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REVIEW ARTICLE

Tocotrienols and Cancer: From the State of the Art to Promising Novel Patents

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Abstract: ***Background***: Tocotrienols (TTs) are vitamin E derivatives naturally occurring in several plants and vegetable oils. Like Tocopherols (TPs), they comprise four isoforms, α, β, γ and δ, but unlike TPs, they present an unsaturated isoprenoid chain. Recent studies indicate that TTs provide important health benefits, including neuroprotective, anti-inflammatory, anti-oxidant, cholesterol lowering and immunomodulatory effects. Moreover, they have been found to possess unique anti-cancer properties.

Objective: The purpose of this review is to present an overview of the state of the art of TTs role in cancer prevention and treatment, as well as to describe recent patents proposing new methods for TTs isolation, chemical modification and use in cancer prevention and/or therapy.

Methods: Recent literature and patents focusing on TTs anti-cancer applications have been identified and reviewed, with special regard to their scientific impact and novelty.

Results: TTs have demonstrated significant anti-cancer activity in multiple tumor types, both *in vitro* and *in vivo*. Furthermore, they have shown synergistic effects when given in combination with standard anti-cancer agents or other anti-tumor natural compounds. Finally, new purification processes and transgenic sources have been designed in order to improve TTs production, and novel TTs formulations and synthetic derivatives have been developed to enhance their solubility and bioavailability.

Conclusion: The promising anti-cancer effects shown by TTs in several preclinical studies may open new opportunities for therapeutic interventions in different tumors. Thus, clinical trials aimed at confirming TTs chemopreventive and tumor-suppressing activity, particularly in combination with standard therapies, are urgently needed.

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1. INTRODUCTION

The term "nutraceutical" was coined in 1989 by Dr. Stephen De Felice, MD founder and chairman of the Foundation for Innovation in Medicine, who defined it as a "food, or parts of food, that provide medical or health benefits, including the prevention and treatment of disease". Thus, nutraceuticals may refer to naturally nutrient-rich or biologically active foods and herbs, such as blueberries or soybeans, or they may be specific food components or isolated nutrients and phytochemicals with medicinal properties, such as ω-3 fatty acids, polyphenols, flavonoids, carotenoids and vitamins [1].

Vitamin E family consists of eight members, α-, β-, γ- and δ-Tocopherols (TPs) and the corresponding four Tocotrienols (TTs). TTs, in particular, were demonstrated to possess specific health-promoting and/or disease-preventing properties in different pathologies, associated with their powerful neuroprotective, anti-inflammatory, anti-oxidant, cholesterol lowering and immunomodulatory potentials [2]. It has also been observed that TTs, particularly γ and δ isoforms, can exert a potent anti-cancer activity in different tumors [3]. The molecular mechanisms of these anti-proliferative properties remain to be fully elucidated and they appear to involve several intracellular pathways [4, 5] (Fig. **1**).

This review aims to describe the anti-tumor effects of TTs based on the experimental evidence so far available, as well as to offer a selection of published patents in the last years, which provide new methods for the production and use of TTs in cancer prevention and/or treatment.

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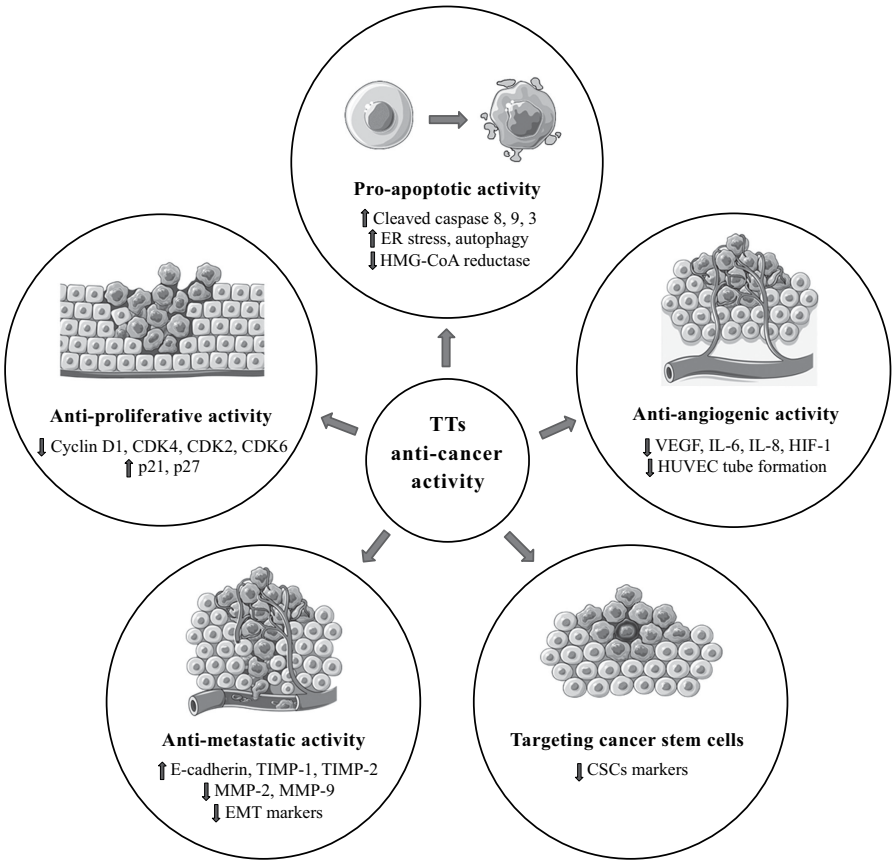

Fig. (1). Molecular mechanisms of Tocotrienols (TTs) anti-cancer activity.

2. TOCOTRIENOLS

Vitamin E was discovered by Herbert Evans and Katharine Bishop in 1922 [6] and isolated by Evans and Gladys Emerson in 1935 [7]. Its hydrophobic components are divided into two groups: α-, β-, γ- and δ-TPs and α-, β-, γ- and δ-TTs. The critical chemical structural difference between them is that the chromanol ring, common to both groups, is linked to a saturated isoprenoid side chain in TPs and to an unsaturated isoprenoid side chain in TTs. Moreover, each isoform of both TPs and TTs differs from others in the number and position of methyl groups on the chromanol ring: the α and β isomers are trimethylated, the γ isomers are dimethylated and the δ isomers are monomethylated.

The metabolism of TTs is still not clear. They are absorbed in the small intestine in the presence of bile salts and transported to body tissues through the blood after α -TP transport protein-mediated sequestration into liver lipoproteins. However, TTs seem to have lower affinity than TPs for α-TP transport protein, and various studies have pointed out that TPs can interfere with TTs benefits by lowering their intestinal absorption and increasing their catabolism in the liver [8, 9]. Despite these observations, preclinical trials have reported high activity of these compounds after oral administration, without significant side effects and toxicity [10, 11]. High bioavailability and safety of TTs were also demonstrated in healthy human subjects and patients with breast and pancreatic cancer [12-14].

In nature, TTs are present in many plants, cereals, seeds, nuts and grains, as well as in the oils derived from them. They are particularly abundant in palm oil, which represents the main source of TTs and in particular of γ-TT [15], annatto (*Bixa orellana* L.) seeds, which contain about 150mg δ-TT/100g dry seeds with no TPs [16], and rice bran, containing high levels of α- and γ-TT [17, 18]. Other sources of TTs are wheat germ, grapefruit, hazelnuts, olive oil, sunflower oil and flaxseed oil [19, 20].

2.1. Tocotrienols in Cancer

In recent years, TTs have emerged as one of the most effective class of natural compounds for preventing and ameliorating cardiovascular and neurodegenerative diseases, as well as hyperlipidemia, inflammation, diabetes and osteoporosis [21-24]. In addition, they have demonstrated promising potential as anti-cancer agents, particularly in breast, cervix, colon, gastric, lung, skin, pancreatic and prostate cancer.

2.1.1. Tocotrienols in Breast Cancer

γ-TT was found to exert both anti-proliferative and pro-apoptotic activity in breast cancer cells. In particular, it was shown to reduce the levels of cyclin D1 and Cyclin-Dependent Kinases (CDK) 2, 4 and 6 and to increase the expression of CDK inhibitors [25, 26]; to suppress both the PI3K/Akt/mTOR and the Ras/Raf/MEK/ERK signaling pathways and to decrease c-Myc levels [27]; to induce intrinsic apoptosis accompanied by cytochrome *c* release, mitochondrial membrane depolarization, caspase activation, DNA fragmentation and poly(ADP-ribose) polymerase cleavage [28, 29]; to trigger the extrinsic apoptotic pathway by activation of caspase-8 [30].

δ-TT was also demonstrated to exert potent anti-tumor effects in mammary cancer cells, by reducing proliferation through downregulation of the HMG-CoA reductase activity and inhibition of cholesterol synthesis [31] and by inducing oxidative stress-related mitochondrial apoptosis [32].

The Human Epidermal Growth Factor Receptor 2 (HER-2) is a receptor tyrosine-protein kinase that in humans is encoded by the erbB-2 gene and normally promotes breast cell proliferation. Amplification of this oncogene occurs in 30% of breast tumors, thus representing an important biomarker and target for therapy [33]. Alawin *et al.* demonstrated that HER-2 receptors and TTs specifically accumulate in breast cancer cell lipid raft microdomains. Moreover, they found that TTs profoundly alter the composition of the lipid rafts, with subsequent disruption of their integrity and inactivation (due to reduced dimerization and phosphorylation) of the associated HER-2 receptors and of the downstream signaling pathways [34].

Almost 70% of human breast cancers are estrogen-dependent and estrogen receptor-positive. TTs were shown to promote the nuclear translocation of the anti-proliferative Estrogen Receptor (ER) β and to decrease the tumorigenic ERα expression [35]. On the other hand, Khallouki *et al.* suggested that δ-TT can induce cytotoxic effects in breast cancer cells independently of their ER status [31].

A proper protein folding and processing are essential for the maintenance of cellular homeostasis, and two important mechanisms involved in its control are the Unfolded Protein Response (UPR), activated in case of Endoplasmic Reticulum (ER) stress, and autophagy. The ER stress is a cellular process that can be triggered by various physiological conditions and pathological insults, as well as by different synthetic and natural drugs: Cells initially respond with a defensive UPR, accompanied by an increase in the ER protein folding capacity and in the ER-Associated Protein Degradation (ERAD) machinery; however, in case of severe or prolonged stress, unfolded and misfolded proteins may exceed the ER capacity and accumulate in its lumen, leading to the activation of a set of pro-death programs, such as the double-stranded RNA-dependent Protein Kinase PKR-Like ER Kinase (PERK)/Eukaryotic Initiation Factor 2α (eIF2α)/Activating Transcription Factor 4 (ATF4)/C/EBP homologous protein (CHOP) pathway and the Inositol-Requiring Enzyme 1α (IRE1α)/c-Jun N-Terminal Kinase (JNK)/p38 MAPK cascade [36]. Autophagy is an evolutionarily conserved catabolic process that is used to deliver cytoplasmic material, such as damaged organelles and protein aggregates, to the lysosome for degradation: it is characterized by the formation of double-membrane vesicles, the autophagosomes, that fuse with lysosomes for cytoplasmic cargo recycling, and it is mediated by two proteins, microtubule-associated proteins 1A/1B light chain 3B, commonly known as LC3, and p62 (also called SQSTM1) [37]. Pharmacological targeting of these two molecular pathways has been recently proposed as an effective treatment strategy for tumors [38, 39]. Different natural compounds were reported to induce ER stress- and autophagy-mediated death in cancer cells [40, 41]: among them, γ-TT specifically triggered both of these pro-apoptotic pathways in breast cancer cells [42-46].

Angiogenesis, the process that leads to new blood vessel formation from pre-existing vasculature, and metastatization, the spread of cancer cells from the tissue of origin to new areas of the body, are two main steps in tumor growth and progression. In breast cancer, TTs exhibited both anti-metastatic and anti-angiogenic properties, associated with the inhibition of Met/hepatocyte growth factor receptor and Rac1/WAVE2 cascade and the downregulation of VEGF expression, respectively [47-50].

It is known that Cancer Stem Cells (CSCs) represent only a small subpopulation of cancer cells within a tumor mass. However, their self-renewal ability and their capacity to differentiate into the entire heterogeneous tumor cell bulk make them important therapeutic targets. Moreover, they appear to be implicated in the resistance to standard cancer treatments, also promoting tumor recurrence and metastasis [51]. Many natural compounds were found to target CSCs [52], and TTs were shown to specifically eliminate the breast CSCs subpopulation, alone or in combination with simvastatin [53, 54].

In +SA mammary tumor cells TTs treatment synergistically increased the anti-cancer activity of synthetic drugs, such as tyrosine kinase inhibitors (erlotinib and gefitinib), statins (simvastatin, mevastatin and lovastatin) and the COX-2 selective nonsteroidal anti-inflammatory agent celecoxib, through suppression of HERB2-4 receptors expression levels and inhibition of Akt and MAPK pathways [55-57]. Furthermore, in multidrug-resistant MCF-7/ADR cells γ-TT significantly reduced the expression of P-gp, leading to enhanced accumulation of doxorubicin in cells and subsequent G2/M cell cycle arrest and apoptosis [58]. Similar synergistic effects were shown by TTs when given in combination with several natural compounds: in murine malignant mammary epithelial cells, the co-treatment with sesamin, a lignan contained in sesame seeds and oil, not only improved TTs bioavailability by reducing their metabolic degradation but also exhibited a synergistic inhibitory effect on the EGF-dependent proliferative pathway [59-61]; the addition of a polyphenol, such as Epigallocatechin Gallate (EGCG), found in green tea, or resveratrol, present in berries and grapes, potentiated the γ-TT-induced downregulation of cyclin D1 and Bcl-2 expression in MCF-7 human breast cancer cells, and the triple combination of these compounds synergistically up-regulated the expression of NAD(P)H Quinone Dehydrogenase 1 (NQO1), an enzyme activated in case of redox imbalance [62]; a combination of TTs and oridonin caused a significant additive effect in decreasing +SA cell viability through suppression of Akt/mTOR signaling and elevation of apoptosis (caspase-3 and Bax/Bcl-2 ratio) and autophagy (Atg and Beclin-1) markers [63].

It should be noted that also semisynthetic redox-silent TT oxazine derivatives were found to successfully inhibit breast tumor growth, both *in vitro* and *in vivo* [64-66]. In particular, they successfully counteracted the $CoCl_2$-induced increase of HIF-1α levels, with a parallel inactivation of the Akt/mTOR pathway and of its downstream targets p70S6K and eIF-4E1. In addition, TT oxazine derivative treatment resulted in a blockade of the $CoCl_2$-mediated VEGF overexpression.

A five-year double-blinded and placebo-controlled clinical trial was conducted in 250 women with early breast cancer to investigate the TTs adjuvant potentials when given in combination with tamoxifen [67]. The patients, 40-60 years old, with either stage I or II estrogen receptor-positive breast cancer, were non-randomly assigned to two groups: The treatment group was administered 400mg/day TRF plus tamoxifen while the control group was given placebo plus tamoxifen. The 5-year breast cancer-specific survival was 98.3% in the treatment group and 95% in the control group, while the 5-year disease-free survival was 86.7% and 83.3%, respectively. The mortality risk was 60% lower in the TRF group versus controls but it was not statistically significant, probably as a result of the small sample size of the experiment.

2.1.2. Tocotrienols in Cervical Cancer

TTs were reported to inhibit HeLa cervical cancer cell proliferation through downregulation of the expression of cell cycle-related proteins, such as cyclin D3, p16 and CDK6. Moreover, the induction of HeLa cell death by TTs appeared to be associated with the upregulation of Interleukin-6 (IL-6) [68].

Comitato *et al.* demonstrated that γ- and δ-TT induce apoptosis in cervical cancer cells, by triggering molecular signals associated with ER stress, such as IRE-1α phosphorylation, XBP-1 alternative splicing and CHOP enhanced transcription [69]. Furthermore, they observed significant Ca^{2+} release from the ER membranes to the cytoplasm, as well as an interesting modulation of isoprenoid, sterol and steroid biosynthesis and metabolism, with SCD, LPIN and SREBPF1-2 downregulation.

γ-TT was found to specifically target Src Homology 2 Domain-Containing Phosphatase 2 (SHP2) and the RAS/ERK signaling pathway in spheres from cervical cancer, inhibiting the CSCs subpopulation growth [70].

2.1.3. Tocotrienols in Colon Cancer

In RKO human colon cancer cells, a TRF preparation triggered intrinsic apoptosis correlated with p53 and caspases activation, Bax/Bcl-2 ratio modulation, chromatin condensation, DNA fragmentation and cell membrane shrinkage [71]. Moreover, γ-TT was reported to profoundly alter sphingolipid metabolism in HCT-116 cells through suppression of dihydroceramide desaturase activity and activation of sphingomyelin hydrolysis, ultimately leading to autophagy and apoptosis [72].

Paraptosis is a type of programmed cell death characterized by cytoplasmic vacuolization, particularly by ER dilatation and mitochondrial swelling. Various studies have shown that several natural compounds, such as celastrol, paclitaxel, ophiobolin A and curcumin, can induce this non-apoptotic cell death in different types of tumors [73]. It has been recently demonstrated that also TTs can trigger paraptosis in SW620 and HCT-8 human colon carcinoma cells through inhibition of the Wnt signaling pathway and downregulation of c-jun, cyclin D1 and β-catenin levels [74, 75].

A synergistic antitumor activity of γ-TT and different synthetic and natural anti-cancer agents was observed: the co-treatment with capecitabine synergistically decreased Ki-67, cyclin D1, NF-κB, CXCR4 and MMP-9 expression levels in a nude mouse xenograft model of human colorectal cancer [76]; addition of atorvastatin to tocotrienol treatment enhanced the disruption of RhoA signal transduction in HT29 and HCT116 human colon cancer cells, and a triple combination with celecoxib resulted in a synergistic induction of G(0)/G(1) phase cell cycle arrest and apoptosis [77]; 6-gingerol, the bioactive constituent of ginger, potentiated the γ-TT pro-apoptotic activity in HT29 and SW837 human colorectal cancer cell lines, inducing caspase-3 activation and significant morphological changes, such as cell shrinkage and pyknosis [78].

δ-TT was shown to suppress hypoxia-induced VEGF, IL-8 and COX-2 synthesis in DLD-1 human colorectal adenocarcinoma cells [79], as well as to inhibit tube formation, migration and adhesion of HUVEC cells grown in DLD-1 conditioned medium [80]. These results were also confirmed by *in vivo* experiments [80, 81].

Rice bran is not only enriched in δ-TT but also in ferulic acid. Eitsuka *et al.* demonstrated that co-treatment with both these natural compounds significantly increased the intracellular concentration and anti-tumor activity of δ-TT in DLD-1 cells, suggesting that ferulic acid improves the bioavailability as well as the therapeutic effectiveness of δ-TT [82]. In particular, this combination treatment was shown to synergistically down-regulate the expression of Human Telomerase Reverse Transcriptase (hTERT), the catalytic subunit of telomerase, thus suppressing its proliferative activity.

2.1.4. Tocotrienols in Gastric Cancer

γ-TT was first demonstrated to induce intrinsic apoptosis in human gastric cancer cells through the suppression of the MAPK signaling [83, 84].

γ-TT was also found to exert potent anti-metastatic and anti-angiogenic activity in gastric cancer. In particular, it inhibited cell migration and invasion capability, by reducing the expression of the matrix metalloproteinases MMP-2 and MMP-9 and by increasing the levels of Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) and TIMP-2 [85], and significantly counteracted the hypoxia-mediated HIF-1α overexpression and VEGF synthesis by modulation of the ERK signaling pathway [86]. Furthermore, it significantly decreased the expression of VEGFR-2 in HUVEC cells grown in a conditioned medium of gastric adenocarcinoma cells [87].

In addition to its pro-apoptotic, anti-metastatic and anti-angiogenic effects, γ-TT was reported to enhance the antitumor activity of capecitabine in human gastric cancer cell lines, as well as in nude mice xenografted with human gastric cancer cells [88].

2.1.5. Tocotrienols in Lung Cancer

MicroRNAs (miRNAs) are endogenous, ~22 nucleotides, non-coding RNAs that play key regulatory roles in animals and plants by inducing transcriptional silencing through mRNAs cleavage or translational arrest. miRNAs may function as either oncogenes or tumor suppressors (oncomirs), depending on the specific cancer type [89]. Ji *et al*. observed that δ-TT could inhibit nonsmall cancer cell growth and in-

vasiveness through upregulation of miR-34a, which resulted in decreased expression of Notch-1 and its downstream targets, such as Hes-1, cyclin D1, survivin and Bcl-2 [90]. Moreover, they found that δ-TT exhibited a significant synergistic anti-cancer effect when given in combination with cisplatin. This was related to a reduction of the NF-κB DNA binding activity and to an increase in cleaved caspase-3 and PARP expressions [91].

TTs were also shown to potentiate lovastatin-mediated cell growth arrest in the A549 human lung carcinoma cell line [92].

2.1.6. Tocotrienols in Pancreatic Cancer

Anti-proliferative and pro-apoptotic effects of δ-TT, mediated by $p27^{Kip1}$-dependent cell cycle arrest [93], inhibition of HMG-CoA reductase activity [94] or HER2 [95] and EGR-1/Bax pathway activation [96], were shown in pancreatic cancer cells. Similar results were obtained after γ-TT treatment [97].

TTs significantly suppressed the invasive behavior through downregulation of specific epithelial-mesenchymal transition (EMT) biomarkers (such as N-cadherin, vimentin and MMP9) in L3.6pl and Mia PaCa-2 cells, both *in vitro* and *in vivo* [98].

δ-TT successfully targeted and eliminated Pancreatic Ductal Adenocarcinoma (PDAC) stem-like cells, decreasing the expression of CSCs self-renewal-promoting transcription factors, Oct4 and Sox2, and delaying tumor onset in mice [98].

Promising results were reported in pancreatic cancer cells treated with polyethylene glycol (350 and 1000) succinate derivatives of TTs [99]. In addition, *in vitro* studies pointed out that entrapment of gemcitabine/γ-tocotrienol or paclitaxel/γ-tocotrienol lipid conjugates into nanoemulsions significantly enhanced their anti-tumor effects when compared to the free drug [100, 101].

Springett *et al.* investigated δ-TT efficacy and safety in patients with PDAC [14]. In this Phase I preoperative clinical trial, 25 patients were given crescent oral doses of δ-TT (from 200 to 3200mg) daily for 13 days before surgery and one dose on the day of surgery. Except for one case of drug-related grade 1 diarrhea registered at the higher daily dose level, the treatment was well tolerated, with no dose-limiting toxicity. δ-TT exhibited an effective half-life of about 4 hours, rapidly reaching bioactive levels in blood and inducing apoptosis-associated caspase-3 cleavage in cancer cells. In particular, the most effective δ-TT dose was between 400 and 1600mg.

2.1.7. Tocotrienols in Prostate Cancer

TTs were reported to exert anti-proliferative and pro-apoptotic effects in different prostate cancer cell lines by targeting several signaling pathways, such as Phosphoinositide-3 Kinase (PI3K)/Akt/mTOR, Signal Transducer and Activator of Transcription (STAT), transforming growth factor β (TFGβ) receptor and NF-κB cascades, as well as cyclins and the cell cycle inhibitors p27 and p21 [102-106].

It has been shown that γ-TT significantly decreases the expression of CD133 and CD44 CSCs markers in PC-3 and DU145 castration-resistant prostate cancer cells, also suppressing their anchorage-independent growth and spheroidogenic ability. Moreover, γ-TT pre-treatment of prostate cancer cells resulted in the inhibition of tumor initiation after their inoculation in nude mice. Finally, despite the high resistance of CD133-positive PC-3 cells to docetaxel treatment, they were as sensitive to γ-TT as the CD133-depleted population [107]. Similar studies were performed by Lee *et al.*, who confirmed the γ-TT ability to specifically target the CSCs subpopulation in different prostate cancer cell lines and mouse models, leading to a significant suppression of the proliferation of the castration-resistant tumors [108].

Recent evidence suggests that also δ-TT can inhibit the proliferation of prostate CSCs under hypoxic conditions, by specifically inactivating the HIF-1α cascade [109].

In prostate cancer, TTs were reported to potentiate the anti-tumor activity of lovastatin *in vitro* [110]. A combination treatment with δ-TT and geranylgeraniol was also shown to synergistically suppress the viability of DU145 prostate cancer cells, inducing a significant downregulation of the expression of HMG-CoA reductase, as well as an interesting reduction in membrane K-RAS protein levels [111].

2.1.8. Tocotrienols in Skin Cancer

We recently demonstrated that δ-TT induces ER-stress-mediated apoptosis in human melanoma cells *in vitro* and in tumors *in vivo*, through the activation of the PERK/p-eIF2α/ATF4/CHOP, IRE1α and caspase-4 ER stress-related branches [112]. Moreover, we observed that, unlike vemurafenib, it can selectively target and eliminate the melanoma ABCG2-positive CSCs subpopulation, successfully inducing disaggregation of A375 melanospheres and reducing the spheroid formation ability of sphere-derived cells [113].

γ-TT also exhibited apoptosis-inducing and invasion-suppressing activity in malignant melanoma cells. In particular, it was reported to inhibit NF-κB, EGF-R and Id family proteins, to activate the JNK signaling pathway, to decrease different mesenchymal markers levels and to restore E-cadherin and γ-catenin expression [114].

The Aryl hydrocarbon Receptor (AhR) is a ligand-activated transcription factor, which controls many biological and physiological processes in response to aromatic hydrocarbons, such as cellular proliferation and differentiation, tissue development, immune and toxic response and skin barrier homeostasis. Upon ligand binding to AhR, the activated complex translocates to the cell nucleus, where it forms a functional heterodimer with the AhR nuclear translocator (ARNT), with subsequent interaction with DNA and transcriptional activation of several target genes (in particular p21 and Bax) by binding to the xenobiotic responsive element [115]. Yamashita *et al.* demonstrated that γ-TT induces AhR expression in a dose-dependent manner in B16 mouse melanoma cells and enhances its sensitivity to baicalein, a flavone particularly abundant in the roots of *Scutellaria baicalensis* and *Scutellaria lateriflora*, which can inhibit tumor cell growth by acting as a ligand of AhR and thus upregulating p21 and Bax levels [116].

Synergistic antitumor activity of TTs and lovastatin was evidenced in murine B16 melanoma cells, as well as in C57BL6 mice bearing B16 xenografts [92].

Improved anti-proliferative effectiveness against A431 and SCC-4 human keratinocyte cancer cells *in vitro* was shown by a hybrid-nanoemulsified TTs delivery system and it was associated with better yield in physicochemical parameters, as well as stability in chemical and structural composition [117]. Interestingly, an orally administered γ-TT nanoformulation also exhibited enhanced radioprotection compared to γ-TT alone in CD2F1 mice exposed to total body γ radiation [118].

Transferrin receptors are frequently expressed in tumor cells, thus representing a potential target for the delivery of anti-cancer drugs into the tumor mass. Karim *et al.* reported that α-TT entrapped in transferrin-bearing multilamellar vesicles possesses potent growth-suppressing activity in A431 human epidermoid carcinoma cancer cells and B16-F10 murine melanoma cells. Moreover, the intravenous administration of these vesicles to mice bearing A431 and B16-F10 tumors successfully inhibited cancer progression, without visible side effects [119].

2.1.9. Tocotrienols in Other Tumors

Anti-proliferative, pro-apoptotic, anti-metastatic and anti-angiogenic effects of TTs were also reported in other cancer cell types.

In bladder cancer cells, δ-TT treatment suppressed cell proliferation and colony formation capacity by triggering G1 phase arrest and intrinsic apoptosis, accompanied by upregulation of p21, p27 and Bax expression and downregulation of cyclin D1, Bcl-2, Bcl-xL and Mcl-1 levels, ultimately leading to caspase-3 and PARP cleavage. Moreover, δ-TT induced ETK inactivation and SHP-1 increase, resulting in the inhibition of the STAT3 pathway. Importantly, low doses of δ-TT sensitized human bladder cancer cells to the anti-tumor activity of gemcitabine [120].

Tan *et al.* demonstrated that γ-TT can act as a BH3 mimetic in SH-SY5Y neuroblastoma cells, by binding to the hydrophobic groove of Bcl-2 and triggering Bax- and caspase-9-mediated apoptosis [121].

TTs were shown to suppress cell viability in different leukemic cell lines and blasts from patients, by inducing loss of mitochondrial membrane potential, release of cytochrome *C* and cleavage of Bid and caspases. Interestingly, normal mononuclear cells were spared by TTs-induced cytotoxic effects [122].

γ-TT triggered apoptotic cell death in human T-cell lymphoma via both the intrinsic and extrinsic pathways. In particular, the tocotrienol treatment resulted in mitochondrial ROS production, calcium cytoplasmic accumulation, JNK phosphorylation and changes in the Bax/Bcl-2 ratio, as well as in Fas and FasL upregulation and caspase-8 activation [123].

In human hepatocellular carcinoma cells, TTs exerted potent anti-proliferative effects correlated with a significant decrease of the expression levels of H-RAS, Grb2, Sos-1, p-Src and p-Shc and subsequent inhibition of the RAS-RAF-MEK-ERK downstream signaling pathway [124]. Moreover, γ-TT significantly reduced the angiogenesis-mediated growth of human hepatocellular carcinoma in an orthotopic mouse model by suppressing the AKT/mTOR [125] pathway (Table **1)** [65-125].

Table 1. ***In Vivo*** **and Clinical Studies on Tocotrienols (TTs) in Cancer Therapy/Prevention.**

Tumor Type	Current Studies	References
Breast cancer	• Two *in vivo* studies demonstrating the anti-proliferative and anti-angiogenic effects of redox-silent TT oxazine derivatives • One clinical trial aimed at investigating the TTs adjuvant potentials when given in combination with tamoxifen	[65-67]
Colon cancer	• One *in vivo* study showing the synergistic anti-cancer effects of TTs and capecitabine • Two *in vivo* studies demonstrating the anti-angiogenic properties of TTs	[76, 80, 81]
Gastric cancer	• One *in vivo* study showing the synergistic anti-cancer effects of TTs and capecitabine	[88]
Lung cancer	• One *in vivo* study showing the synergistic anti-cancer effects of TTs and lovastatin	[92]
Pancreatic cancer	• One *in vivo* study demonstrating the pro-apoptotic properties of TTs • One *in vivo* study demonstrating the anti-invasive properties of TTs • One clinical trial conducted to test TTs efficacy and safety	[14, 97, 98]
Prostate cancer	• Two *in vivo* studies demonstrating the anti-proliferative properties of TTs • Two *in vivo* studies demonstrating the specific CSCs-targeting ability of TTs	[103, 106-108]
Skin cancer	• One *in vivo* study demonstrating the pro-apoptotic properties of TTs • One *in vivo* study demonstrating the specific CSCs-targeting ability of TTs • One *in vivo* study demonstrating the chemopreventive properties of TTs • One *in vivo* study demonstrating the anti-cancer effects of transferrin-bearing vesicles containing TTs	[112, 113, 118, 119]
Liver cancer	• One *in vivo* study demonstrating the anti-proliferative and anti-angiogenic effects of TTs	[125]

3. PATENTS FOR THE ISOLATION, CHEMICAL MODIFICATION AND USE OF TOCOTRIENOLS IN CANCER PREVENTION/THERAPY

In the last years, novel methods and techniques have been proposed to successfully isolate and exploit TTs in cancer prevention and therapy.

3.1. Isolation and Purification of Tocotrienols

As reported above, TTs are present in several plants and vegetable oils, and many studies have highlighted the need for development of effective isolation and purification processes for the proper preparation of TTs intended for experimental or clinical testing [20, 126, 127].

Red palm oil is the major source of TTs among all edible oils, with TTs representing 70% of total vitamin E derivatives present in it. Different patents provide a method for the extraction of TTs from Palm Fatty Acid Distillates (PFAD), which involves conversion of a PFAD into distilled methyl esters, followed by ion-exchange chromatography and by another step of molecular distillation to get vitamin E components concentrate of more than 90% [128-134]. Some other patents propose to obtain TTs from Crude Palm Oil (CPO) by converting CPO to methyl esters, with subsequent removal of the esters by distillation to yield carotene-rich and vitamin E-rich fraction [135-137]. Conversion of CPO into methyl esters, followed by chromatographic separation in the presence of solvents in order to obtain carotene-rich, vitamin E-rich and sterol-rich fractions, has also been suggested [138]. Finally, two novel inventions disclose the use of supercritical fluids, such as SC-CO_2, in the adsorption/desorption chromatography isolation/separation of TTs from CPO [139, 140].

High levels of TTs can be easily obtained from rice bran through its ultrasound-assisted saponification and subsequent recrystallization or chromatography separation of the saponified organic phase content, as shown in some new patents [141, 142]. This process also offers the possibility to successfully extract ferulic acid from the aqueous layer obtained in the above separation process, by precipitation of its alkali salt through addition of an acid, such as diluted sulfuric or hydrochloric acid [142].

TTs isolation from annatto seeds has been described in a recent invention [143]. This process presents many advantages. First of all, the amount of δ-TT present in the byproduct solution of *Bixa Orellana* L. seeds is higher than that found in other common sources, such as palm or rice bran oil. Moreover, and also in contrast to palm and rice bran oils, *Bixa Orellana* L. seeds extract essentially does not contain α-TP, which can have a mitigating effect on the therapeutic properties of δ-TT, as discussed above. Finally, the annatto solution is also a convenient source of geranylgeraniol, that can be administered in combination with TTs in order to potentiate their anti-tumor effects.

A novel patent highlights a method for producing a tocotrienol-rich composition by transesterification of coconut palm oil, followed by distillation of the reaction product and adsorption treatment of the residue with an anion exchange resin having a 2-hydroxyethyl dimethylammonium group [144].

A new invention provides a procedure for the production of purified γ- and/or δ-TT from TTs rich oils and distillates [145]. In this process, γ- and/or δ-TT are rapidly isolated from the TTs sources by medium (flash) or low-pressure chromatography, obtaining a fraction with high concentration of γ-TT and/or δ-TT and minimized content of α-isomers. In particular, the γ- and/or δ-TT rich composition contains approximately 95% of TTs, with 10% of γ-TT and 3% of δ-TT, each of which having purity of about 95%.

3.2. Transgenic Sources of Tocotrienols

The use of biotechnological techniques to increase the bioactive components content in plants has become a very popular subject of study and debate in the last decades [146].

A recent patent reports on a method for the production of a transgenic rice plant enriched in δ-TT, whose extract successfully inhibits PC-3 prostate cancer cell growth. The transformed plant is obtained through the introduction in its DNA of the *Homogentisic Acid Geranylgeranyl Transferase* (*HGGT*) gene, which mediates TTs synthesis by catalyzing the condensation of homogentisate and geranylgeranyl diphosphate to form 2-methyl-6-geranylgeranylbenzoquinol. The *HGGT* gene is overexpressed using seeds-specific and constitutive promoters, leading to an increase of δ-TT content of about two times around in the transgenic rice compared to the normal plant [147]. Similar enriched sources of TTs are obtained through the transformation of soybeans and *Perilla frutescens*, an annual plant native to Korean peninsula, Southern China and India [148, 149].

3.3. Tocotrienols as Anti-Cancer Drugs

Recent patents suggest different methods for using TTs as anti-cancer drugs.

There is a recent invention according to which a diet of rice oil extract enriched in TTs can prevent colon cancer in F344 rats treated with the carcinogenic agent azoxymethane. In particular, the TTs treatment is reported to reduce the number and the volume of the aberrant crypt foci in the colonic mucosa of the animals, without causing any significant weight loss or side effect [150].

A new patent proposes γ-TT as a potent inhibitor of melanoma (C32 and G361), prostate cancer (LNCaP and PC-3) and breast cancer (MDA-MB-231 and MCF-7) cell growth both *in vitro* and *in vivo*. The anti-tumor effects of γ-TT were shown to be selective for cancer cells since normal cells, such as the immortalized human prostate epithelial PZ-HPV-7 cells, were not affected by the treatment, and they were associated with decreased cell proliferation and apoptosis induction. In particular, γ-TT-mediated cytotoxicity was correlated with inhibition of IdI and NF-κB via modulation of their upstream regulators Src, Smadl/5/8, Fak and LOX. γ-TT treatment also resulted in the activation of JNK signaling pathway, and its inhibition by the specific inhibitor SP600125 partially restored cell viability. Interestingly, it was also found that γ-TT treatment enhanced the expression of E-cadherin, supporting the idea that γ-TT also possesses anti-metastatic activity in different tumors. Finally, it was demonstrated that γ-TT can down-regulate the expression of stem cell markers (CD133/CD44) [151].

A recent patent provides a method utilizing δ-TT to inhibit pancreatic tumor growth. Results show that δ-TT induces apoptosis and inhibits cell proliferation in human pancreatic ductal carcinoma cell lines (MIA PaCa2, SW1990, BXPC3)

in vitro as well as in MIA PaCa2 cells xenografted in nude mice. In particular, δ-TT induces caspases activation, decreases p-Akt, p-MAPK and Ki-67 levels and increases p27 levels. Immortalized human pancreatic ductal epithelial cells, HPDE 6C7, were treated under identical conditions, showing that δ-TT cytotoxic effects are selective for pancreatic cancer cells [152].

It should be noted that different inventions have evidenced the cholesterol-lowering (due to HMG-CoA reductase inactivation and mevalonate pathway inhibition) and the anti-oxidant (correlated with ROS suppression and block of superoxide release from tumor-associated neutrophils) properties of TTs as well as their anti-angiogenic (consisting in the arrest of endothelial cell multiplication and lumen formation) and immune-regulatory (associated with an increase in the total antibody titer) activity, suggesting the use of these compounds for cancer treatment [153-155].

3.4. Alternative Routes of Administration and Dosage Forms

As discussed above, TTs are usually administered *per os*. However, many studies have been focused on the development of new TTs formulations, and several patents have proposed alternative routes of administration and dosage forms for TTs delivery to target tissues.

There is a new patent that demonstrates that the transdermal application of a gel containing δ-TT is very suitable and effective for the treatment of patients with breast cancer. Interestingly, δ-TT was observed to accumulate at higher concentrations in the adipose tissue of the benign breast lumps in comparison to the malignant lumps, and this could be due to its antioxidant activity and in particular to its ability to quench free radicals and to regulate peroxidative reactions [156].

There is a recent invention according to which a nanodroplet containing a tocotrienol (e.g. δ-TT), a tocopherol or tocotrienol covalently bonded to a polyalkylene glycol (α-tocopheryl-polyethyleneglycol-1000-succinate), a poloxamer (PLURONIC® P188) and a polyalkylene glycol (PEG400) exerts a cytotoxic effect on NCI-H460 human lung cancer cells subcutaneously injected in nude mice. Moreover, it exhibits a synergistic anti-tumor effect in combination with docetaxel, and the severe weight loss caused by standard chemotherapy in the animals is reduced by simultaneous treatment with δ-TT nanodroplets, thus demonstrating their chemoprotective effects [157].

The Australian inventor Tong has found that the administration of TTs by a transmucosal delivery route, particularly by sublingual tablets containing a dose of 9mg δ-TT and 1mg γ-TT, provides for enhanced bioavailability when compared with orally administered TTs, proposing it for cancer therapy [158]. Similarly, also the topical administration through the skin and the rectum has been suggested for TTs anti-cancer treatment [159].

3.5. Combinations with Other Compounds

Many inventors have tested one or more TTs in combination with other compounds in different pathologies, including cancer.

A synergistic anti-tumor effect was observed when cancer cells and xenografts were co-treated with γ-TT and a standard chemotherapeutic agent, such as docetaxel or dacarbazine [151]. Similar results were also obtained after co-treatment with δ-TT and 5-fluorouracil (5-FU) in pancreatic tumor cells [152].

A novel patent reports on a method for the prevention and treatment of neoplastic diseases and particularly of breast cancer, showing that a combination of citrus limonoids (limonin or nomilin), citrus flavonoids (nobiletin or tangeretin) and TTs (α-, γ- or δ-TT), with or without conventional anti-tumor therapy (tamoxifen), synergistically inhibits MCF-7 and MDA-MB-231 cell proliferation [160].

Another invention for breast cancer treatment evidences that curcumin synergistically enhances the anti-proliferative effect of a TRF preparation on MCF-7 human breast cancer cells *in vitro* [161].

A recent patent provides a method using a composition comprising γ-TT and lovastatin or β-ionone to synergistically inhibit the growth of B16 melanoma cells, also after their implantation in nude mice [162].

It has also been demonstrated that a combination of TTs with lovastatin and/or genistein can synergistically suppress prostate cancer (LNCaP, DU145 and PC-3) cell growth [163].

A new patent proposes the use of TTs gelatin capsules, to be administered orally and on a daily basis, for prostate and breast cancer treatment. Each capsule can contain 200mg of γ-TT, 75mg of δ-TT, 100mg of turmeric extract and 200mg of saw palmetto extract for prostate cancer therapy, and 100mg of γ-TT, 25mg of δ-TT, 100mg of turmeric extract and 50mg of L-ascorbic acid for breast cancer therapy. The compositional dosage can also include 100ml of fermented noni juice and 100ml of mangosteen juice, which can be administered orally separated from the gelatin capsules [164].

It has been recently suggested by an interesting patent that pharmaceutical compositions containing TTs and Met receptor tyrosine kinase inhibitors can exert a synergistic inhibitory effect on tumor cell growth. In cancer cells, Met can be constitutively activated via overexpression, mutations and/or interaction with other membrane receptors, such as the HER/ErbB family members. Upregulation of the Met pathway can ultimately lead to increased tumor cell proliferation, reduced susceptibility to apoptosis and enhanced angiogenesis and metastasis. Combinations of γ-TT and SU11274, a specific Met receptor tyrosine kinase inhibitor, synergistically inhibit the growth of +SA mammary tumor cells [165].

It has been shown by a novel patent that metalloproteins, including lactoferrin, transferrin, ovotransferrin, ceruloplasmin and metallo-thionein, can stabilize and enhance the bio-functional activity of TTs. Importantly, the synergism between metallo-proteins and TTs also promotes the intestinal transfer and the ultimate bioavailability of TTs for physiological functions [166].

Finally, different inventions present novel TTs antioxidant formulations that can be used to inhibit oxidative damage and to treat and prevent disorders caused by free radicals, including cancer. The antioxidant compositions comprise: a combination of α-, β-, y- or δ-TT or TTs mixture plus a radical scavenger recycler (α-lipoic acid, ascorbic acid, ascorbic acid salts or ascorbyl palmitate) and optionally a radical formation inhibitor (pyruvate) [167]; a combination of δ-TT with green tea extract or pure epicatechin [168]; a combination of asthaxantin, betacarotene, lutein and a mix-

ture of TTs (56% of γ-TT, 30% of α-TT, 13% of δ-TT and 1% of other TTs including β-TT) [169].

3.6. Synthetic Derivatives

The first model of anti-cancer tocotrienol synthetic derivative was provided by Kanazawa *et al.*, who proposed the linoleic acid α-TT ester as a preventive agent for cancer metastasis. Since then, several methods have been developed for the synthesis and use of TT derivatives [170].

There is a recent patent that indicates γ-TT succinate as a potent inhibitor of cancer cell growth, successfully suppressing the proliferation of liver cancer (Hep 3B), lung cancer (NIH-H69), colon cancer (HT29), prostate cancer (DU145), cutaneous melanoma (A375), ocular melanoma (OCMI), leukemia (HL60) and breast cancer (MCF7 and MD-231) cells [171].

A new patent reports that different ester derivatives of TTs exert both anti-proliferative and anti-invasive activity in +SA, MCF7 and MDA-MB-231 cell lines [172, 173].

A recent invention provides a method for synthetizing and using TTs ether derivatives. The novel compounds were shown to trigger apoptotic cell death of human breast cancer (MDA-MB-435, MDA-MB-231 and MCF-7), prostate cancer (PC-3, DU145 and LnCaP), ovarian tumor (C-170), cervical tumor (ME-180), endometrial carcinoma (RL-95-2), leukemia (HL-60), colon cancer (HT-29 and DLD-1) and lung cancer (A-549) cells via activation of the TGF-β, stress-activated protein kinase and Fas/FasL signaling pathways. Moreover, they did not induce apoptosis of Normal Human Mammary Epithelial Cells (HMECs) and of immortalized but non-tumorigenic MCF-10A [174] mammary cells Table **2** [20, 128-174].

Table 2. Patents for the Isolation, Chemical Modification and Use of Tocotrienols (TTs) in Cancer Prevention/Therapy.

Patent Type	Methods	References
Isolation and purification	• From red palm oil: 1) extraction from plant fatty acid distillates, conversion into distilled methyl esters, ion-exchange chromatography, molecular distillation; 2) conversion of crude palm oil to methyl esters, distillation or chromatographic separation; 3) adsorption/desorption chromatography from crude palm oil with supercritical fluids (i.e. SC-CO_2) • From rice bran: ultrasound-assisted saponification and recrystallization or chromatography separation • From coconut palm oil: transesterification, distillation and adsorption treatment with anion exchange resin • From TTs rich oils or distillates: flash or low-pressure chromatography	[20, 128-145]
Transgenic sources	Rice plant, soybeans and *Perilla Frutescens* transformed through introduction of the *Homogentisic Acid Geranylgeranyl Transferase* (*HGGT*) gene	[146-149]
TTs as anti-cancer drugs	• Anti-proliferative activity • Pro-apoptotic activity • Anti-metastatic activity • Anti-angiogenic activity • Cancer stem cells-targeting activity • Cholesterol-lowering activity • Anti-oxidant activity • Immune-regulatory activity	[150-155]
Alternative routes of administration and dosage forms	• Transdermal gel • Subcutaneous nanodroplets • Sublingual tablets	[156-159]
Combinations	• Docetaxel or dacarbazine • Fluorouracil • Citrus limonoids and flavonoids • Curcumin • Lovastatin or β-ionone • Lovastatin and/or genistein • Turmeric extract and palmetto extract or L-ascorbic acid • Turmeric extract, palmetto extract, fermented noni juice and mangosteen juice • Met receptor tyrosine kinase inhibitors • Metallo-proteins: lactoferrin, transferrin, ovotransferrin, ceruloplasmin and metallothionein • α-Lipoic acid, ascorbic acid, ascorbic acid salts, ascorbyl palmitate and pyruvate • Green tea extract or pure epicatechin • Asthaxantin, betacarotene and lutein	[151, 152, 160-169]
Synthetic derivatives	• Linoleic acid α-TT ester • γ-TT succinate • Ester derivatives • Ether derivatives	[170-174]

CONCLUSION

The present work provides a review of recent literature and patents on the anti-cancer activity of TTs.

Several studies were conducted to investigate the effects of TTs on cancer growth and progression and to clarify the molecular mechanisms underlying these effects.

More recent studies focus on the development of new TTs synthetic derivatives, formulations and combinations, with the aim to open new windows of novel chemopreventive/therapeutic interventions.

The translation of these findings into the clinical setting still represents a great challenge and requires further investigation.

CURRENT & FUTURE DEVELOPMENTS

In recent years, interest in vitamin E-derived TTs has considerably increased owing to their unique health benefits and non-toxic nature.

Many *in vitro* and *in vivo* experiments have demonstrated the anti-tumor effects of TTs (especially γ- and δ-TT) in several cancer cell lines and mouse models through the inhibition of cell proliferation and induction of apoptosis. Moreover, TTs have been shown to possess important anti-angiogenic and anti-metastatic properties and to specifically target and eliminate cancer stem cells.

The ability to act through multiple molecular pathways, such as by HMG-CoA reductase downregulation, growth factor receptors signaling cascades inhibition and ER stress and autophagy induction, makes these compounds ideal for sensitizing cancer cells to standard chemotherapeutic agents and to other anti-cancer natural compounds, as evidenced by different preclinical trials.

Based on these promising results, various inventions have been focused on the biotechnological development of novel TTs transgenic sources with the aim to enhance the natural availability of these compounds, and many studies have been conducted to design and synthesize new derivatives and formulations of TTs with the purpose to improve their biological activity.

Despite all these encouraging observations, the clinical data so far available are still incomplete and several important questions remain to be addressed about the role of TTs in cancer patients, especially as regards their pharmacokinetics and bioavailability. Thus, clinical trials aimed to clarify the TTs anti-tumor effectiveness are urgently needed.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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