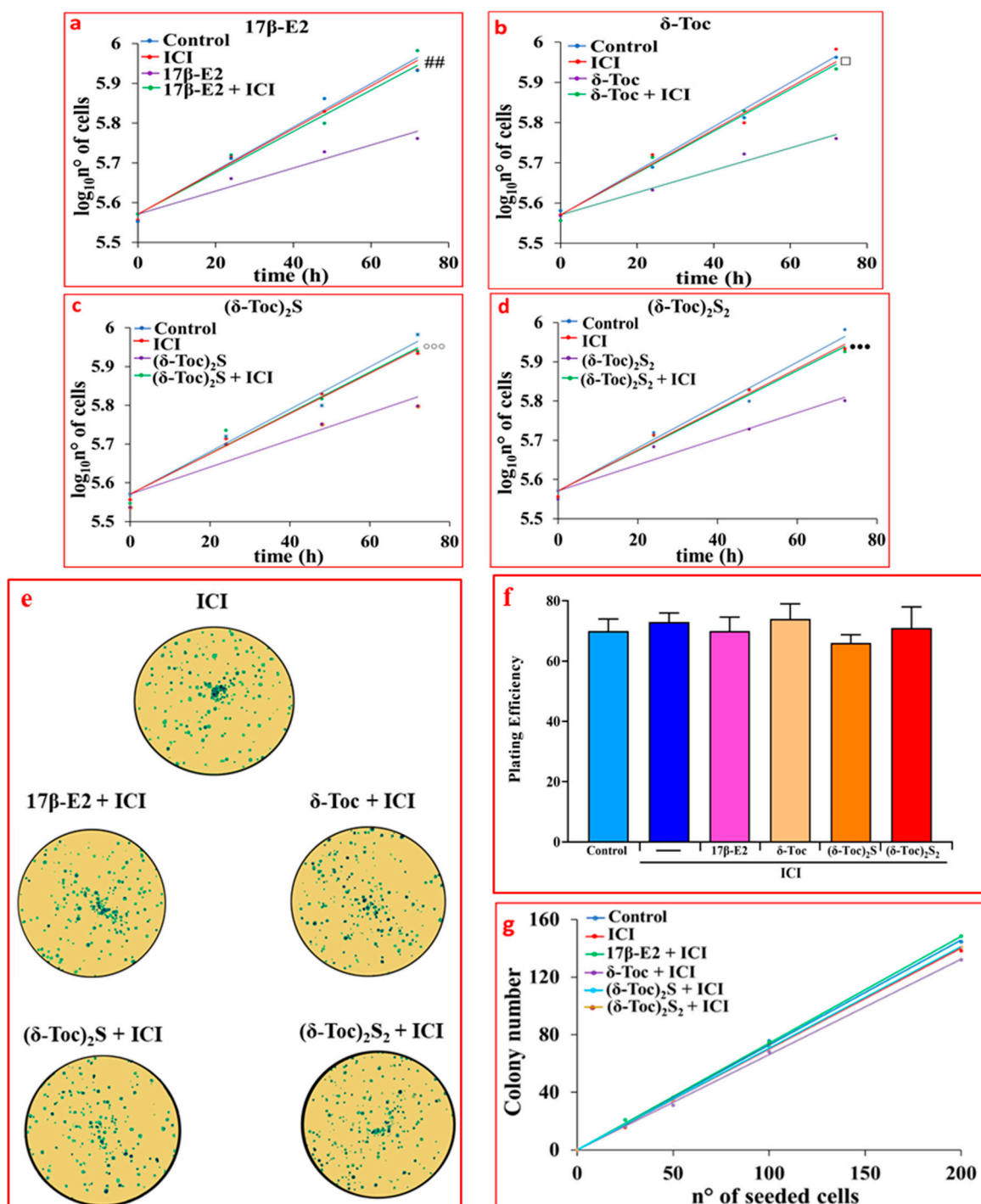


Figure 3. Proliferation (a–d) and colony formation assay (e–g) of HCT8-β8 treated with 17β-E2, natural δ-Toc, or semi-synthetic (δ-Toc)₂S and (δ-Toc)₂S₂, in the presence of ICI 182,780. HCT8-β8 cells, cultured in 0.5% charcoal-stripped FBS were pretreated for 1 h with 10 μM ICI 182,780 (ICI) and untreated (control) or treated with 10 nM 17β-E2, 250 μN δ-Toc, 12.5 μN (δ-Toc)₂S, or 50 μN (δ-Toc)₂S₂ for 0, 24, 48, and 72 h to detect proliferation or for 10 days to detect colony formation, as described in Section 4. (a–d) Proliferation values are the mean ± SE of three experiments repeated in tetraplicate and are expressed as the \log_{10} of cell number; (e) Representative colony formation images in 200 cells/well. (f) Plating efficiency values in 200 cell/well are the ratio between number of colonies formed and number of cells seeded × 100, (g) Colony number relative to number of cells seeded (25–200 cells). Plating efficiency and colony number values are the mean ± SE of three independent experiments. ## $p \leq 0.01$ compared to 17β-E2; □ $p \leq 0.001$ compared to δ-Toc; ○○○ $p \leq 0.05$ compared to (δ-Toc)₂S; ●●● $p \leq 0.05$ compared to (δ-Toc)₂S₂.

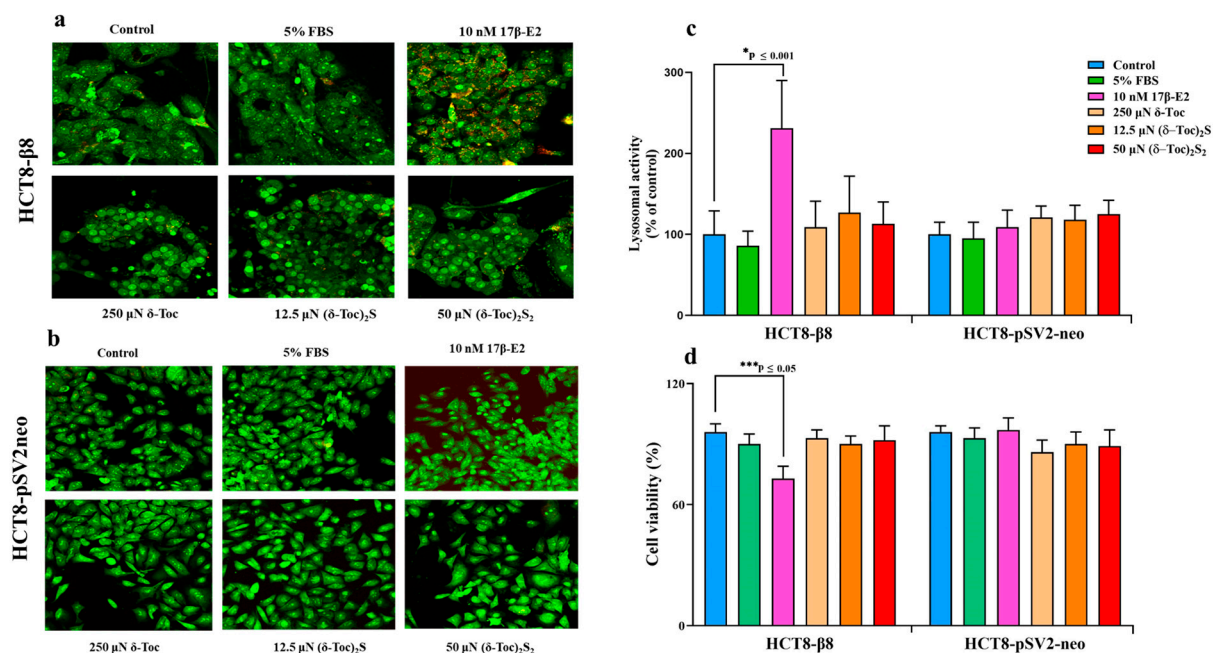


Figure 4. Microscopy observation (a,b), quantitative evaluation of lysosomal activity (c), and trypan blue dye exclusion test (d) in HCT8-β8 and HCT8-pSV2neo cells treated with 17β-E2, natural δ-Toc, or semi-synthetic (δ-Toc)₂S and (δ-Toc)₂S₂. HCT8-β8 and HCT8-pSV2neo cells were cultured in 5% FBS; or in 0.5% charcoal-stripped FBS and untreated (control) or treated with 17β-E2, δ-Toc, (δ-Toc)₂S, or (δ-Toc)₂S₂ for 72 h and stained with acridine orange (AO) or trypan blue dye as described in Section 4. (a,b) Representative image from three independent experiments. Fluorescence measured with LSM 900 confocal microscopy shows lysosomes in red-orange (λ_{ex} 405 nm/ λ_{em} 575–700 nm), and nuclei and mitochondria in green (λ_{ex} 488 nm/ λ_{em} 495–560 nm); original magnification: 40×. (c) Quantitative values of lysosomal activity, expressed as red/green fluorescence ratio and reported as percentage of control, are the means \pm SD of three experiments. (d) The percentage of viable cells was calculated as the ratio of unstained cells to total cells \times 100. Values are the means \pm SD of three experiments repeated in triplicate.

2.3. Effect of 17β-E2, Natural δ-Toc, and Semi-Synthetic (δ-Toc)₂S and (δ-Toc)₂S₂ on Apoptosis and Expression of CCND1 and PLK1 Genes in HCT8-β8 Cells

In HCT8-β8 cells after 72 h of treatment, an apoptosis analysis shows that δ-Toc and its sulfur derivatives at the concentrations tested did not induce apoptotic death unlike 17β-E2, which caused a significant apoptotic effect (Figure 5a). These results were in line with the viability results; 1 μM ionomycin, a calcium ionophore that increases intracellular calcium levels and promotes apoptosis [49], was used as a positive control for apoptosis.

To better understand the antiproliferative role of the compounds tested, we also analyzed the regulation of the cell cycle by evaluating the expression of genes such as cyclin D (CCND1), which encodes the cyclin D1 involved in the transition from G1 to S phase, and polo-like kinase 1 (PLK1), which encodes the PLK1 critical for the G2/M transition and mitosis [50,51]. After the treatment of HCT8-β8 with estrogen- and tocopherol-containing compounds for 48 and 72 h, both CCND1 and PLK1 expression were significantly decreased compared to control (Figure 5b,c). After 24 h, all treatments reduced CCND1 and PLK1 expression, but only the PLK1 reduction was significant (Figure 5b,c).

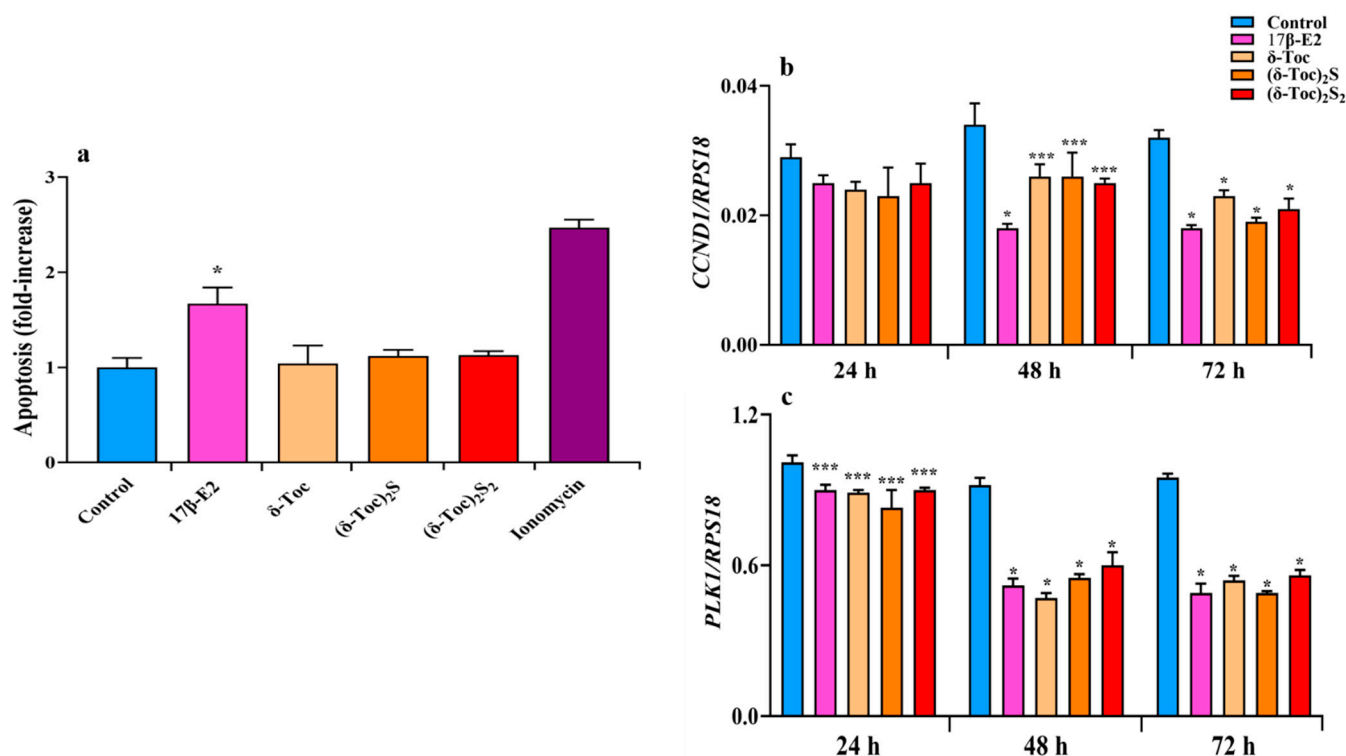


Figure 5. Apoptosis (a) and expression of *CCND1* (b) and *PLK1* (c) genes in HCT8-β8 cells treated with 17β-E2, natural δ-Toc, or semi-synthetic (δ-Toc)₂S and (δ-Toc)₂S₂ and ionomycin. HCT8-β8 cells were cultured in 0.5% charcoal-stripped FBS and untreated (control) or treated with 10 nM 17β-E2, 250 μN δ-Toc, 12.5 μN (δ-Toc)₂S, 50 μN (δ-Toc)₂S₂, or 1 μM ionomycin for 72 h to detect apoptosis or for 24, 48, and 72 h to detect *CCND1* and *PLK1* genes' expression, as described in Section 4. (a) Apoptosis data, relative to mono- and oligonucleosomes released into the cytoplasmic fraction from 10⁴ cells, are expressed as fold increase over the control value, using the absorbance of sample/absorbance of control ratio, and are the mean ± SD of three experiments repeated in triplicate. (b,c) Quantitative real-time qPCR analysis of *CCND1* and *PLK1* was performed and the values, expressed as the number of mRNA molecules of the gene and normalized to ribosomal protein S1 (RPS18) housekeeping gene (*RPS18*) mRNA, are the mean ± SD of three experiments repeated in triplicate. * $p \leq 0.001$; *** $p \leq 0.05$ compared to respective controls.

2.4. Effect of 17β-E2, Natural δ-Toc, and Semi-Synthetic (δ-Toc)₂S and (δ-Toc)₂S₂ on ERβ and ERα Expression in HCT8-β8 and HCT8-pSV2neo Cells

Figure 6a shows that the expression of ERβ in HCT8-β8, treated or not with 10 nM 17β-E2, 250 μN δ-Toc, 12.5 μN (δ-Toc)₂S, or 50 μN (δ-Toc)₂S₂, was significantly upregulated by both 17β-E2 and all tocopherol-containing compounds as compared to untreated cells. However, with 17β-E2, the levels of ERβ expression were significantly higher than those obtained with the tocopherol-containing compounds, whose ERβ mRNA levels were similar and not significantly different from each other (Figure 6a). On the contrary, no change in ERα expression levels was detected in the presence of the treatments (Figure 6c). In HCT8-pSVneo treated with 17β-E2, δ-Toc, (δ-Toc)₂S, or (δ-Toc)₂S₂, the expression of ERβ (Figure 6b) and ERα (Figure 6c) did not change compared to control cells.

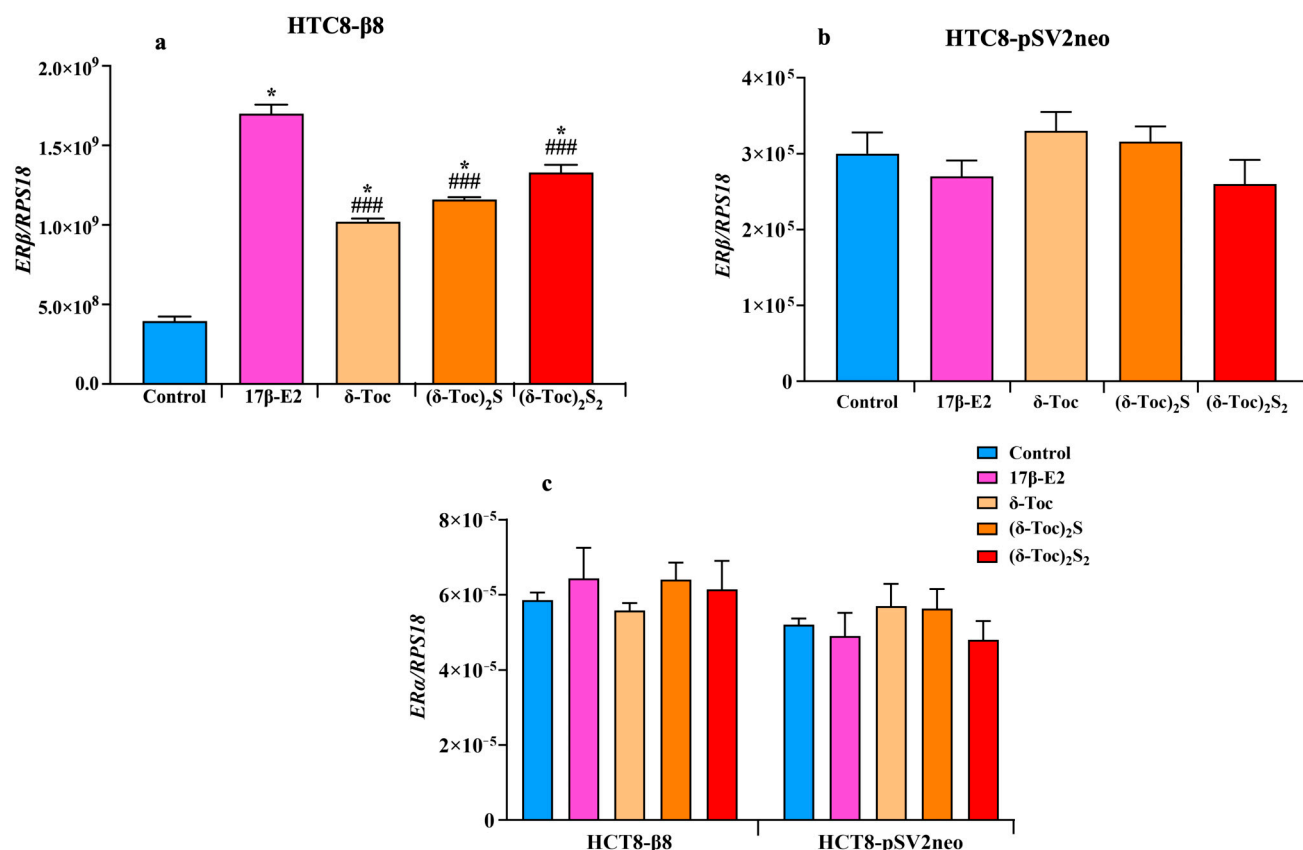


Figure 6. Real-time qPCR analysis of *ERβ* (a,b) or *ERα* (c) genes' expression in HCT8-β8 and HCT8-pSV2neo cells treated with 17β-E2, natural δ-Toc, or semi-synthetic (δ-Toc)₂S and (δ-Toc)₂S₂. HCT8-β8 and HCT8-pSV2neo cells were cultured in 0.5% charcoal-stripped FBS and untreated (control) or treated with 10 nM 17β-E2, 250 μM δ-Toc, 12.5 μM (δ-Toc)₂S, or 50 μM (δ-Toc)₂S₂ for 24 h as described in Section 4. Quantitative real-time qPCR analysis of the *ERβ* (a,b) and *ERα* (c) expression was performed and the values, expressed as the number of mRNA molecules of the gene and normalized to ribosomal protein S1 (RPS18) housekeeping gene (RPS18) mRNA, are the mean ± SD of three experiments repeated in tetraplicate. * $p \leq 0.001$ compared to control; ### $p \leq 0.05$ compared to 17β-E2.

2.5. Effect of 17β-E2, Natural δ-Toc, and Semi-Synthetic (δ-Toc)₂S and (δ-Toc)₂S₂ on Protein ERβ Expression in HCT8-β8 Cells

To demonstrate the presence of ERβ and the validity of our cellular model, ERβ was assessed by immunofluorescence staining in HCT8-β8 treated or not with 10 nM 17β-E2, 250 μM δ-Toc, 12.5 μM (δ-Toc)₂S, and 50 μM (δ-Toc)₂S₂ for 48 and 72 h. A microscopic observation at 48 h of HCT8-β8 showed the presence of ERβ for the control and all the treatments (Figure 7a). The results at 72 h were similar. However, because it is difficult to detect possible variations in ERβ levels under different treatments by immunofluorescence assay, the levels of ERβ under the same experimental conditions were detected by an immunoenzymatic method. Figure 7b shows that the levels of ERβ protein expression were similarly increased by 17β-E2 and all tocopherol-containing compounds.

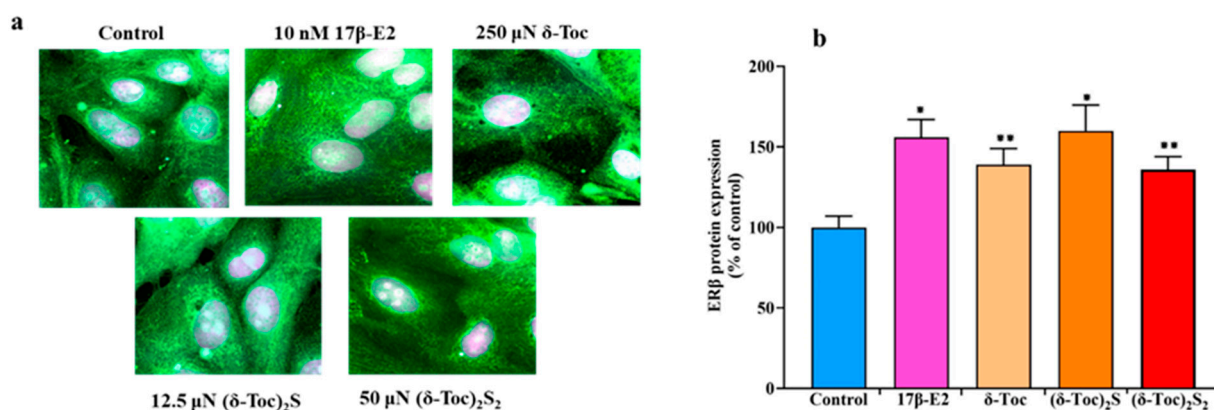


Figure 7. Immunofluorescence staining (a) and protein levels (b) of ERβ in HCT8-β8 treated with 17β-E2, natural δ-Toc, or semi-synthetic (δ-Toc)₂S and (δ-Toc)₂S₂. HCT8-β8 cells, cultured in 0.5% charcoal-stripped FBS, were untreated (control) or treated with 10 nM 17β-E2, 250 μN δ-Toc, 12.5 μN (δ-Toc)₂S, or 50 μN (δ-Toc)₂S₂ for 48 h as described in Section 4. (a) Representative image from three independent experiments. Immunofluorescence staining was performed with Alexa Fluor 488 and propidium iodide. Fluorescence was measured by LSM confocal microscopy in conventional colors: green for ERβ (Alexa Fluor 488) and violet (propidium iodide) for nuclei. Original magnification: 63×. (b) ERβ protein levels were assayed by ELISA kit and the values, normalized on total protein content and expressed as the percentage of control, are the mean ± SD of three experiments repeated in tetraplicate. * $p \leq 0.001$ compared to control; ** $p \leq 0.01$ compared to control.

2.6. Intracellular Redox State During Proliferation of HCT8-β8 Treated or Not with 17β-E2, Natural δ-Toc, and Semi-Synthetic (δ-Toc)₂S, (δ-Toc)₂S₂

Nutrient deprivation, such as serum starvation, promotes alterations of mitochondrial function in cancer cells, resulting in reduced ATP production and increased reactive oxygen species (ROS) levels [52]. In fact, mitochondria are the main source of ROS and a link has been shown between starvation and mitochondrial ROS modulator 1, which is involved in ROS production [53]. Thus, firstly, the intracellular redox state in HCT8-β8 cells was determined by measuring ROS levels in untreated cells cultured in a complete medium (5% FBS) and in a starved medium during proliferation. Figure 8a shows that starvation induced oxidative stress; indeed, ROS levels progressively increased from time 0 to 48 h of proliferation compared to the levels measured in cells grown with 5% FBS. After 72 h of proliferation, ROS levels remained high but decreased to the 24 h levels.

Subsequently, starved cells were treated for 24, 48, and 72 h with 10 nM 17β-E2 and tocopherol-containing compounds at all concentrations (12.5–250 μN) used to detect the proliferation. Figure 8a–d shows that ROS levels in cells treated with 17β-E2 were similar to those in untreated cells (control) at all times. Even with δ-Toc, (δ-Toc)₂S, and (δ-Toc)₂S₂, ROS levels at all concentrations were similar to their respective controls after 24 and 72 h of proliferation (Figure 8b,d). After 48 h of proliferation, the ROS levels were similar to untreated cells with all tocopherol-containing compounds at 12.5 μN and with 25 μN δ-Toc and (δ-Toc)₂S. In contrast, after 48 h of proliferation, the ROS levels were significantly lower compared to the control and similar to those detected after 24 and 72 h of proliferation when cells were treated with 25 μN (δ-Toc)₂S₂ and 50, 100, and 250 μN δ-Toc and its sulfur derivatives (Figure 8c).

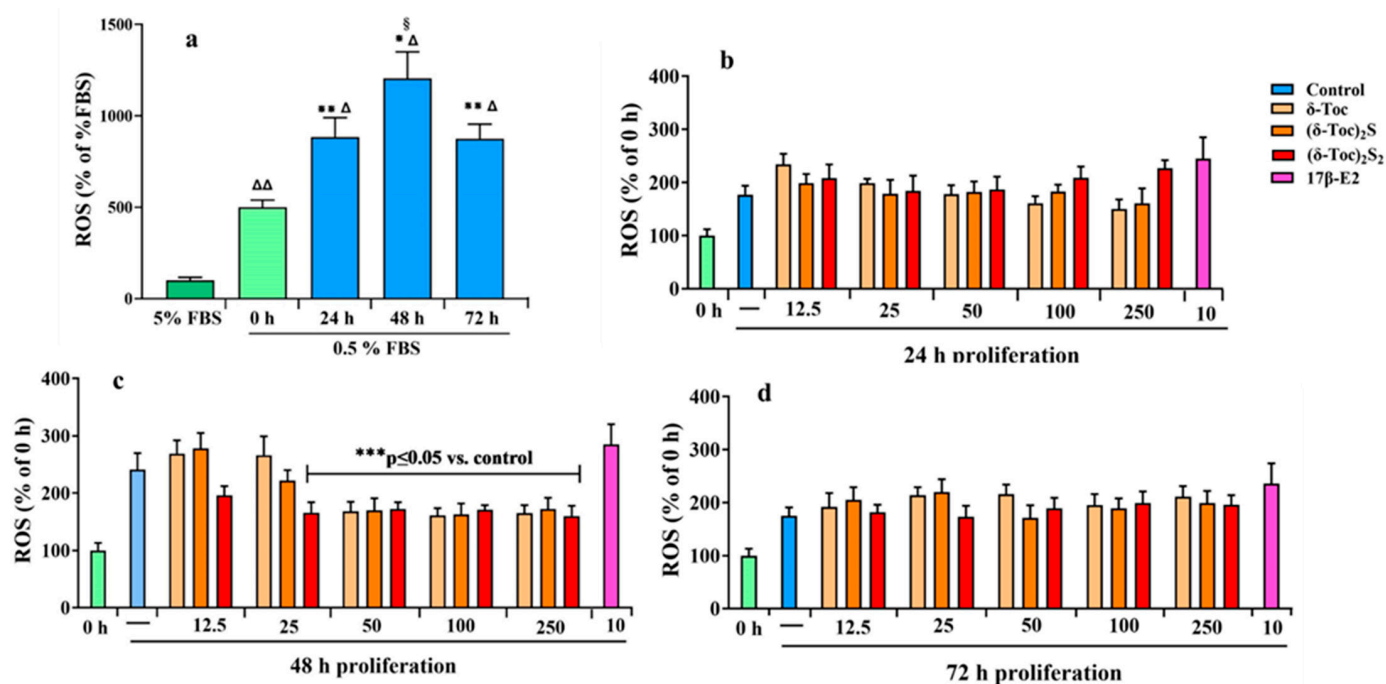


Figure 8. Intracellular ROS production in HCT8- β 8 untreated or treated with 17 β -E2, natural δ -Toc, or semi-synthetic $(\delta$ -Toc) $_2$ S and $(\delta$ -Toc) $_2$ S $_2$ during proliferation. ROS levels were detected by measuring the fluorescence intensity of the intracellular-oxidation-sensitive probe H2DCFDA. **(a)** HCT8- β 8 cells were cultured in 5% FBS or 0.5% charcoal-stripped FBS (0.5% FBS) for 0 (control), 24, 48, and 72 h as described in Section 4. The values, normalized on total protein content and expressed as percentage of 5% FBS, are the mean \pm SD of three experiments repeated in tetraplicate. Δ $p \leq 0.001$; $\Delta\Delta$ $p \leq 0.01$ compared to 5% FBS; $\Delta\Delta\Delta$ $p \leq 0.001$; $\Delta\Delta\Delta\Delta$ $p \leq 0.01$ compared to control (0 h); $\Delta\Delta\Delta\Delta\Delta$ $p \leq 0.05$ compared to 24 and 72 h. **(b–d)** HCT8- β 8 cells were cultured in 0.5% charcoal-stripped FBS for 0 h and untreated (—, control) or treated with 10 nM 17 β -E2 or various concentrations (12.5–250 μ N) of tocopherol-containing compounds for 24, 48, and 72 h as described in Section 4. The values, normalized on total protein content and expressed as percentage of control, are the mean \pm SD of three experiments repeated in tetraplicate. $***p \leq 0.05$ compared to control.

2.7. Docking Calculations

In Table 1, we report the binding affinities (in kcal/mol) computed as described in the Section 4. Experimental binding affinities of 17 β -E2 for ER α ds and ER β were taken from Refs. [54] and [55], respectively. The experimental binding affinity of DPN vs. ER β is taken from Ref. Handa 2022 [56]. The agreement between experimental and computed binding free energies for the estrogen receptor ligands with known affinities is satisfactory. In all cases, the best docking pose is remarkably close (less than 2 Angs. in terms of RMSD) to the corresponding crystallographic configuration, hence lending support to the Autodock-Vina calculations of the activity and binding structures of the tocopherol derivatives. The predicted binding free energy of δ -Toc for ER β is -10.9 kcal/mol, a value that is comparable to that of the 17 β -E2 substrate.

Table 1. Binding free energies (in Kcal/mol) for various ER α and ER β ligands (see text) predicted using Autodock-Vina.

Ligand	PDB	ER α		ER β		
		ΔG_{calc}	ΔG_{exp}	PDB	ΔG_{calc}	ΔG_{exp}
17 β -E2	1A52	-10.2	-13.4	5TOA	-10.5	-13.2
DPN	—	—	—	7XVY	-8.7	-9.0

Table 1. Cont.

Ligand	PDB	ER α		ER β		
		ΔG_{calc}	ΔG_{exp}	PDB	ΔG_{calc}	ΔG_{exp}
δ -Toc	3ERD	−8.4	—	7XVY	−10.9	—
$(\delta$ -Toc) $_2$ S	—	—	—	1HJ1	−9.2	—
$(\delta$ -Toc) $_2$ S $_2$	—	—	—	1HJ1	−9.0	—

— no docking calculations or no experimental binding affinity are available.

2.8. Binding Strength

For $(\delta$ -Toc) $_2$ S and $(\delta$ -Toc) $_2$ S $_2$, we have used the 1HJ1 PDB structure for docking calculations. In the 1HJ1 co-crystal structure, the protein is missing the terminal H12 helix (partly sealing the LBD binding site in the apo form) due to the steric displacement of the anti-estrogen ICI-164,384, a synthetic derivative of estradiol bearing a long aliphatic chain [57,58]. The Vina-predicted dissociation constants of the $(\delta$ -Toc) $_2$ S and $(\delta$ -Toc) $_2$ S $_2$ bound to the H12-lacking ER β 21 receptor are found in the sub-micromolar range as shown in Table 1, in agreement with their observed antiproliferation activity in HCT8- β 8 cells (see Figure 3).

In Figure 9, we finally report the LigPlot-substituted tocopherol derivatives with ER β [59]. In all cases' diagrams for the mono and bi, the binding is overwhelmingly governed by non-polar contacts: out of the 19 contacts for $(\delta$ -Toc) $_2$ S, 15 are of the hydrophobic type. The same holds true for the $(\delta$ -Toc) $_2$ S $_2$ ligand where 14 out of 18 contacts involve hydrophobic side chains.

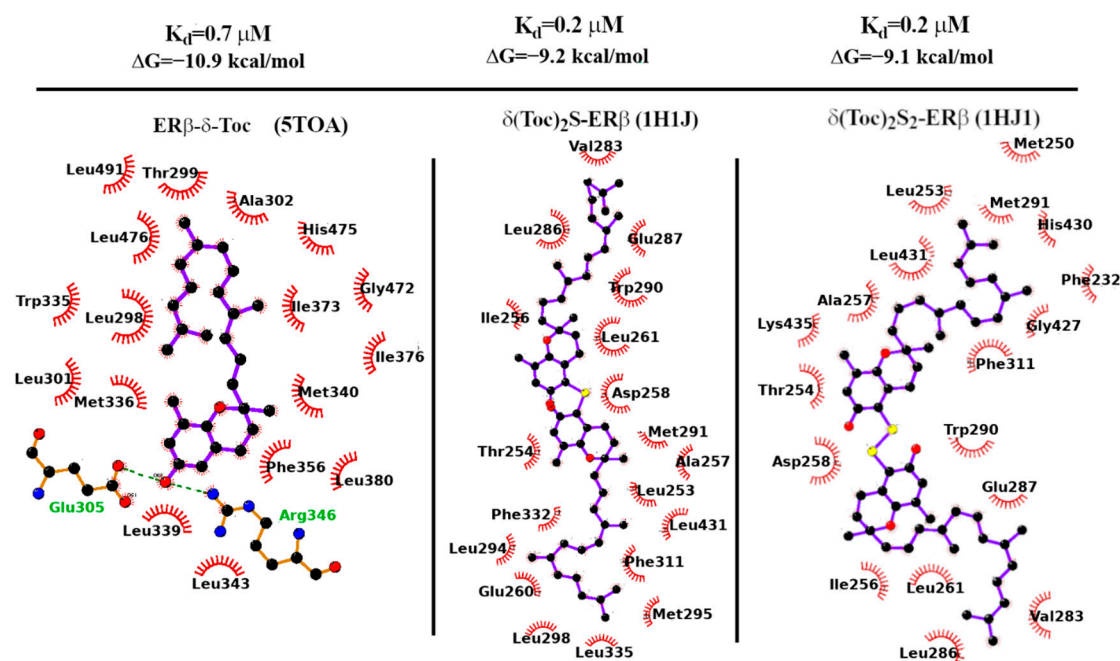


Figure 9. Binding affinity and dissociation constant of δ -Toc, $(\delta$ -Toc) $_2$ S, and $(\delta$ -Toc) $_2$ S $_2$ computed with Autodock-Vina 1.1.2 on the 5TOA and 1HJ1 X-ray structures of ER β .

3. Discussion

The present study demonstrates, for the first time, the antiproliferative role of natural δ -Toc, and semi-synthetic $(\delta$ -Toc) $_2$ S and $(\delta$ -Toc) $_2$ S $_2$ in HCT8- β 8 colorectal cancer cells overexpressing ER β . The structural and functional analogy with 17 β -E2, the lack of the antiproliferative effect in HCT8- β 8 pretreated with ICI 182,780 and HCT8-pSV2neo cells, and the silico approach suggest that tocopherol-containing compounds may exert their

antitumor function by binding to ER β . Furthermore, similar to 17 β -E2, the natural δ -Toc and semi-synthetic (δ -Toc) $_2$ S and (δ -Toc) $_2$ S $_2$ have been shown to upregulate the ER β gene expression in HCT8- β 8 only. In our experiments, the colon adenocarcinoma cell line, HCT8- β 8, transfected to overexpress ER β was used because the ER β -mediated role of phytoestrogens or phenolic compounds in colon cancer has previously been investigated in this cell line [23,24], and colon cancer lines overexpressing ER β have been used to study the anticancer role of this estrogen receptor [21].

The data obtained with δ -Toc and its sulfur derivatives were compared with the effect of 17 β -E2, which has previously been shown to reduce proliferation, upregulate ER β expression in HCT8- β 8, and exert a protective role on colon mucosa to prevent the CRC [23,24,60]. Indeed, estrogens are able to regulate reproduction, signaling pathways and gene transcription in target tissues by binding to their nuclear receptor [1]. ER β is the estrogen receptor that is predominantly expressed in the intestine, where it normally regulates the role of the epithelial barrier and the immune response. One of the roles of ER β is to protect the colon from developing cancer, as a reduced expression of this receptor correlates with the risk and progression of CRC [13]. ER β has been suggested to repress cancer-associated genes involved in cell proliferation and activate the expression of genes controlling cell growth inhibition, apoptosis, and differentiation [61]. Literature data suggest that various natural and synthetic compounds, such as phytoestrogens and xenoestrogens structurally related to estrogen, may play a protective role against CRC by binding to ER β and enhancing its anticancer function [10]. In particular, isoflavones such as genistein and calycosin, and the selective agonist of ER β , diarylpropionitrile, exert their anticancer properties by inhibiting proliferation and increasing apoptosis through ER β in colon cancer cells [23,62,63].

δ -Toc and its sulfur derivatives, in addition to being structurally similar to 17 β -E2, also exhibit an antiproliferative estrogen-like effect in HCT8- β 8, but not in the presence of the ER antagonist ICI 182,780 nor in HCT8-pSV2neo, demonstrating that their effect on cell proliferation is mediated by the binding to ER β . It is noteworthy that both δ -Toc and its sulfur derivatives achieve approximately similar maximal antiproliferative effects, but at different concentrations. In fact, this occurs with 250 μ N δ -Toc, 50 μ N (δ -Toc) $_2$ S $_2$, and 12.5 μ N (δ -Toc) $_2$ S, suggesting that sulfide/disulfide are able to activate the receptor at a lower concentration as compared to δ -Toc. All tocopherol-containing compounds bind to ER β , as demonstrated by in silico simulations, but our results suggest that, because of their different chemical structure, they may have a different intrinsic activity and/or ability to induce the translocation of the receptor–ligand complex into the nucleus. In fact, in silico tests show that the affinity of δ -Toc for ER β is greater than that of (δ -Toc) $_2$ S and (δ -Toc) $_2$ S $_2$, but the antiproliferative effect of δ -Toc is much lower than that of its sulfur derivatives. This could be explained by the fact that the two sulfur compounds, which have two hydrophobic tails, give the receptor–ligand complex greater lipophilicity and facilitate its transfer into the nucleus. Moreover, although the sulfur derivatives of δ -Toc bind to the receptor with the same affinity, (δ -Toc) $_2$ S $_2$ has a lower ability to induce the receptor-mediated cellular response than (δ -Toc) $_2$ S, probably because the presence of the disulfide bond could alter the intrinsic ability of the derivative to activate the receptor and the nuclear translocation of the complex with ER β .

Literature data show a direct interaction of tocopherols with ER α and ER β , but δ -Toc is the component with the highest affinity able to increase the growth of the hormone-sensitive breast cancer cell MCF7, which expresses both ER α and ER β [36]. α -Toc does not affect MCF7 cell proliferation, which is instead reduced by synthetic vitamin E derivatives [39], and it is not involved in ER β nuclear translocation and the expression of ER-dependent genes in these cells [38]. Thus, the effect of vitamin E on cell proliferation depends on its

different isoforms and ER α /ER β ratio, which is high in breast cancer cells and low in colon cancer cells [64,65].

The downregulated expression of the *CCDN1* and *PLK 1* genes, resulting in a slowing of the cell cycle, is consistent with the antiproliferative properties of all compounds tested. A reduced expression of these genes was also observed in the antiproliferative role of the bioflavonoid luteolin in MCF7 cells [66].

δ -Toc and its sulfur derivatives at the concentrations tested have no effect on the cell viability of HCT8- β 8 and HCT8-pSV2neo cells, and do not induce apoptosis in HCT8- β 8 cells. On the contrary, 17 β -E2 exerts a pro-apoptotic function in HCT8- β 8 and reduces the viability in these cells but not in HCT8-pSV2neo cells. The effect of 17 β -E2 on cell death is probably due to the ability of this hormone to influence biological mechanisms involved in cell death under certain conditions [67,68], and our results suggest that it is mediated through ER β . Indeed, it has been shown that 17 β -E2 is only able to induce apoptosis in Rat1 cells transfected to overexpress the ER receptor and not in parental Rat1 cells [69].

δ -Toc, (δ -Toc) $_2$ S, and (δ -Toc) $_2$ S $_2$ significantly increase the transcription of ER β but not that of ER α in HCT8- β 8. This increase in ER β expression is consistent with what has been observed in the presence of 17 β -E2 and phytoestrogens, such as genistein and quercetin, which not only reduce the proliferation but also upregulate the ER β expression in colon cancer cells [23,70]. Moreover, the increase in ER β expression is associated with an increase in ER β protein levels, although a close relationship between ER β and its mRNA levels in the presence of 17 β -E2 and tocopherol-containing compounds was not found. This may be due to the fact that HCT8- β 8 cells already overexpress the receptor and, therefore, not all of the mRNA is translated. In HCT8-pSV2neo, the treatment with 17 β -E2, δ -Toc, and semi-synthetic sulfide and disulfide derivatives does not affect the expression of ER β and ER α , suggesting that the increased transcription of ER β is also mediated by the binding of compounds to ER β .

We hypothesize that δ -Toc and the semi-synthetic sulfide and disulfide derivatives, by binding to ER β and increasing its expression, modulate signaling pathways involved in the inhibition of cell proliferation. In fact, in breast cancer cells, calycosin inhibits cell proliferation by increasing ER β expression, which induces changes in downstream signaling pathways, including the activation of p38 mitogen-activated protein kinase (MAPK) [71]. Bisphenol AF, an environmental contaminant, induces the apoptosis of human granulosa cells, KGN, through both ER β and the ROS-dependent activation of MAPK pathways [72].

Indeed, ROS levels influence cell proliferation [73,74]. However, in our experimental conditions, starvation-induced oxidative stress is not related to the antiproliferative role of tocopherol-containing compounds considering that ROS levels are similar in both untreated and treated cells. δ -Toc and its sulfur derivatives have a low antioxidant capacity which is evident in HCT8- β 8 cells only when a threshold is exceeded. In fact, after 48 h, δ -Toc, (δ -Toc) $_2$ S, and (δ -Toc) $_2$ S $_2$ are able to restore the levels of ROS to those found after 24 or 72 h of proliferation, but not to those of cells cultured in a complete medium (5% FBS). This is in contrast to what is observed in human colon adenocarcinoma epithelial cells, HT29, stimulated with TNF α . In this condition δ -Toc, (δ -Toc) $_2$ S, and (δ -Toc) $_2$ S $_2$ prevent TNF α -induced oxidative stress, and only δ -Toc exhibits pro-oxidant activity at high concentrations [75]. Moreover, 17 β -E2, which attenuates oxidative stress in the starved osteocyte-like cell line MLO-Y4 and induces ROS production through ER β activation in TCam-2 seminoma cells [76–78], does not affect starvation-induced ROS production in HCT8- β 8 cells. These data demonstrate that the role of vitamin E, its sulfur derivative, and 17 β -E2 on the intracellular redox state depends on the cell type and experimental conditions.

4. Materials and Methods

4.1. Cell Culture and Treatments

Human colon adenocarcinoma HCT8 cells, obtained from the American Type Culture Collection (Rockville, MD, USA), were stably transfected with the mammalian expression vector pCXN2-hER β (HCT8- β 8) or a control pSV2neo vector (HCT8-pSV2neo) [22]. Similar to HCT8-pSV2neo, HCT8- β 8 cells exhibited a polygonal shape with few extensions and a well-defined cytoskeleton when cultured in RPMI 1640 medium (Lonza Group, Basel, Switzerland) with 10% FBS; they grow in islets before reaching a packed confluence of approximately 3×10^5 cells/cm² (Figure 10).

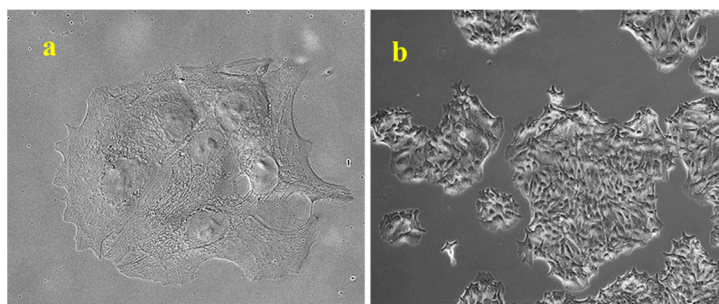


Figure 10. Representative observation of microscopic morphology of HCT8- β 8 cells grown in RPMI 1640 with 10% FBS. (a) Cells observed in phase contrast, original magnification: 10 \times . (b) Cells observed in Nomarski differential interference contrast (DIC), original magnification: 63 \times .

Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ in RPMI-1640 medium supplemented with 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 100 μ g/mL penicillin, 100 μ g/mL streptomycin, 800 μ g/mL di geneticin (G418; Invitrogen from Thermo Fisher Scientific Inc., Waltham, MA USA) (growth medium), and 10% FBS. The cells were then seeded at a density of 0.35×10^3 cells/cm² in dishes or multiwell plates, as appropriate for the experiments, and cultured in growth medium supplemented with 5% FBS (complete medium). After 72 h, the complete medium was replaced with growth medium with 0.5% charcoal-stripped FBS (Biological Industries, Kibbutz Beit Haemek, Israel) (starved medium) for 24 h (0 h). Subsequently, cells were cultured in starved medium and treated with 10 nM 17 β -E2, used as a control for antiproliferative effect, or different concentrations (12.5–250 μ N) of δ -Toc and the semisynthetic, (δ -Toc)₂S and (δ -Toc)₂S₂, for further appropriate time periods (h).

Solutions of δ -Toc, (δ -Toc)₂S, (δ -Toc)₂S₂, and 17- β -E2 in ethanol were diluted in starved medium in order to achieve the required concentration. Then, 1% ethanol was added to the respective untreated cells. All reagents used in our experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted.

4.2. Bis- δ -Tocopheryl Sulfide (δ -Toc)₂S and bis- δ -Tocopheryl Disulfide (δ -Toc)₂S₂ Synthesis

Bis- δ -tocopheryl disulfide (δ -Toc)₂S and bis- δ -tocopheryl disulfide (δ -Toc)₂S₂ were synthesized as previously described [44,79].

4.3. Cell Proliferation Analysis

Proliferation experiments were performed in cells seeded in a 60 mm tissue culture dishes as described above. HCT8- β 8 cells were grown in starved medium for 24 h (0 h) and then treated or not with the 17 β -E2 or tocopherol-containing compounds for a further 24, 48, and 72 h (proliferation times). In other experiments, test compounds were added to HCT8- β 8 pretreated for 1 h with 10 μ M ICI 182,780 (fulvestrant) (Merck KGaA, Darmstadt, Germany). Cells were detached with a trypsin/EDTA solution and counted using a Bürker

hemocytometer (Labor Optik, Lancing, UK) at the indicated times during the logarithmic phase of cell growth to avoid the contact inhibition phenomenon. Cell counts were plotted as linear regressions with \log_{10} of cell number on the y -axis and time on the x -axis, and results were expressed numerically as population-doubling time in h as mean \pm SE.

4.4. Colony Formation Assay

Cells cultured for 72 h in complete medium were detached and seeded in 6-well plates at a density of 25, 50, 100, and 200 cells/well. After 12 h, the time required for cells to attach to the wells, complete medium was replaced with starved medium and 10 nM 17 β -E2, 250 μ N δ -Toc, 12.5 μ N (δ -Toc) $_2$ S, and 50 μ N (δ -Toc) $_2$ S $_2$ were added for 10 days to HCT8- β 8 pretreated or not with 10 μ M ICI 182,780 or to HCT8-pSV2neo. The colonies formed were fixed with 4% paraformaldehyde for 10 min, washed three times with H $_2$ O, and stained with 1% toluidine blue for 5 min. The colonies were then washed three times with H $_2$ O and counted with a stereomicroscope WILD M3 (WILD Heerbrugg AG, Heerbrugg, Switzerland) at 6.4 \times magnification. Only colonies with more than 50 cells were included in the analysis. Images were acquired using LSM-900 confocal microscope (Carl Zeiss, Oberkochen, Germany) in brightfield with 2.5 magnification at a density of 200 seeded cells per well by panning, and each image is derived from the sum of a composition of an average of 64 individual images.

4.5. Cell Viability Analysis

Cell viability was determined in HCT8- β 8 and HCT8-pSV2neo seeded in 24-well plates and treated as described above. Lysosome formation was detected by AO staining in cells cultured in complete medium, to verify the absence of lysosomal activity, or in starved medium and untreated (control) or treated with 10 nM 17 β -E2, 250 μ N δ -Toc, 12.5 μ N (δ -Toc) $_2$ S, and 50 μ N (δ -Toc) $_2$ S $_2$ for 24, 48, and 72 h. The cells were washed three times with phosphate-buffered saline (PBS) and incubated in 1.5×10^{-5} Mol/L AO solution in PBS for 5 min in the dark at room temperature. Then, after three washes with PBS, starved medium was added to the cells to prevent cell damage during microscopic observation.

Cells were observed with an LSM-900 confocal microscope (Carl Zeiss, Oberkochen, Germany) using laser excitation at 488 nm (λ_{em} 495–560 nm) to stain cytoplasm and nucleus with orthochromatic green fluorescence and laser excitation at 405 nm (λ_{em} 575–700 nm) to stain lysosomes with metachromatic red fluorescence. Images were acquired using ZEN 3.1 software (Zeiss). Lysosomal activity was quantified by the red/green fluorescence ratio for each treatment and data were expressed as percentage of the control.

For the trypan blue exclusion test, cells were detached and a small volume of cell suspension was mixed with an equal volume of trypan blue dye. Dead (stained) and live (unstained) cells were counted in a Burkert hemocytometer. The ratio of the number of unstained cells to the total number of cells \times 100 was used to express the percentage of viable cells.

4.6. Cell Apoptosis Assay

HCT8- β 8 were seeded in 24-well plates and treated or not with 10 nM 17 β -E2, 250 μ N δ -Toc, 12.5 μ N (δ -Toc) $_2$ S, 50 μ N (δ -Toc) $_2$ S $_2$, and 1 μ M ionomycin for 72 h as described above. Apoptosis was detected by using Cell Death detection ELISApplus kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instruction. Mono- and oligonucleosomes released from 10^4 cells were detected using two monoclonal antibodies directed against DNA and histones and data are expressed as fold increase over controls using the following ratio: absorbance of sample/absorbance of control.

4.7. RNA Isolation and Real-Time qPCR

Total RNA was isolated from cells seeded in 6-well plates and treated or not with 10 nM 17 β -E2, 250 μ N δ -Toc, 50 μ N (δ -Toc) $_2$ S, and 50 μ N (δ -Toc) $_2$ S $_2$ for 24 h of proliferation as described above. RNA was isolated using Qiazol Lysis Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions and quantified by UV absorbance. Reverse transcription was performed using the Quantitect Reverse-Transcription Kit followed by treatment with ribonuclease-free deoxyribonuclease I (Qiagen). Quantitative polymerase chain reaction (qPCR) was performed using the Kapa Probe Fast qPCR kit (Kapa Biosystems Inc., Wilmington, MA, USA) according to the manufacturer's instructions. Briefly, reactions consisting of 1 μ L cDNA, 10 μ L KAPA PROBE FAST qPCR Master Mix, 2 μ L gene specific primers (10 μ mol/L), 1 μ L TaqMan Probe (5 μ mol/L), and 6 μ L RNase-free H $_2$ O were heated at 95 $^{\circ}$ C for 5 min and amplified by 35 cycles of 95 $^{\circ}$ C for 10 s, and 60 $^{\circ}$ C for 30 s using a Rotor-Gene Q (Qiagen). The results obtained were normalized to the ribosomal protein S1 (RPS18) housekeeping gene (RPS18). The following primers and Taq-Man probes were used as listed in Table 2.

Table 2. Primers and TaqMan used for quantitative real-time PCR (RT-qPCR).

Gene	Oligonucleotides	Sequence (5' \rightarrow 3')	Amplicon Size (bp)	T _{ann} ($^{\circ}$ C)
ER β	Forward primer	TCGCCAGTTATCACATCTGTATGCGG	95	65
	Reverse Probe *	GTGTCTCTCTGTTTACAGGTAAGGTGTG FTCCCGGTGZTGAAGCAAGATCGCTAGAAQ		
ER α	Forward primer	GACTATGCTTCAGGCTACCATTA	120	60
	Reverse Probe *	GGCTGGACACATATAGTCGTTAT FTCTCTTGAAZGAAGGCCTTGCAGCCQ		
CCND1	Forward primer	CTGTGCATCTACACCGACAA	86	55
	Reverse Probe *	AGGTTCCACTTGAGCTTGT FAGCTCCATTZTGCAGCAGCTCCTCQ		
PLK1	Forward primer	CACAGTGTCAATGCCTCCAA	125	55
	Reverse Probe *	AGGCCGTACTTGTCCGAATA FATCTTCTGGZGTCAGCAAGTGGGTGQ		
RPS18	Forward primer	AATCCGTTGACTCCGACCTTC	179	69
	Reverse Probe *	ACAGTACAGCCGCATCTTC FCCACATCGCZTCAGACACCATGGGQ		

* TaqMan probes in which F, the reporter fluorochrome [6-carboxyfluorescein], Z, the internal ZEN quencher, and Q, the quencher fluorochrome (Iowa Black FQ), are present. bp, base pairs of amplicon size; T_{ann}, annealing temperature.

4.8. Immunofluorescence Staining of ER β

HCT8- β 8 were seeded in 24-well plates and treated or not with 10 nM 17 β -E2, 250 μ N δ -Toc, 12.5 μ N (δ -Toc) $_2$ S, or 250 μ N (δ -Toc) $_2$ S $_2$ as described above. After 48 and 72 h of treatment, cells were fixed for 10 min with 4% paraformaldehyde and permeabilized with 0.2% Triton 100 \times at 37 $^{\circ}$ C for 30 min in humidified air with 5% CO $_2$. After three washes with PBS, the cells were treated with RNase diluted 1/1000 with 2% bovine serum albumin (BSA)/PBS at 37 $^{\circ}$ C in humidified air with 5% CO $_2$ to block non-specific sites and degrade RNA. Cells were washed three times with PBS and were incubated overnight at 4 $^{\circ}$ C with the primary antibody for ER β (Cell Signaling, Danvers, MA, USA). After removal of the primary antibody with three washes with PBS, the secondary antibody (Goat anti-Rabbit IgG (H + L) superclonal secondary antibody, Alexa Fluor 488, Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA) was added in the dark for 45 min at room temperature. The cells were washed with PBS and the nuclei were counterstained with propidium iodide

for 5 min at room temperature. After washing with H₂O, the cells were observed by laser scanning confocal microscopy (LSM 900, ZEISS) at 63 \times .

4.9. ER β Protein Assay

ER β protein level expression was detected in cell lysates from HCT8- β 8 seeded in 12-well plates and treated with 10 nM 17 β -E2, 250 μ N δ -Toc, 50 μ N (δ -Toc)₂S, and 50 μ N (δ -Toc)₂S₂ for 48 and 72 h as described above, using a Human ER β ELISA kit (Elabscience, Houston, TX, USA), according to the manufacturer's instructions. Briefly, cells were detached with trypsin and centrifuged for 5 min at 1000 \times g. The pellet containing the cells was washed three times, resuspended in cold PBS, and sonicated until the cells were completely lysed. The cell lysates were then centrifuged at 1500 \times g for 10 min at 4 °C to remove cell fragments and the supernatants were used for the assay. Data, normalized on total protein content, were expressed as a percentage of the ER β level detected in the control.

4.10. Intracellular ROS Assay

Intracellular ROS production was measured, as previously described [80], in HCT8- β 8 cells seeded in 12-well plates and treated as described above. Specifically, the ROS assay was performed in HCT8- β 8 cells cultured in complete medium, and in starved medium for 24 h (0 h). Additionally, ROS were detected at proliferation times in cells cultured in starved medium and treated or not with 10 nM 17 β -E2 or tocopherol-containing compounds at all concentrations used. Cell permeant 2'-7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Invitrogen, Carlsbad, CA, USA) is a fluorogenic compound that is deacetylated inside cells to a non-fluorescent compound. Upon oxidation by ROS, this compound is converted to the fluorescent compound 2'-7'-dichlorodihydrofluorescein. Then, 5 mg/L H2DCF-DA was added to the cells 30 min before the end of each treatment. HCT8- β 8 cells were lysed in a buffer containing 50 mM Tris/HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 100 mM NaF, and 2 mM EGTA, after washing with PBS. Lysed cells were centrifuged at 10,000 \times g for 10 min and fluorescence was detected in a Fluoroskan AscentFL microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 518 nm. Data were normalized to total protein content and expressed as percent ROS measured in complete medium or ROS at 0 h.

4.11. Protein Assay

Bicinchoninic acid solution (BCA) protein reagent assay (Thermo Fisher Scientific, Waltham, MA, USA) and BSA as standard were used to determine protein concentration as previously described [81].

4.12. In Silico Approach for the Prediction of the Binding of Tocopherols to ER β

The 3D structure of the Ligand Binding domain (LBD) of human ER β in complex with diarylpropionitrile (DPN) was obtained from the PDB files 7XVY [48], 1HJ1 [82], and 5TOA in complex with estradiol [83]. The 3D structure of the Ligand Binding domain (LBD) of human ER α in complex with diethylstilbestrol (DSB) was obtained from the PDB file 3ERD [84], and PDB file 1A52 in complex with estradiol [85]. The 3D structure of the compounds (δ -Toc)₂S, (δ -Toc)₂S₂, α -Toc, and δ -Toc were generated using the OpenBabel suite from the corresponding isomeric SMILES codes [86]. The docking calculations were performed using the Autodock-Vina code assuming a rigid receptor for the substrate 17 β -E2 and for the ligands (R)DPN and DSB [87]. For the bulky tocopherol derivatives, the residues GLU353, LEU384, LEU391, ARG394, LEU424, and LEU525 of ER α , and the residues GLU305, MET336, LEU339, PHE356, ILE376, HIS475, and LEU476 of ER β were assumed to be flexible. Flexible residues have been selected by analyzing the best docked

pose of the substrate 17 β -E2 with ER α / β , as those amino acids having at least one atom of the side chain within 3.8 Å of any atom of 17 β -E2. When using a rigid receptor, for the docking calculation, we retained the default Autodock setting (40 \times 40 \times 40 points with a grid spacing of 0.375 Å, yielding a cubic box of 15 Å, and 50 minimization rounds). When dealing with a flexible receptor, as for the case of the tocopherol derivatives, we used 60 \times 60 \times 60 points with a grid spacing of 0.375 Å, with a cubic box of 22.5 Å, and 70 minimization rounds. The cubic box was centered in all cases at the center of mass of 17 β -E2 in the best docking pose.

4.13. Statistical Analysis

As a first step, the normality of the replicates and the homoscedasticity of the groups were verified using the Lilliefors and Levene tests, respectively.

The statistical significance of the differences between the media in cell viability, cell apoptosis, RT-qPCR, ER β and ER α proteins, and intracellular RO assays was determined by one-way ANOVA analysis and by post hoc Bonferroni's multiple comparison test with a predefined experimental probability $\alpha_T = 0.05$ using GraphPad Prism10 software (GraphPad Software, San Diego, CA, USA).

Statistical analysis of cell proliferation and colony formation was performed as follows: (a) goodness-of-fit of each linear regression plot was verified by linearity test carried out using one way ANOVA, and (b) statistical differences between treated groups and control were analyzed by parallelism test performed in Excel by one-way ANOVA followed by post hoc Bonferroni's multiple-comparison test with a predefined experimental probability $\alpha_T = 0.05$.

5. Conclusions

This study demonstrates, for the first time, that natural δ -Toc and semisynthetic δ -tocopheryl sulfide (δ -Toc) $_2$ S and disulfide (δ -Toc) $_2$ S $_2$, similar to 17 β -E2, are able to reduce proliferation and increase the ER β expression in human colon adenocarcinoma HCT8 cells engineered to overexpress ER β but not in HCT8-pSV2neo. In addition, the lack of the antiproliferative effect of the compounds tested in HCT8- β 8 treated with ICI 182,780 and the in silico assay support the hypothesis that δ -Toc, (δ -Toc) $_2$ S, and (δ -Toc) $_2$ S $_2$ act through their binding to ER β . No involvement of the intracellular redox state in the antiproliferative role of the compounds was demonstrated. All tocopherol-containing compounds at the concentrations tested are not pro-apoptotic and do not affect cell viability but downregulate the expression of genes involved in cell cycle regulation. The results obtained suggest that both δ -Toc and its sulfur derivatives may play a role preventive in colorectal cancer through ER β . However, (δ -Toc) $_2$ S and (δ -Toc) $_2$ S $_2$ are able to exert their protective effect at lower concentrations. Taken together, these data may provide the basis for new anticancer therapeutic strategies. Further studies are needed to identify the signaling pathways involved in the ER β -mediated antiproliferative effects of tocopherol-containing compounds.

Author Contributions: Conceptualization, T.I., S.M., and M.L.B.; methodology, I.F., G.P., K.V., A.B., C.A., S.D., and R.Z.; software, R.Z., C.A., S.D., and P.P.; validation, T.I., S.M., C.V., and F.R.; formal analysis, C.V., F.R., R.Z., G.P., K.V., A.B., and I.F.; investigation, I.F., G.P., C.V., K.V., A.B., S.D., C.A., and R.Z.; resources, T.I., S.M., F.R., P.P., M.L.B., and C.V.; data curation, R.Z., I.F., P.P., T.I., S.M., F.R., and C.V.; writing—original draft preparation, T.I., P.P., G.P., I.F., and R.Z.; writing—review and editing, T.I., R.Z., C.V., G.P., P.P., M.L.B., and S.M.; supervision, M.L.B.; funding acquisition, T.I., F.R., C.V., M.L.B., and S.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by grants from the Ministero dell’ Istruzione, dell’ Università e della Ricerca (RICATEN22 and RICATEN23) to T.I., S.M., F.R., and C.V., and from the Fondazione Italiana Ricerca sulle Malattie dell’ Osso (F.I.R.M.O. Onlus) (M.L.B.). This research was also supported by the MUR—Progetto Dipartimenti di Eccellenza 2023–2027 (DICUS 2.0) to the Department of Chemistry “Ugo Schiff” of the University of Florence.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets analyzed in the current study are not publicly accessible, but they are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Fuentes, N.; Silveyra, P. Estrogen receptor signaling mechanisms. *Adv. Protein Chem. Struct. Biol.* **2019**, *116*, 135–170. [[CrossRef](#)] [[PubMed](#)]
2. Patel, S.; Homaei, A.; Raju, A.B.; Meher, B.R. Estrogen: The necessary evil for human health, and ways to tame it. *Biomed. Pharmacother.* **2018**, *102*, 403–411. [[CrossRef](#)] [[PubMed](#)]
3. Thomas, C.; Gustafsson, J. The different roles of ER subtypes in cancer biology and therapy. *Nat. Rev. Cancer* **2011**, *11*, 597–608. [[CrossRef](#)]
4. Caruntu, C.; Mirica, A.; Roșca, A.E.; Mirica, R.; Caruntu, A.; Tampa, M.; Matei, C.; Constantin, C.; Neagu, M.; Badarau, A.I.; et al. The role of estrogens and estrogen receptors in melanoma development and progression. *Acta Endocrinol.* **2016**, *12*, 234–241. [[CrossRef](#)]
5. Lewoniewska, S.; Oscilowska, I.; Forlino, A.; Palka, J. Understanding the Role of Estrogen Receptor Status in PRODH/POX-Dependent Apoptosis/Survival in Breast Cancer Cells. *Biology* **2021**, *10*, 1314. [[CrossRef](#)] [[PubMed](#)]
6. Chaudhri, R.A.; Hadadi, A.; Lobachev, K.S.; Schwartz, Z.; Boyan, B.D. Estrogen receptor- α 36 mediates the anti-apoptotic effect of estradiol in triple negative breast cancer cells via a membrane-associated mechanism. *Biochim. Biophys. Acta (BBA)—Mol. Cell Res.* **2014**, *1843*, 2796–2806. [[CrossRef](#)]
7. Caiazza, F.; Ryan, E.J.; Doherty, G.; Winter, D.C.; Sheahan, K. Estrogen receptors and their implications in colorectal carcinogenesis. *Front. Oncol.* **2015**, *5*, 19. [[CrossRef](#)]
8. Edvardsson, K.; Nguyen-Vu, T.; Kalasekar, S.M.; Pontén, F.; Gustafsson, J.-Å.; Williams, C. Estrogen receptor β expression induces changes in the microRNA pool in human colon cancer cells. *Carcinogenesis* **2013**, *34*, 1431–1441. [[CrossRef](#)]
9. Topi, G.; Ehrnström, R.; Jirstrom, K.; Palmquist, I.; Lydrup, M.-L.; Sjölander, A. Association of the oestrogen receptor beta with hormone status and prognosis in a cohort of female patients with colorectal cancer. *Eur. J. Cancer* **2017**, *83*, 279–289. [[CrossRef](#)]
10. Williams, C.; Di Leo, A.; Niv, Y.; Gustafsson, J.-Å. Estrogen receptor beta as target for colorectal cancer prevention. *Cancer Lett.* **2016**, *372*, 48–56. [[CrossRef](#)]
11. Rudolph, A.; Toth, C.; Hoffmeister, M.; Roth, W.; Herpel, E.; Schirmacher, P.; Brenner, H.; Chang-Claude, J. Colorectal Cancer Risk Associated with Hormone Use Varies by Expression of Estrogen Receptor- β . *Cancer Res.* **2013**, *73*, 3306–3315. [[CrossRef](#)] [[PubMed](#)]
12. Hases, L.; Indukuri, R.; Birgersson, M.; Nguyen-Vu, T.; Lozano, R.; Saxena, A.; Hartman, J.; Frasier, J.; Gustafsson, J.; Katajisto, P.; et al. Intestinal estrogen receptor beta suppresses colon inflammation and tumorigenesis in both sexes. *Cancer Lett.* **2020**, *492*, 54–62. [[CrossRef](#)] [[PubMed](#)]
13. Maingi, J.W.; Tang, S.; Liu, S.; Ngenya, W.; Bao, E. Targeting estrogen receptors in colorectal cancer. *Mol. Biol. Rep.* **2020**, *47*, 4087–4091. [[CrossRef](#)] [[PubMed](#)]
14. Sasso, C.V.; Santiano, F.E.; Arboccó, F.C.V.; Zyla, L.E.; Semino, S.N.; Guerrero-Gimenez, M.E.; Creydt, V.P.; Fontana, C.M.L.; Carón, R.W. Estradiol and progesterone regulate proliferation and apoptosis in colon cancer. *Endocr. Connect.* **2019**, *8*, 217–229. [[CrossRef](#)]
15. Mahbub, A.A. Therapeutic Strategies and Potential Actions of Female Sex Steroid Hormones and Their Receptors in Colon Cancer Based on Preclinical Studies. *Life* **2022**, *12*, 605. [[CrossRef](#)]
16. Indukuri, R.; Jafferli, M.H.; Song, D.; Damdimopoulos, A.; Hases, L.; Zhao, C.; Archer, A.; Williams, C. Genome-wide estrogen receptor β chromatin binding in human colon cancer cells reveals its tumor suppressor activity. *Int. J. Cancer* **2021**, *149*, 692–706. [[CrossRef](#)]
17. Saleiro, D.; Murillo, G.; Benya, R.V.; Bissonnette, M.; Hart, J.; Mehta, R.G. Estrogen receptor- β protects against colitis-associated neoplasia in mice. *Int. J. Cancer* **2012**, *131*, 2553–2561. [[CrossRef](#)]

18. Lozano-Herrera, S.J.; Luna-Bárceñas, G.; Guevara-González, R.G.; Campos-Vega, R.; Solís-Sáinz, J.C.; Hernández-Puga, A.G.; Vergara-Castañeda, H.A. Fermentation extract of naringenin increases the expression of estrogenic receptor β and modulates genes related to the p53 signalling pathway, miR-200c and miR-141 in human colon cancer cells exposed to BPA. *Molecules* **2022**, *27*, 6588. [\[CrossRef\]](#)
19. Luceri, C.; Femia, A.P.; Tortora, K.; D'ambrosio, M.; Fabbri, S.; Fazi, M.; Caderni, G. Supplementation with phytoestrogens and insoluble fibers reduces intestinal carcinogenesis and restores ER- β expression in Apc-driven colorectal carcinogenesis. *Eur. J. Cancer Prev.* **2020**, *29*, 27–35. [\[CrossRef\]](#)
20. Giroux, V.; Bernatchez, G.; Carrier, J.C. Chemopreventive effect of ER β -Selective agonist on intestinal tumorigenesis in Apc^{Min/+} mice. *Mol. Carcinog.* **2011**, *50*, 359–369. [\[CrossRef\]](#)
21. Hartman, J.; Edvardsson, K.; Lindberg, K.; Zhao, C.; Williams, C.; Ström, A.; Gustafsson, J.A. Tumor repressive functions of estrogen receptor beta in SW480 colon cancer cells. *Cancer Res.* **2009**, *69*, 6100–6106. [\[CrossRef\]](#)
22. Martineti, V.; Picariello, L.; Tognarini, I.; Carbonell Sala, S.; Gozzini, A.; Azzari, C.; Mavilia, C.; Tanini, A.; Falchetti, A.; Fiorelli, G.; et al. ERbeta is a potent inhibitor of cell proliferation in the HCT8 human colon cancer cell line through regulation of cell cycle components. *Endocr. Relat. Cancer.* **2005**, *12*, 455–469. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Pampaloni, B.; Palmini, G.; Mavilia, C.; Zonefrati, R.; Tanini, A.; Brandi, M.L. In vitro effects of polyphenols on colorectal cancer cells. *World J. Gastrointest. Oncol.* **2014**, *6*, 289–300. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Bernini, R.; Carastro, I.; Palmini, G.; Tanini, A.; Zonefrati, R.; Pinelli, P.; Brandi, M.L.; Romani, A. Lipophilization of Hydroxytyrosol-Enriched Fractions from *Olea europaea* L. Byproducts and Evaluation of the in Vitro Effects on a Model of Colorectal Cancer Cells. *J. Agric. Food Chem.* **2017**, *65*, 6506–6512. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Azzi, A.; Stocker, A. Vitamin E: Non-antioxidant roles. *Prog. Lipid Res.* **2000**, *39*, 231–255. [\[CrossRef\]](#)
26. Violi, F.; Nocella, C.; Loffredo, L.; Carnevale, R.; Pignatelli, P. Interventional study with vitamin E in cardiovascular disease and meta-analysis. *Free. Radic. Biol. Med.* **2021**, *178*, 26–41. [\[CrossRef\]](#)
27. Germano, B.C.d.C.; de Moraes, L.C.C.; Neta, F.I.; Fernandes, A.C.L.; Pinheiro, F.I.; Rego, A.C.M.D.; Filho, I.A.; de Azevedo, E.P.; Cavalcanti, J.R.L.d.P.; Guzen, F.P.; et al. Vitamin E and Its Molecular Effects in Experimental Models of Neurodegenerative Diseases. *Int. J. Mol. Sci.* **2023**, *24*, 11191. [\[CrossRef\]](#)
28. Mosca, A.; Crudele, A.; Smeriglio, A.; Braghini, M.R.; Panera, N.; Comparcola, D.; Alterio, A.; Sartorelli, M.R.; Tozzi, G.; Raponi, M.; et al. Antioxidant activity of Hydroxytyrosol and Vitamin E reduces systemic inflammation in children with paediatric NAFLD. *Dig. Liver Dis.* **2020**, *53*, 1154–1158. [\[CrossRef\]](#)
29. Ungurianu, A.; Zandirescu, A.; Nițulescu, G.; Margină, D. Vitamin E beyond Its Antioxidant Label. *Antioxidants* **2021**, *10*, 634. [\[CrossRef\]](#)
30. McIntyre, B.S.; Briski, K.P.; Gapor, A.; Sylvester, P.W. Antiproliferative and apoptotic effects of tocopherols and tocotrienols on preneoplastic and neoplastic mouse mammary epithelial cells. *Proc. Soc. Exp. Biol. Med.* **2000**, *224*, 292–301. [\[CrossRef\]](#)
31. Wang, H.; Yan, W.; Sun, Y.; Yang, C.S. δ -Tocotrienol is the most potent vitamin E form in inhibiting prostate cancer cell growth and inhibits prostate carcinogenesis in Pten^{P-/-} mice. *Cancer Prev. Res.* **2022**, *15*, 233–245. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Neuzil, J.; Weber, T.; Schröder, A.; Lu, M.; Ostermann, G.; Gellert, N.; Mayne, G.C.; Olejnicka, B.; Nègre-Salvayre, A.; Střicha, M.; et al. Induction of cancer cell apoptosis by α -tocopheryl succinate: Molecular pathways and structural requirements. *FASEB J.* **2001**, *15*, 403–415. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Weber, T.; Lu, M.; Andera, L.; Lahm, H.; Gellert, N.; Fariss, M.W.; Korinek, V.; Sattler, W.; Ucker, D.S.; Terman, A.; et al. Vitamin E succinate is a potent novel antineoplastic agent with high selectivity and cooperativity with tumor necrosis factor-related apoptosis-inducing ligand (Apo2 ligand) in vivo. *Clin. Cancer Res.* **2002**, *8*, 863–869. [\[PubMed\]](#)
34. Yang, C.S.; Luo, P.; Zeng, Z.; Wang, H.; Malafa, M.; Suh, N. Vitamin E and cancer prevention: Studies with different forms of tocopherols and tocotrienols. *Mol. Carcinog.* **2020**, *59*, 365–389. [\[CrossRef\]](#)
35. Anstead, G.M.; Carlson, K.E.; Katzenellenbogen, J.A. The estradiol pharmacophore: Ligand structure-estrogen receptor binding affinity relationships and a model for the receptor binding site. *Steroids* **1997**, *62*, 268–303. [\[CrossRef\]](#)
36. Khallouki, F.; de Medina, P.; Caze-Subra, S.; Bystricky, K.; Balaguer, P.; Poirot, M.; Silvente-Poirot, S. Molecular and Biochemical Analysis of the Estrogenic and Proliferative Properties of Vitamin E Compounds. *Front. Oncol.* **2016**, *5*, 287. [\[CrossRef\]](#)
37. Comitato, R.; Nesaretnam, K.; Leoni, G.; Ambra, R.; Canali, R.; Bolli, A.; Marino, M.; Virgili, F. A novel mechanism of natural vitamin E tocotrienol activity: Involvement of ERbeta signal transduction. *Am. J. Physiol. Endocrinol. Metab.* **2009**, *297*, E427–E437. [\[CrossRef\]](#)
38. Comitato, R.; Leoni, G.; Canali, R.; Ambra, R.; Nesaretnam, K.; Virgili, F. Tocotrienols activity in MCF-7 breast cancer cells: Involvement of ERbeta signal transduction. *Mol. Nutr. Food Res.* **2010**, *54*, 669–678. [\[CrossRef\]](#)
39. Gok, S.; Kuzmenko, O.; Babinskyi, A.; Severcan, F. Vitamin E derivative with modified side chain induced apoptosis by modulating the cellular lipids and membrane dynamics in MCF7 cells. *Cell Biochem. Biophys.* **2021**, *79*, 271–287. [\[CrossRef\]](#)
40. Bak, M.J.; Furmanski, P.; Shan, N.L.; Lee, H.J.; Bao, C.; Lin, Y.; Shih, W.J.; Yang, C.S.; Suh, N. Tocopherols inhibit estrogen-induced cancer stemness and OCT4 signaling in breast cancer. *Carcinogenesis* **2018**, *39*, 1045–1055. [\[CrossRef\]](#)

41. Chen, J.X.; Liu, A.; Lee, M.J.; Wang, H.; Yu, S.; Chi, E.; Reuhl, K.; Suh, N.; Yang, C.S. δ - and γ -tocopherols inhibit pHIP/DSS-induced colon carcinogenesis by protection against early cellular and DNA damages. *Mol. Carcinog.* **2017**, *56*, 172–183. [[CrossRef](#)] [[PubMed](#)]
42. Guan, F.; Li, G.; Liu, A.B.; Lee, M.J.; Yang, Z.; Chen, Y.K.; Lin, Y.; Shih, W.; Yang, C.S. δ - and γ -tocopherols, but not α -tocopherol, inhibit colon carcinogenesis in azoxymethane-treated F344 rats. *Cancer Prev. Res.* **2012**, *5*, 644–654. [[CrossRef](#)] [[PubMed](#)]
43. Zaveri, N.; Jiang, F.; Olsen, C.; Polgar, W.; Toll, L. Small-molecule agonists and antagonists of the opioid receptor-like receptor (ORL1, NOP): Ligand-based analysis of structural factors influencing intrinsic activity at NOP. *AAPS J.* **2005**, *7*, E345–E352. [[CrossRef](#)] [[PubMed](#)]
44. Viglianisi, C.; Vasa, K.; Tanini, D.; Capperucci, A.; Amorati, R.; Valgimigli, L.; Baschieri, A.; Menichetti, S. Ditocopheryl Sulfides and Disulfides: Synthesis and Antioxidant Profile. *Chemistry* **2019**, *25*, 9108–9116. [[CrossRef](#)]
45. Wagner, L.; Howe, K.; Philbrick, K.A.; Maddalozzo, G.F.; Kuah, A.F.; Wong, C.P.; Olson, D.A.; Branscum, A.J.; Iwaniec, U.T.; Turner, R.T. Effects of Alcohol and Estrogen Receptor Blockade Using ICI 182,780 on Bone in Ovariectomized Rats. *Alcohol. Clin. Exp. Res.* **2019**, *43*, 2301–2311. [[CrossRef](#)]
46. Kimmins, S.; Russell, G.L.; Lim, H.C.; Hall, B.K.; MacLaren, L.A. The effects of estrogen, its antagonist ICI 182, 780, and interferon-tau on the expression of estrogen receptors and integrin α V β 3 on cycle day 16 in bovine endometrium. *Reprod. Biol. Endocrinol.* **2003**, *1*, 38. [[CrossRef](#)]
47. Kavčič, N.; Pegan, K.; Turk, B. Lysosomes in programmed cell death pathways: From initiators to amplifiers. *Biol. Chem.* **2016**, *398*, 289–301. [[CrossRef](#)]
48. Strober, W. Trypan blue exclusion test of cell viability. *Curr. Protoc. Immunol.* **2001**, *21*, A.3B.1–A.3B.2.
49. Gil-Parrado, S.; Fernández-Montalván, A.; Assfalg-Machleidt, I.; Popp, O.; Bestvater, F.; Holloschi, A.; Knoch, T.A.; Auerswald, E.A.; Welsh, K.; Reed, J.C.; et al. Ionomycin-activated calpain triggers apoptosis. A probable role for Bcl-2 family members. *J. Biol. Chem.* **2002**, *277*, 27217–272126. [[CrossRef](#)]
50. Mizushima, S.; Sasanami, T.; Ono, T.; Matsuzaki, M.; Kansaku, N.; Kuroiwa, A. Cyclin D1 gene expression is essential for cell cycle progression from the maternal-to-zygotic transition during blastoderm development in Japanese quail. *Dev. Biol.* **2021**, *476*, 249–258.
51. Liu, Z.; Sun, Q.; Wang, X. PLK1, A Potential Target for Cancer Therapy. *Transl. Oncol.* **2017**, *10*, 22–32. [[CrossRef](#)]
52. White, E.Z.; Pennant, N.M.; Carter, J.R.; Hawsawi, O.; Odero-Marrah, V.; Hinton, C.V. Serum deprivation initiates adaptation and survival to oxidative stress in prostate cancer cells. *Sci. Rep.* **2020**, *10*, 12505. [[CrossRef](#)] [[PubMed](#)]
53. Lee, S.B.; Kim, J.J.; Kim, T.W.; Kim, B.S.; Lee, M.S.; Yoo, Y.D. Serum deprivation-induced reactive oxygen species production is mediated by Romo1. *Apoptosis* **2010**, *15*, 204–218. [[CrossRef](#)]
54. Rivera-Guevara, C.; Pérez-Alvarez, V.; García-Becerra, R.; Ordaz-Rosado, D.; Morales-Ríos, M.S.; Hernández-Gallegos, E.; Cooney, A.J.; Bravo-Gómez, M.E.; Larrea, F.; Camacho, J. Genomic action of permanently charged tamoxifen derivatives via estrogen receptor- α . *Bioorganic Med. Chem.* **2010**, *18*, 5593–5601. [[CrossRef](#)]
55. Meyers, M.J.; Sun, J.; Carlson, K.E.; Marriner, G.A.; Katzenellenbogen, B.S.; Katzenellenbogen, J.A. Estrogen receptor-beta po-tency-selective ligands: Structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J. Med. Chem.* **2001**, *44*, 4230–4251. [[CrossRef](#)] [[PubMed](#)]
56. Handa, C.; Yamazaki, Y.; Yonekubo, S.; Furuya, N.; Momose, T.; Ozawa, T.; Furuishi, T.; Fukuzawa, K.; Yonemochi, E. Evaluating the correlation of binding affinities between isothermal titration calorimetry and fragment molecular orbital method of es-trogen receptor beta with diarylpropionitrile (DPN) or DPN derivatives. *J. Steroid Biochem. Mol. Biol.* **2022**, *222*, 106152. [[CrossRef](#)] [[PubMed](#)]
57. Kuiper, G.G.; Carlsson, B.; Grandien, K.; Enmark, E.; Häggblad, J.; Nilsson, S.; Gustafsson, J.A. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **1997**, *138*, 863–870. [[CrossRef](#)]
58. Ruff, M.; Gangloff, M.; Wurtz, J.M.; Moras, D. Estrogen receptor transcription and transactivation: Structure-function rela-tionship in DNA- and ligand-binding domains of estrogen receptors. *Breast Cancer Res.* **2000**, *2*, 353–359. [[CrossRef](#)]
59. Wallace, A.C.; Laskowski, R.A.; Thornton, J.M. LIGPLOT: A program to generate schematic diagrams of protein-ligand inter-actions. *Protein Eng.* **1995**, *8*, 127–134. [[CrossRef](#)]
60. Song, C.-H.; Kim, N.; Lee, S.M.; Nam, R.H.; Choi, S.I.; Kang, S.R.; Shin, E.; Lee, D.H.; Lee, H.-N.; Surh, Y.-J. Effects of 17 β -estradiol on colorectal cancer development after azoxymethane/dextran sulfate sodium treatment of ovariectomized mice. *Biochem. Pharmacol.* **2019**, *164*, 139–151. [[CrossRef](#)]
61. Treeck, O.; Diepolder, E.; Skrzypczak, M.; Schüler-Toprak, S.; Ortmann, O. Knockdown of estrogen receptor β increases pro-liferation and affects the transcriptome of endometrial adenocarcinoma cells. *BMC Cancer* **2019**, *19*, 745.
62. Zhao, X.; Li, X.; Ren, Q.; Tian, J.; Chen, J. Calycosin induces apoptosis in colorectal cancer cells, through modulating the ER β /MiR-95 and IGF-1R, PI3K/Akt signaling pathways. *Gene* **2016**, *591*, 123–128. [[CrossRef](#)] [[PubMed](#)]

63. Hsu, H.-H.; Kuo, W.-W.; Ju, D.-T.; Yeh, Y.-L.; Tu, C.-C.; Tsai, Y.-L.; Shen, C.-Y.; Chang, S.-H.; Chung, L.-C.; Huang, C.-Y. Estradiol agonists inhibit human LoVo colorectal-cancer cell proliferation and migration through p53. *World J. Gastroenterol.* **2014**, *20*, 16665–16673. [\[CrossRef\]](#)
64. Sotoca, A.M.; van den Berg, H.; Vervoort, J.; van der Saag, P.; Ström, A.; Gustafsson, J.A.; Rietjens, I.; Murk, A.J. Influence of cellular ERalpha/ERbeta ratio on the ERalpha-agonist induced proliferation of human T47D breast cancer cells. *Toxicol. Sci.* **2008**, *105*, 303–311. [\[PubMed\]](#)
65. Ditonno, I.; Losurdo, G.; Rendina, M.; Pricci, M.; Girardi, B.; Ierardi, E.; Di Leo, A. Estrogen Receptors in Colorectal Cancer: Facts, Novelties and Perspectives. *Curr. Oncol.* **2021**, *28*, 4256–4263. [\[CrossRef\]](#) [\[PubMed\]](#)
66. Markaverich, B.M.; Shoulars, K.; Rodriguez, M.A. Luteolin Regulation of Estrogen Signaling and Cell Cycle Pathway Genes in MCF-7 Human Breast Cancer Cells. *Int. J. Biomed. Sci.* **2011**, *7*, 101–111. [\[PubMed\]](#) [\[PubMed Central\]](#)
67. Patel, S.; Kilburn, B.; Imudia, A.; Armant, D.R.; Skafar, D.F. Estradiol Elicits Proapoptotic and Antiproliferative Effects in Human Trophoblast Cells1. *Biol. Reprod.* **2015**, *93*, 74. [\[CrossRef\]](#)
68. Seilicovich, A. Cell Life and Death in the Anterior Pituitary Gland: Role of Oestrogens. *J. Neuroendocr.* **2010**, *22*, 758–764. [\[CrossRef\]](#)
69. Lee, Y.J.; Renaud, R.A.; Friedrich, T.C.; Gorski, J. Estrogen causes cell death of estrogen receptor stably transfected cells via apoptosis. *J. Steroid Biochem. Mol. Biol.* **1998**, *67*, 327–332.
70. Das, P.K.; Saha, J.; Pillai, S.; Lam, A.K.; Gopalan, V.; Islam, F. Implications of estrogen and its receptors in colorectal carcinoma. *Cancer Med.* **2022**, *12*, 4367–4379. [\[CrossRef\]](#)
71. Chen, J.; Hou, R.; Zhang, X.; Ye, Y.; Wang, Y.; Tian, J. Calycosin suppresses breast cancer cell growth via ERβ-dependent regulation of IGF-1R, p38 MAPK and PI3K/Akt pathways. *PLoS ONE* **2014**, *9*, e91245.
72. Huang, M.; Li, X.; Jia, S.; Liu, S.; Fu, L.; Jiang, X.; Yang, M. Bisphenol AF induces apoptosis via estrogen receptor beta (ERβ) and ROS-ASK1-JNK MAPK pathway in human granulosa cell line KGN. *Environ. Pollut.* **2020**, *270*, 116051. [\[CrossRef\]](#)
73. Li, H.; Zhang, Q.; Zhang, Y.; Zhang, S.; Zhang, R.; Chen, D.; Shi, J.; Xu, J.; Li, L. Nonivamide inhibits proliferation of human corneal epithelial cells by inducing cell cycle arrest and oxidative stress. *Toxicology* **2023**, *500*, 153674. [\[CrossRef\]](#) [\[PubMed\]](#)
74. Martindale, J.L.; Holbrook, N.J. Cellular response to oxidative stress: Signaling for suicide and survival. *J. Cell. Physiol.* **2002**, *192*, 1–15. [\[CrossRef\]](#)
75. Domazetovic, V.; Falsetti, I.; Viglianisi, C.; Vasa, K.; Aurilia, C.; Stio, M.; Menichetti, S.; Iantomasi, T. Protective Role of Natural and Semi-Synthetic Tocopherols on TNFα-Induced ROS Production and ICAM-1 and Cl-2 Expression in HT29 Intestinal Epithelial Cells. *Antioxidants* **2021**, *10*, 160. [\[CrossRef\]](#) [\[PubMed\]](#)
76. Domazetovic, V.; Fontani, F.; Marcucci, G.; Iantomasi, T.; Brandi, M.L.; Vincenzini, M.T. Estrogen inhibits starvation-induced apoptosis in osteocytes by a redox-independent process involving association of JNK and glutathione S-transferase P1-1. *FEBS Open Bio.* **2017**, *7*, 705–718. [\[CrossRef\]](#)
77. Mann, V.; Huber, C.; Kogianni, G.; Collins, F.; Noble, B. The antioxidant effect of estrogen and Selective Estrogen Receptor Modulators in the inhibition of osteocyte apoptosis in vitro. *Bone* **2006**, *40*, 674–684. [\[CrossRef\]](#)
78. Panza, S.; Santoro, M.; De Amicis, F.; Morelli, C.; Passarelli, V.; D'aquila, P.; Giordano, F.; Cione, E.; Passarino, G.; Bellizzi, D.; et al. Estradiol via estrogen receptor beta influences ROS levels through the transcriptional regulation of SIRT3 in human seminoma TCam-2 cells. *Tumor Biol.* **2017**, *39*, 1010428317701642. [\[CrossRef\]](#)
79. Viglianisi, C.; Bonardi, C.; Ermini, E.; Capperucci, A.; Menichetti, S.; Tanini, D. Selenosilane-promoted selective mild transformation of N-thiophthalimides into symmetric disulfides. *Synthesis* **2019**, *51*, 1819–1824.
80. Catarzi, S.; Romagnoli, C.; Marcucci, G.; Favilli, F.; Iantomasi, T.; Vincenzini, M.T. Redox regulation of ERK1/2 activation induced by sphingosine 1-phosphate in fibroblasts: Involvement of NADPH oxidase and platelet-derived growth factor receptor. *Biochim. Biophys. Acta* **2011**, *1810*, 446–456.
81. Smith, P.K.; Krohn, R.I.; Hermanson, G.T.; Mallia, A.K.; Gartner, F.H.; Provenzano, M.D.; Fujimoto, E.K.; Goeke, N.M.; Olson, B.J.; Klenk, D.C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76–85. [\[PubMed\]](#)
82. Pike, A.C.; Brzozowski, A.; Walton, J.; Hubbard, R.E.; Thorsell, A.-G.; Li, Y.-L.; Gustafsson, J.; Carlquist, M. Structural Insights into the Mode of Action of a Pure Antiestrogen. *Structure* **2001**, *9*, 145–153. [\[CrossRef\]](#)
83. Souza, P.C.T.; Textor, L.C.; Melo, D.C.; Nascimento, A.S.; Skaf, M.S.; Polikarpov, I. An alternative conformation of ERβ bound to estradiol reveals H12 in a stable antagonist position. *Sci. Rep.* **2017**, *7*, 3509. [\[CrossRef\]](#)
84. Shiau, A.K.; Barstad, D.; Loria, P.M.; Cheng, L.; Kushner, P.J.; Agard, D.A.; Greene, G.L. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **1998**, *95*, 927–937.
85. Tanenbaum, D.M.; Wang, Y.; Williams, S.P.; Sigler, P.B. Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 5998–6003. [\[PubMed\]](#)

-
86. O'Boyle, N.M.; Banck, M.; James, C.A.; Morley, C.; Vandermeersch, T.; Hutchison, G.R. Open babel: An open chemical toolbox. *J. Cheminform.* **2011**, *3*, 33. [[CrossRef](#)]
 87. Trott, O.; Olson, A.J. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2010**, *31*, 455–461. [[CrossRef](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.