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REVIEW ARTICLE

Anticancer properties of tocotrienols: a review of cellular mechanisms and molecular targets

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ABSTRACT

Vitamin E is composed of two groups of compounds: α-, β-, γ- and δ-tocopherols (TPs), and the corresponding unsaturated tocotrienols (TTs). TTs are found in natural sources such as red palm oil, annatto seeds and rice bran. In the last decades, TTs (specifically, γ-TT and δ-TT) have gained interest due to their health benefits in chronic diseases, based on their antioxidant, neuroprotective, cholesterol-lowering, anti-inflammatory activites. Several *in vitro* and *in vivo* studies pointed out that TTs also exert a significant antitumor activity in a wide range of cancer cells. Specifically, TTs were shown to exert antiproliferative/proapoptotic effects and to reduce the metastatic/angiogenic properties of different cancer cells; moreover, these compounds were reported to specifically target the subpopulation of cancer stem cells, known to be deeply involved in the development of resistance to standard therapies. Interestingly, recent studies pointed out that TTs exert a synergistic antitumor effect on cancer cells when given in combination with either standard antitumor agents (i.e., chemotherapeutics, statins, 'targeted' therapies) or natural compounds with anticancer activity (i.e., sesamin, EGCG, resveratrol, ferulic acid). Based on these observations, different TT synthetic derivatives and formulations were recently developed and demonstrated to improve TT water solubility and to reduce TT metabolism in cancer cells, thus increasing their biological activity. These promising results, together with the safety of TT administration in healthy subjects, suggest that these compounds might represent a new chemopreventive/anticancer treatment (i.e., in combination with standard therapies) strategy. Clinical trials aimed at confirming this antitumor activity of TTs are needed.

1 | INTRODUCTION

Nutraceuticals are chemicals naturally found in foods (functional foods) or in dietary supplements that have general health beneficts; they include vitamins, polyphenols, ω-3 fatty acids, probiotics, aminoacids and soy derivatives (Rautiainen et al., 2016; Santini et al., 2017; Sauer and Plauth, 2017; Schwingshackl et al., 2017).

Vitamin E was first discovered as a fat-soluble vitamin associated with antioxidant properties and involved in the control of the reproductive functions (Evans and Bishop, 1922). It exists in eight hydrophobic compounds ('tococromanols'), named tocopherols (TPs) and tocotrienols (TTs). The term 'tocopherol' derives from the Greek language (i.e., tocos: child birth; pheros: to bear; ol: alcohol). On the other hand, the term 'tocotrienol' (i.e., TPs isoforms with three double bonds in the isoprenoid side chain) was first proposed by Bunyan and coworkers in 1961 (Bunyan et al., 1961).

TPs and TTs are divided into two groups: α, β, γ and δ TPs and the corresponding isomers α, β, γ and δ TTs. Their chemical structure is composed of a chromanol ring which is linked to a isoprenoid side chain at the C2 position; this chain is saturated in TPs and unsaturated in TTs (with three double bonds at positions 3', 7' and 11'). The unsaturated isoprenoid side chain of TTs may be responsible for the better distribution of these isomers in the cell membranes and their high penetration into tissues with saturated fatty layers (Peh et al., 2016; Suzuki et al., 1993). The four isoforms of both TPs and TTs differ dependently on the degree and position of methyl groups: the α and β isomers are trimethylated, while the γ isomers are dimethylated and the δ are monomethylated on the chromanol ring. The structure of the four TTs isoforms is shown in Figure 1.

Vitamin E members are absorbed in the small intestine and bile salts are necessary for this absorption. The presence of the α-tocopherol transport protein (α-TTP) in liver cells is responsible for the packaging of these compounds (mainly α-tocopherol) into lipoproteins and the subsequent transportation to body tissues through the blood (Hosomi et al., 1997). Tissue uptake for both TPs and TTs may then occur with the involvement of lipoprotein lipases or by receptor-mediated lipoprotein endocytosis (Ahsan et al., 2014). Based on the observation that TTs have a low affinity for α-TTP and undergo a rapid catabolism in the liver, it has been questioned for many years whether orally administered TTs can reach the different tissues (Birringer et al., 2002; Cardenas and Ghosh, 2013; Hosomi et al., 1997; Peh et al., 2016; Traber, 2007). Moreover, TPs have been reported to interfere with TTs cellular uptake both *in vitro* (Shibata et al., 2010) and *in vivo* (Ikeda et al., 2003). However, pre-clinical observations reported effective health benefits and safety after their oral administration, suggesting the bioavailability of these compounds (Khan et al., 2010; Khanna et al., 2005). Moreover, the bioavailability of TTs has been reported in healthy humans, supporting that these compounds may reach their target tissues through alternative pathways despite their low affinity for α-TTP (Fu et al., 2014; Qureshi et al., 2016). This clearly supports the existence of specific mechanisms for the absorption and transport of these vitamin E isoforms.

Since 1980s and 1990s, TTs attracted a great attention for their health benefits in preventing or treating chronic diseases, such as cardiovascular and neurodegenerative diseases and osteoporosis (Abdul-Majeed et al., 2013; Abdul-Majeed et al., 2015; Chin and Ima-Nirwana, 2015; Kanchi et al., 2017; Khanna et al., 2006; Parker et al., 1993; Pathak et al., 2016; Sen et al., 2004). In addition, TTs have attracted great interest for their anticancer effects (Ahsan et al., 2014; Cardenas and Ghosh, 2013; Chin et al., 2016; Henderson et al., 2012; Peh et al., 2016).

This review provides the state of the art on TTs anticancer properties, based on the experimental, preclinical and clinical evidence so far available. The molecular mechanisms of the antitumor activity of these compounds, as well as their effectiveness in combination treatments are discussed. The potential increased antitumor effects of new synthetic TTs derivatives or novel formulations is also addressed.

2 | NATURAL SOURCES OF TOCOTRIENOLS

TTs were first isolated from the latex of the rubber plant *Hevea brasilensis* (Willd. ex A.Juss.) Müll.Arg. (Whittle et al., 1966); later, it became consistently clear that TTs are present in different plant sources, particularly in palm oil, annatto (*Bixa orellana L.*) seeds and rice bran (Ahsan et al., 2015; Shahidi and de Camargo, 2016).

Among all edible oils, red palm oil represents the richest source of tocotrienols, particularly γ-TT (about 60% of total tocotrienols) (Ng et al., 2004). In palm oil, tocotrienols and tocopherols represent 70% and 30% of vitamin E derivatives (Tocotrienol Rich Fraction, TRF), respectively.

Bixa orellana is a small tree originating from the tropical region of the Americas. This tree is mainly known as the source of annatto, a natural orange-red condiment that can be obtained from its seeds. The annatto seeds are widely used in traditional dishes in Central and South America, in Mexico, and in the Caribbean; annatto extracts are also used as an industrial colorant for foods to add color (yellow or orange color) to many products such as cheese, butter, popcorn and cakes. Interestingly, annatto (*Bixa orellana*) seeds are the only vegetable source of TTs with virtually no tocopherols present. More importantly, δ-TT (140-147 mg/100 gr dry seeds) accounts for almost 90% of TTs in these seeds, with γ-TT accounting for only 10 % of total TTs. No α-TT can be found in annatto seed extracts (Raddatz-Mota et al., 2017).

Another source of TTs is rice bran, containing about 41% of α-TT and 59% of γ-TT; no or very low levels of δ-TT can be found in this oil (Ahsan et al., 2015; Goufo and Trindade, 2014; Krager et al., 2015; Min et al., 2011). Additional sources of TTs include wheat germ, halzenuts, olive oil, grape fruit, flax seed oil and sunflower oil (Ahsan et al., 2015; Shahidi and de Camargo, 2016).

Tocotrienol-rich fraction (TRF), as well as the percentage of the different TT isoforms present in the most relevant food sources, are summarized in Table 1.

3 | ANTICANCER PROPERTIES OF TOCOTRIENOLS: *IN VITRO* AND *IN VIVO* STUDIES

In 1986, it was reported that dietary consumption of palm oil reduce the development of mammary tumors (induced by carcinogens) in rats (Sylvester et al., 1986). These results were later confirmed by *in vitro* studies reporting that palm oil-derived TRF (tocotrienol-rich fraction) exerts an antiproliferative effect on mammary tumor cells (McIntyre et al., 2000; Shah et al., 2003).

Since then, several studies were reported pointing out that TTs, specifically γ- and δ-TT, are endowed with a significant anticancer activity against different tumors (Aggarwal et al., 2010; Cardenas and Ghosh, 2013; Chin et al., 2016; Henderson et al., 2012; Malavolta et al., 2016; Meganathan and Fu, 2016; Peh et al., 2016). In addition to their antioxidant and antiinflammatory properties, the anticancer effects of these compounds were also shown to be related to their interaction with different intracellular signaling pathways involved in the mechanisms of proliferation, apoptosis, angiogenesis and metastasis (Galli and Azzi, 2010; Kannappan et al., 2012; Miyazawa et al., 2008; Nesaretnam, 2008; Sailo et al., 2018; Shanmugam et al., 2017; Sylvester et al., 2014; Zingg, 2015).

3.1 | Antiproliferative Activity

TTs were shown to exert antiproliferative effects on a wide range of tumor cells, through modulation of the activity of different intracellular signaling pathways. Most of these studies were performed on human breast cancer cells (Sylvester et al., 2014). γ-TT was found to reduce the expression of proteins involved in cell cycle progression, such as cyclin D1 and the cyclin-dependent kinases (CDK) CDK4, CDK2 and CDK6 in mammary cancer cells (Hsieh et al., 2010; Samant et al., 2010). At the same time, the vitamin E derivative was shown to increase the expression of CDK inhibitors and to reduce the phosphorylation of the Rb (retinoblastoma) protein (Hsieh et al., 2010; Samant et al., 2010). The antiproliferative effects of TTs were also analyzed in prostate cancer cells. It was reported that a TRF preparation exerts a significant growth inhibition on prostate cancer cells (but not in normal epithelial cells), through G1 arrest (Srivastava and Gupta, 2006). More specifically, TTs were shown to suppress proliferation and induced apoptosis in prostate cancer cells by affecting the expression/activity of different targets, such as NF-κB (nuclear factor-kappa B), PI3K (phosphoinositide-3 kinase)/Akt, STAT (signal transducer and activator of transcription), TFGβ (transforming growth factor β) receptor, cyclins, as well as the cell cycle inhibitors p27 and p21 (Barve et al., 2010; Campbell et al., 2011; Sugahara et al., 2015; Yap et al., 2008). Interestingly, Huang and coworkers (Huang et al., 2017) reported that a tocotrienol mixture inhibits the growth of the human prostate VCaP cell line, in a dose-dependent manner. TTs (δ-TT being more effective than γ-TT) exert this anticancer effect by

increasing the expression of p21 and p27; this effect was associated with increased H3K9 acetylation levels at the proximal promoter regions of both CDI inhibitors and with reduced expression of HDACs (histone deacethylases). Thus, TTs can suppress tumor growth by blocking the cell cycle at the G1/S transition phase, at least partially, through epigenetic mechanisms.

Antiproliferative effects of TTs mediated by cell cycle regulation were reported for other cancer cell types such as pancreatic (Hodul et al., 2013; Hussein and Mo, 2009; Kunnumakkara et al., 2010), cervical (HeLa) (Wu and Ng, 2010), lung (Ji et al., 2012a), colon (Shibata et al., 2015) and bladder (Ye et al., 2015) cancer cells.

The family of EGF receptors, which is composed of four types of receptors (ErbB1/HER1, ErbB2/HER2, ErbB3/HER3, ErbB4/HER4) is known to be deeply involved in the control of cell proliferation (Appert-Collin et al., 2015) through different intracellular signaling pathways, such as the PI3K/Akt/mTOR, MAPK (mitogen-activated protein kinase), and JAK (Janus kinase)/STAT signaling cascades (Hynes and Lane, 2005; Laurent-Puig et al., 2009; Yarden and Sliwkowski, 2001). For this reason, ErbB proteins are now considered effective molecular targets in anticancer therapy (Arteaga and Engelman, 2014; Filippi et al., 2017).

In mammary cancer cells, γ-TT was reported to inhibit EGF-dependent activation of mitogenic pathways by reducing the ErbB/HER receptor autophosphorylation, thus suppressing the activity of the PI3K/Akt signaling pathway and the transcriptional activity of the nuclear factor NF-κB (Shah et al., 2003). In line with these observations, γ-TT was found to significantly decrease human breast cancer cell proliferation by reducing both the PI3K/Akt/mTOR and the Ras/Raf/MEK/ERK signaling pathways; this results in the decrease of c-Myc levels due to its ubiquitination and degradation (Parajuli et al., 2015a). Interestingly, γ-TT also suppressed the activity of the PI3K/Akt/mTOR pathway, responsible for a rewiring of the breast cancer cell metabolism, through a decrease of the aerobic glycolysis (Parajuli et al., 2015b). TT treatments were found to reduce the development of mammary tumors in ErbB2 transgenic mice and to induce apoptosis and senescence-like growth arrest of cancer cells (Pierpaoli et al., 2013).

Similar observations were reported in pancreatic (Shin-Kang et al., 2011) and in hepatocellular cancer cells (Burdeos et al., 2016).

TTs were shown to reduce cell proliferation also by affecting the post-translational modification of proteins involved in the mitogenic signaling pathways. In particular, TT affect isoprenylation of these proteins based on their ability to inhibit HMG-CoA (hydroxymethylglutaryl-coenzyme A) reductase activity by post-transcriptional downregulation and metabolic degradation. HMG-CoA reductase is the rate-limiting enzyme in cholesterol synthesis in the mevalonate pathway. This pathway produces different farnesyl and geranylgeranyl intermediates known to be involved in the post-translational modifications of small G proteins (i.e. Ras) and of αβγ-G protein subunits, thus allowing their anchoration (i.e., activation) to the plasma membrane. δ-TT reduced the proliferation of breast and pancreatic cancer cells

through the down regulation of HMG-CoA reductase activity (Hussein and Mo, 2009; Khallouki et al., 2015).

The antiproliferative activity of TTs was further supported by preclinical studies in nude mice (Kunnumakkara et al., 2010; Manu et al., 2012; Selvaduray et al., 2010; Sylvester et al., 1986; Yap et al., 2010) (Aggarwal et al., 2013; Huang et al., 2017; Montagnani Marelli et al., 2016; Zhang et al., 2015).

3.2 | Proapoptotic Activity

TTs were reported to induce apoptosis in different cancer cells, by triggering both the extrinsic and intrinsic apoptosis pathways. Pure vitamin E-derived TTs as well as a TRF preparation were found to induce the intrinsic apoptosis in human breast cancer cells (Loganathan et al., 2013; Takahashi and Loo, 2004; Viola et al., 2013). In colon carcinoma RKO cells, a TRF preparation induced mitochondrial apoptosis through activation of p53, followed by a significant increase of the Bax/Bcl-2 ratio, associated with downstream activation of caspase-9 and caspase-3 (Agarwal et al., 2004). The intrinsic apoptosis pathway was also shown to mediate the anticancer activity of TTs in hematological (Inoue et al., 2011), pancreatic (Wang et al., 2015), and neuroblastoma (Tan et al., 2016) cell lines.

TTs were shown to trigger the extrinsic apoptotic pathway. In mammary tumors, TTs decreased the levels of of FLIP (FLICE-inhibitory protein), an apoptosis inhibitory protein that inhibits caspase-8, although this occurred without the involvement of surface death receptors (Shah and Sylvester, 2004; Sylvester and Ayoub, 2013).

Interestingly, both the extrinsic and the intrinsic apoptosis pathways can be activated by TTs. For instance, γ-TT induces apoptosis in human T-cell lymphoma through mitochondrial ROS production and calcium release, changes in the Bax/Bcl-2 ratio and loss of mitochondrial membrane potential; it also upregulates surface expression of Fas and FasL, thus triggering caspase-8 activation (Wilankar et al., 2011). Moreover, it was shown that γ-TT sensitizes colon cancer cells to the proapoptotic activity of TRAIL (a member of the tumor necrosis factor superfamily) and induces the expression of the TRAIL death receptors DR-4 and DR-5. This effect was mediated by the expression of p53 and Bax, proteins of the intrinsic apoptosis pathway (Kannappan et al., 2010).

In breast cancer cells, it has been proposed that TTs pro-apoptotic activity involves estrogen receptor beta (ERbeta) signaling (Comitato et al., 2010). This study shows that, in MCF-7 breast cancer cells expressing both ERalpha and ERbeta, treatments with tocotrienol rich fraction from palm oil (PTRF) or purified γ-TT,t increase ERbeta nuclear translocation and significantly inhibits ERalpha expression and complete disappearing of the protein from the nucleus. Moreover, PTRF treatment induces ER-dependent genes expression (macrophage inhibitory cytokine-1, early growth response-1 and Cathepsin D) and this is inhibited by the ER inhibitor, ICI 182.780, and induces DNA fragmentation (Comitato et al., 2010) .

A signaling pathway involved in the apoptosis process is also the so called endoplasmic reticulum (ER) stress. The ER stress is a cellular process that is triggered by different conditions leading to an imbalance in intracellular homeostasis. Different physiological and pathological conditions can induce ER stress, severely impairing protein folding; on the other hand, ER stress can also be induced by several compounds of synthetic or natural origins (Foufelle and Fromenty, 2016; Schonthal, 2013; Wang and Kaufman, 2016). Initially, cells react to ER stress with the so called unfolded protein response (UPR), a defensive process, known to be aimed at restoring homeostasis, through the enhancement of the protein folding capacity (Halperin et al., 2014). However, in conditions of severe stress, misfolded proteins accumulate in the ER and this triggers a number of prodeath programs (Schonthal, 2013). Double-stranded RNA-dependent protein kinase PKR-like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α) and activating transcription factor 6 (ATF6) are the most important proteins known to act as stress sensors in the ER (Parmar and Schroder, 2012). In physiological conditions, these proteins are associated (i.e., inactivated) with the chaperone BiP (immunoglobulin-heavy-chain-binding protein, also known as GRP78) protein. However, in conditions of severe ER stress, BiP dissociates from the sensors, leading to their activation; each of these sensors is coupled with a specific cytosolic pathway and each pathway converges to apoptosis (Hiramatsu et al., 2015; Maurel et al., 2015). In particular, the ATF4 transcription factor pathway, activated by the PERK/eIF2α (eukaryotic translational initiation factor2α), stimulates the expression of the proapoptotic protein CHOP (C/EBP homologous protein, also called GADD153). IRE1α leads to downstream activation of the JNK (c-Jun N-terminal kinase)/p38 MAPK, CHOP and caspase-4 pathways (Hiramatsu et al., 2015; Maurel et al., 2015; Schonthal, 2012).

Based on these data, pharmacological targeting of ER stress is now considered an effective therapeutic strategy to treat tumors (Maurel et al., 2015; Schonthal, 2012; Schonthal, 2013). Different natural compounds were shown to induce ER stress-mediated death in cancer cells (Pereira et al., 2015). In mouse mammary tumor cells, γ-TT induced apoptosis through the activation of the PERK/eIF2α/ATF4/CHOP pathway and of caspase-4 (Wali et al., 2009a). In breast cancer cells, γ-TT was shown to increase the expression of CHOP, leading to the upregulation of the death receptor DR5 hrough the JNK and p38 MAPK kinases (Park et al., 2010). The IRE1α pathway was also shown to be activated after γ-TT treatment in breast cancer cells (Patacsil et al., 2012). By means of *in vitro* and *in vivo* studies, we reported that δ-TT exerts a proapoptotic effect in human melanoma cells, while sparing normal melanocytes. In melanoma cells, δ-TT exerted its antitumor activity through the PERK/p-eIF2α/ATF4/CHOP, IRE1α and

caspase-4 ER stress-related branches (Montagnani Marelli et al., 2016). Similar results were reported in cervical cancer cells (Comitato et al., 2016).

It is now well established that autophagy may mediate the apoptotic activity of drug-induced ER stress pathways in cancer cells. In mouse and human mammary tumor cells, γ-TT triggered autophagy through: increased conversion of the microtubule associated protein 1A/1B-light chain 3 from LC3B-I (its cytosolic form) to LC3B-II (its lipidated form) and increased beclin-1 levels (Tiwari et al., 2014). In addition, in breast cancer cells, γ-TT was found to induce apoptosis by triggering both the ER stress and the early phase (LC3B-II, Beclin-1) and late phase (cathepsin-D, LAMP-1) autophagy pathways (Tiwari et al., 2015a). These observations demonstrate that both ER stress and autophagy are concurrently activated by TTs and together mediate their effects in inducing apoptosis of cancer cells. However, further studies are required to definitely confirm the role of autophagy (prodeath vs. prosurvival) in the antitumor activity of TTs (Tran et al., 2015).

3.3 | Antimetastatic Activity

Metastasis involves dissemination of tumor cells from the primary tumor to distant organs and subsequent growth in the new tissue microenvironment. Invasion of the extracellular matrix, formation of new blood vessels from a preexisting vasculature (angiogenesis) and colonization of distant organs are deeply involved in the metastatic process. Recent evidence demonstrates that several plant-derived dietary agents (nutraceuticals), including TTs, can exert their antitumor activity also by targeting these processes, possibly due to their antiinflammatory properties (De Silva et al., 2016; Gupta et al., 2010; Weng and Yen, 2012). γ-TT was reported to suppress the invasive ability of prostate cancer cells. γ-TT also induced up-regulation of E-cadherin (involved in the cell-cell adhesion mechanisms) (Yap et al., 2008) and decreased the expression of the matrix metalloproteinase MMP-9 in pancreatic cancer cells both *in vitro* and *in vivo* (Kunnumakkara et al., 2010). γ-TT significantly reduced gastric adenocarcinoma cell migration and matrigel invasion, by down-regulation of the matrix metalloproteinases MMP-2 and MMP-9 and up-regulation of TIMP-1 (tissue inhibitors of metalloproteinase-1) and TIMP-2 (Liu et al., 2010). A similar antimetastatic activity was observed in melanoma (Chang et al., 2009), lung (NSCLC) (Ji et al., 2012b), and gastric cancer cells (Manu et al., 2012).

The epithelial-to-mesenchymal transition (EMT) is well recognized as a typical feature of cancer progression and a potential target of novel therapies. TTs inhibit the EMT process in breast cancer cells through inhibition of HGF (hepatocyte growth factor)-dependent activation of Met (the HGF receptor) (Sylvester, 2014) and activation of the canonical Wnt signaling pathway (Ahmed et al., 2016). The antimetastatic activity of TTs was also reported for the δ-TT isoform. In particular, Husain and coworkers (Husain et al., 2017) demonstrated that, in pancreatic ductal adenocarcinoma

(PDAC) cells *in vitro* and tumors *in vivo*, δ-TT significantly inhibits migration, invasion, and the expression of several biomarkers of EMT.

3.4 | Antiangiogenic Activity

An additional anticancer mechanism of TTs is related to their antiangiogenic properties. Angiogenesis is the process of new capillary sprouting (neovascularization) from pre-existing blood vessels, responsible for the delivery of oxygen and nutrients to the tumor microenvironment. Tumor cells synthesize and secrete angiogenesis-related proteins, such as VEGF, FGF and EGF that are the responsible, together with their receptors, for the expression of angiogenic genes in endothelial cells and for the ability of these cells to form new vessels. The VEGF/VEGFR axis is the major factor responsible for neoangiogenesis in tumors; thus, it is well recognized as a key molecular target for anticancer agents, both standard therapeutics and dietary food components (Gupta et al., 2010; Shanmugam et al., 2017).

TTs were shown to reduce the angiogenic pathways in both tumor and endothelial cells. Palm tocotrienols downregulated the expression of VEGF in murine mammary cancer cells (Selvaduray et al., 2010). Similar results were obtained in preclinical studies showing that TRF significantly reduces serum VEGF levels in mice bearing mammary tumor xenografts (Selvaduray et al., 2012; Weng-Yew et al., 2009).

A major driver to tumor angiogenesis is hypoxia: low oxygen levels lead to activation of hypoxia-inducible factors (HIFs), a family of transcription factors responsible for the regulation of genes involved in glycolysis and angiogenesis. TTs were reported to decrease hypoxia-induced VEGF secretion in liver hepatocellular and colorectal adenocarcinoma cancer cells; in this study, δ-TT inhibited hypoxia-induced HIF-1α production, thus leading to a suppression of VEGF and IL-8 expression (Shibata et al., 2008a). γ-TT was shown to reduce HIF-1α accumulation and VEGF paracrine secretion in human gastric adenocarcinoma cells induced by cobalt(II) chloride, an hypoxia mimic, via ERK signaling pathway (Bi et al., 2010).

TTs exert their antiangiogenic activity also by directly targeting endothelial cells. The proliferation of HUVEC (human umbilical vein cells) cells was reported to be reduced by TRF treatment (Weng-Yew et al., 2009); TTs counteracted the VEGF- and FGF-induced HUVEC cell proliferation, with an order of potency of δ->β->γ->α-TT. These compounds also inhibited new blood vessel formation in *in vivo* angiogenic models (Nakagawa et al., 2007; Siveen et al., 2014). Moreover, γ-TT inhibited VEGF-induced autophosphorylation of VEGFR-2 in HUVEC cells through abrogation of the Akt/mTOR signaling pathway (Siveen et al., 2014). Furthermore, in endothelial cells, TTs significantly reduced the expression of two pro-angiogenic cytokines, IL-8 and IL-6, with δ-TT being more effective than TRF or γ-TT (Selvaduray et al., 2012).

The direct interaction between TTs and the proangiogenic activity of cancer cells was also addressed. δ-TT significantly inhibited colon cancer cell-induced tube formation, migration, and adhesion of HUVEC cells (Shibata et al., 2008b); δ-TT also suppressed VEGFR expression and signaling in HUVEC cells ultimately leading to caspase activation. *In vivo* experiments further confirmed the anti-angiogenic activity of the

vitamin E isomer (Shibata et al., 2009). Similar results were reported by Li and coworkers (Li et al., 2011) showing that γ-TT inhibits the angiogenesis process of HUVEC cells induced by the conditioned medium of gastric adenocarcinoma cells; this effect is mediated by downregulation of VEGFR-2 expressed on these cells.

These data support the notion that TTs might be considered an effective strategy to interfere with tumor progression based on their antimetastatic/antiangiogenic properties.

3.5 | Targeting Cancer Stem Cells

Tumors are a mixture of malignant stem cells (cancer stem cells, CSCs) and their differentiated daughter cells. According to the hierarchical model of tumor progression, CSC is a tumor cell that has the capacity for self-renewal, the ability to generate all heterogeneous tumor cell lineages, giving rise to the bulk of the tumor mass and to recapitulate continuous tumor growth (Clarke et al., 2006). CSCs are identified on their ability to generate tumor spheres when cultured in suspension conditions, to give rise to the heterogeneous original tumor when inoculated in nude mice, to possess high invasive behavior, and to express specific surface markers (Nagare et al., 2017). It is now well accepted that CSCs play a major role in the development of resistance to standard cancer therapies, thereby contributing to disease relapse after an initial response (Abbaszadegan et al., 2017; Eun et al., 2017). So far, different therapeutic approaches specifically targeting the CSCs subpopulation have been developed for different tumors (Agliano et al., 2017; Ahmed et al., 2017). Interestingly, natural compounds previously shown to possess anticancer activity were also reported to specifically target CSCs (Chen et al., 2017; McCubrey et al., 2017; Siddappa et al., 2017; Siveen et al., 2017; Torquato et al., 2017).

Luk and coworkers (Luk et al., 2011) reported that γ-TT downregulates the expression of prostate CSCs markers (CD133/CD44) in castration-resistant prostate cancer cells (PC-3 and DU145) and hamper the spheroid formation ability of these cells. In addition, pretreatment of PC-3 cells with γ-TT was found to suppress the tumor initiation ability of the cells when inoculated in nude mice. More importantly, CD133-enriched PC-3 cells, highly resistant to docetaxel treatment, were as sensitive to γ-TT treatment as the CD133-depleted population. In line with these data, Lee and coworkers (Lee et al., 2013) demonstrated that prostate cancer (PCa) patients receiving androgen-deprivation therapy display an increased PCa stem/progenitor cell population; similarly, treatment of PCa cells with antiandrogens induces an increase of the stem/progenitor cell subpopulation. These data demonstrate that the standard antiandrogen therapy in PCa might result in an undesired expansion of stem/progenitor cell population, explaining why this therapy fails in most PCa patients. Using different human PCa cell lines and mouse models, these authors concluded that targeting PCa stem/progenitor cells with γ-TT results in a significant suppression of the tumors in the castration-resistant stage.

TTs were also found to specifically target the CSCs subpopulation in breast cancer. In particular, it was shown that chemoresistant breast cancer cells are enriched in CSCs and express elevated levels of STAT-3 signaling mediators, which contribute to CSCs enrichment. Treatment of these cells with γ-TT, either alone or in combination with simvastatin, efficiently eliminated enriched CSCs and suppressed expression of STAT-3 signaling mediators. Data demonstrate that γ-TT and simvastatin, alone or in combination, are able to eliminate CSCs in drug resistant breast cancer cells (Gopalan et al., 2013). Similar results were reported in triple negative breast (Xiong et al., 2016) and in colon and cervical cancer cells (Gu et al., 2015). The vitamin E-derived δ-TT isoform was shown to selectively inhibit PDAC stem-like cells. In these cells, δ-TT inhibited the viability, survival, self-renewal, and expression of Oct4 and Sox2 transcription factors. Furthermore, in an orthotopic xenograft model of human PDAC stem-like cells, δ-TT significantly delayed the growth and metastases of gemcitabine-resistant PDAC human stem-like cells (Husain et al., 2017).

More recently, we reported that a subpopulation of autofluorescent cells expressing the ABCG2 stem cell marker is present in human melanospheres; δ-TT specifically target this CSCs subpopulation (Marzagalli et al., 2018).

Altogether, since TTs have been shown to be safe and to reach bioactive levels in humans, these data suggest that these compounds may represent effective agents in targeting CSCs; this may account for their anticancer and chemosensitizing effects reported in different studies.

The molecular mechanisms of the anticancer activity of TTs are summarized in Figure 2.

4 | ORIGIN OF TOCOTRIENOL INTRACELLULAR SIGNALING IN CANCER CELLS

As discussed above, TTs exert their anticancer activity by triggering different intracellular signaling pathways; however, the precise origin of these signals is still unknown. Vitamin E signaling was proposed to originate within the different lipid environments of the cell, both at the plasma and organelle membranes, where it is delivered to specific subcellular targets (Galli and Azzi, 2010; Saito et al., 2009).

The trafficking and subcellular localization of vitamin E, α-TP in particular, was shown to be regulated by cytosolic proteins that bind with their hydrophobic domains the vitamin derivative. These proteins, Sec14p-like proteins, are prototype components of the cell vitamin E regulation system that may play also a key role in the signaling of this vitamin and other lipids (Zingg et al., 2008). However, these observations were reported for TPs but not for TTs, responsible for the vitamin E anticancer activity.

As underlined above, ErbB proteins are a family of tyrosine kinase receptors. Based on their key role in the mechanisms of tumor growth and development, these receptors are considered effective molecular targets in anticancer therapy (Arteaga and Engelman, 2014; Filippi et al., 2017).

Lipid rafts are specialized rigid microdomains located within the plasma membrane that are enriched with cholesterol and sphingolipids. They are also associated with specific proteins, such as caveolins, flotillins, palmitoylated proteins, and nonreceptor tyrosine kinases; based on their composition, they are resistant to detergent solubilization and can be easily isolated from the surrounding plasma membrane (Chamberlain, 2004; Pike, 2009). Lipid rafts are deeply involved in ErbB activation and intracellular signal transduction. The anticancer effects of TTs, γ-TT in particular, were shown to be associated with the suppression of HER2 signaling (Shah and Sylvester, 2005; Tiwari et al., 2014); based on this observation, Alawin and coworkers (Alawin et al., 2016) investigated the effects of γ-TT on HER2 activation within the lipid raft microdomains in HER2-positive breast cancer cells. Treatment with γ-TT significantly inhibited cancer cell growth, through a decreased HER2 dimerization and phosphorylation. Both phosphorylated HER2 and γ-TT were found to accumulate exclusively within the lipid raft microdomains. Cotreatment of the cells with a compound that disrupts lipid raft integrity (hydroxypropyl-β-cyclodextrin, HPβCD) significantly reduced γ-TT cytotoxicity as well as its accumulation in the lipid raft microdomains. These results demonstrate that γ-TT accumulates in lipid raft microdomains directly leading to their disruption (thus interfering with growth factor receptor dimerization/activation) to exert its cytotoxic effects in breast cancer cells (Alawin et al., 2016). More recently, γ-TT was reported to suppress the activation of HER3 and HER4 growth factor receptors in lipid rafts microdomains in breast cancer cells through downregulation of the release of heregulin-containing exosomes (Alawin et al., 2017).

TTs were also shown to exert their anticancer effects through their direct binding to specific molecular targets. In particular, these compounds directly bind to Src and HMG-CoA reductase, thus inhibiting their role in tumor development (Aggarwal et al., 2010; Upadhyay and Misra, 2009).

5 | SYNERGISTIC ANTICANCER PROPERTIES OF TOCOTRIENOLS WITH STANDARD TREATMENTS OR NATURAL COMPOUNDS

5.1 | Chemotherapeutic Drugs

The major burden of standard cancer therapies is represented by the development of drug resistance and by the serious side effects often associated with these treatments. TTs were shown to possess anticancer activity against a wide range of tumors cells. Moreover, the bioavailability and safety of these compounds were demonstrated in healthy subjects (Fu et al., 2014; Qureshi et al., 2016) and in pancreatic cancer patients (Springett et al., 2015). Based on these observations, several studies were performed to investigate whether TTs might exert a synergistic antitumor activity in cancer cells when given in combination with anticancer compounds (i.e., standard chemotherapeutic agents, dietary components), with the aim to increase their efficacy in killing these cells (Eitsuka et al., 2016b).

In non-small lung cancer cells, δ-TT was demonstrated to synergize with cisplatin in inducing the suppression of cell viability, migration and invasiveness (Ji et al., 2012b). Manu and coworkers (Manu et al.,

2012) reported that γ-TT, in addition to its antiproliferative/antimetastatic activity on gastric cancer cells, chemosensitizes these cells to the antitumor activity of capecitabine, both *in vitro* and *in vivo*, in nude mice bearing gastric cancer cell xenografts. More recently, similar observations were reported for γ-TT in colorectal tumor cells (Prasad et al., 2016).

As discussed above, the ErbB receptor family is deeply involved in the mechanisms underlying tumor growth and progression. Erlotinib and gefitinib are well known inhibitors (tyrosine kinase inhibitors, TKI) of ErbB1 based on their ability to compete with ATP for binding to the intracellular catalytic domain of this receptor, thus inhibiting its activation (Yuan et al., 2014). Unfortunately, inactivation of ErbB1 leads to heterodimerization (i.e., activation) of other ErbB receptors, thus allowing tumor cells to escape from TKI anticancer activity. For this reason, combination treatments have been considered an interesting and effective therapeutic strategy to overcome the development of cancer cell resistance to TKIs. TTs were shown to exert their antitumor activity, at least partially, through inhibition of the ErbB receptor activation and their associated signaling pathways. It was reported that treatment of +SA mammary tumor cells with γ-TT synergistically increases the anticancer/proapoptotic activity of both erlotinib and gefitinib; this effect was mediated by a decrease in the expression levels of ErbB2-4 receptors and in their downstream Akt and STAT signaling (Bachawal et al., 2010). However, further studies would be needed to definitely assess the efficacy of combination treatments based on both natural TTs and tyrosine kinase inhibitors.

HMG-CoA reductase is the rate-limiting enzyme in cholesterol synthesis in the mevalonate pathway (Goldstein and Brown, 1990). As discussed above, this pathway produces different intermediates that are involved in the post-translational modifications of proteins, such as small G proteins (i.e., Ras) and αβγ-G protein subunits, thus allowing their anchoration to the plasma membrane and subsequent activation. Down-regulation of HMG-CoA reductase inhibits the activation of these proteins, thus interfering with cancer cell proliferation, apoptosis and metastasis. Thus, statins (lovastatin, simvastatin, atorvastatin, mevastatin), through their ability to competitively inhibit HMG-CoA reductase and to induce its post-transcriptional downregulation and metabolic degradation, were shown to suppress the growth of a wide range of cancer cells by inducing cell cycle arrest and apoptosis (Demierre et al., 2005). However, their use is limited due to their severe side effects (muscle pain and damage), ultimately leading to rhabdomyolysis that can cause liver damage, kidney failure and death (Thibault et al., 1996). Moreover, chronic use of statins often leads to a compensatory upregulation of this enzyme (Wali et al., 2009b). δ-TT is known to inhibit cancer cell growth by interfering with the HMG-CoA reductase activity (Hussein and Mo, 2009; Khallouki et al., 2015). Based on these observations, cotreatment with statins and TTs has been thought to induce a synergistic/additive anticancer activity. In prostate cancer cells, γ-TT was reported to potentiate the anticancer activity of lovastatin (Mo and Elson, 2004). A combination treatment with TTs and lovastatin synergistically inhibited the growth of murine melanoma, human prostate cancer and human lung adenocarcinoma cell xenografts in nude mice (McAnally et al., 2007). Wali and coworkers reported that a combination of γ-TT and a statin (simvastatin, mevastatin or lovastatin) sinergistically decrease the

proliferation of +SA mouse mammary epithelial cells, through the suppression of the MAPK, PI3K/Akt, JNK and p38 MAPK pathways (Wali and Sylvester, 2007). A synergistic antitumor activity of γ-TT and statins, atorvastatin and lovastatin, was also demonstrated in colon cancer and melanoma cells, respectively (Fernandes et al., 2010; Yang et al., 2010).

More recently, it has been reported that a concurrent delivery of a TRF preparation and simvastatin by lipid nanoemulsions significantly potentiates their antitumor activity against human breast cancer cells, both estrogen-dependent and estrogen-independent (Alayoubi et al., 2013). Interestingly, Gopalan and coworkers demonstrated that a combination of γ-TT and simvastatin is able to eliminate the cancer stem cell subpopulation in drug resistant human breast cancer cells (Gopalan et al., 2013). A synergistic effect of combined treatment with γ-TT and statins has been reported also in malignant mesothelioma cells (Tuerdi et al., 2013).

Synergistic antitumor effects were shown in +SA mouse mammary epithelial cells after a combination treatment with γ-TT and celecoxib, a non-steroidal anti-inflammatory drug that specifically inhibits cycooxygenase-2 (COX-2). The synergistic antiproliferative activity of the two compounds was found to reduce COX-2, Akt and NF-κB levels and to decrease PGE2 synthesis, through suppression of HERB2-4 tyrosine kinase receptor levels (Shirode and Sylvester, 2011).

5.2 | Natural Compounds

TTs were widely shown to exert a synergistic anticancer activity also when given in combination with different natural dietary compounds (Eitsuka et al., 2016b).

Sesamin is a lignan that can be found in sesame seeds and flax; interestingly, it has been reported to inhibit metabolic degradation of TTs, thus improving their bioavailability (Sontag and Parker, 2002). Akl and coworkers (Akl et al., 2013; Akl et al., 2012) found that cotreatment of mammary tumor cells with γ-TT and sesamin synergistically inhibit cell proliferation by arresting the cell cycle progression in the G1/S transition phase and by interfering with ErbB receptor activation and its downstream signaling pathways (MAPK, PI3K/Akt, JAK/STAT and NF-κB).

Epigallocatechin gallate (EGCG) is a plant-derived flavonoid, belonging to the flavanol subclass, mainly found in green tea (Manach et al., 2004). EGCG is well known for its cancer preventing activity through its ability to interfere with the EGFR intracellular signaling pathways (Shimizu et al., 2008), to induce ER stress, and to modulate gene expression, both by means of a direct effect on transcription factors or by indirect epigenetic mechanisms (Naponelli et al., 2017). Hsieh and Mu (Hsieh and Wu, 2008) investigated the effects of a combination treatment with γ-TT and EGCG on breast cancer cell growth. They found that the two natural compounds synergistically decrease cell proliferation by reducing cell cycle- and apoptosis-related proteins. This combination treatment also upregulated the expression of Nrf2 (nuclear factor erythroid 2-related factor 2), a transcription factor that regulates the transcription of different cytoprotective (antioxidant) genes, such as NQ01

(NAD(P)H quinone dehydrogenase 1) (Dinkova-Kostova and Talalay, 2010). Similar results were observed by cotreating cancer cells with γ-TT and resveratrol, a stilbene mainly found in grapes and red wine, that is associated with anticancer activity due to its ability to downregulate the expression of HMG-CoA reductase and to potentiate the antiproliferative effects of statins (Cho et al., 2008; Wong et al., 2011).

Rice bran is a source of different bioactive compounds, such as TTs and ferulic acid. The bioavailability of both TTs and ferulic acid after oral administration was clearly demonstrated (Khan et al., 2010; Khanna et al., 2005; Zhao et al., 2003). It was shown that ferulic acid can potentiate the growth inhibitory effects of δ-TT in a wide range of cancer cells, including prostate, breast and pancreatic cancer cells. This synergistic effect was attributed by an increased intracellular level of the TT due to a suppression of its metabolism induced by ferulic acid (Eitsuka et al., 2014). More recently, Eitsuka and coworkers (Eitsuka et al., 2016a) reported that a combination treatment with δ-TT and ferulic acid synergistically inhibits cellular telomerase activity. In particular, cotreatment with the two compounds downregulated the expression of telomerase reverse transcriptase (TERT), the catalytic subunit of telomerase, demonstrating that the activity of this enzyme is regulated at the transcriptional level.

Additional natural compounds that have been shown to possess a synergistic anticancer activity when coadministered with TTs include: 6-gingerol, inducing colorectal cancer cell apoptosis in combination with γ-TT (Yusof et al., 2015); oridonin, synergistically triggering apoptosis with γTT in mammary cancer cells through induction of autophagy (Tiwari et al., 2015b); geranylgeraniol, exerting a significant antitumor activity on castration-resistant DU145 prostate cancer cells when coadministered with δ-TT, by potentiating the suppression of HMG-CoA reductase induced by the vitamin E derivative (Yeganehjoo et al., 2017). However, while the safety of TTs in humans has been demonstrated, the safety of some of these compounds still has to be defined based on their ability to affect the functions of different tissues/organs. For instance, geranylgeraniol was reported to stimulate testosterone production in testis-derived tumor cells (Ho et al., 2016) and to counteract the antitumor activity of statins (pitavastatin) in drug-resistant ovarian cancer cells (de Wolf et al., 2017).

Taken together, these observations suggest that properly formulated TT-combination treatments, with both standard anticancer or natural compounds, might represent an novel strategy in cancer preventive or therapeutic interventions (i.e., in combination with standard therapies), avoiding development of drug resistance and reducing toxic effects of standard treatments (Table 2).

6 | NOVEL SYNTHETIC DERIVATIVES AND FORMULATIONS OF TOCOTRIENOLS

During the last few years, several semisynthetic derivatives of tocotrienols were prepared, with the aim to improve their water solubility and to reduce their metabolism in cancer cells, thus increasing their biological

activity. As underlined by Behery and coworkers, the structure of tocotrienols can be divided into three domains. Domain I is the phytyl side chain, responsible for the docking of TTs into the lipophilic bilayer of the membranes; domain II is the chroman ring, which is usually not modified in synthetic TT derivatives; and domain III, the phenolic OH group, responsible for the antioxidant activity of TTs (Behery et al., 2013). During the last decade, this third domain has been modified by esterification, etherification or carbamoylation leading to the synthesis of several tocotrienol analogs demonstrated to possess an improved anticancer activity (Behery et al., 2010; Elnagar et al., 2010). In particular, esterification of TTs converts them into redox-silent compounds demonstrated to undergo a slow hydrolytic process (and subsequent release of the native compounds), and characterized by higher chemical stability and bioactivity (Behery et al., 2010; Elnagar et al., 2010; Neuzil et al., 2007). Similar observations were reported by Gagic et al., reporting an improved stability of aminoacid esters of γ-TT in human plasma (Gagic et al., 2016).

As discussed above, TTs have a lower affinity for α-TTP and undergo liver metabolism and biliary excretion, raising the question whether orally administered TTs can reach the different tissues. This low affinity for α-TTP seems to be related to the rigidity of the tail structure (domain I) that is higher in TTs than in TPs (due to the presence of double bonds). Based on this observation, TT derivatives, the tocoflexols, were developed with a more flexible tail that might be responsible for a higher affinity for α-TTP and, consequently, for an improved distribution to the different target tissues. Preliminary data demonstrate that, even after the modification of the side chain, these compounds are able to maintain the antioxidant properties of TTs (Compadre et al., 2014).

By structural modification of the domain II (by electrophilic substitution reactions), several redox-silent TT (particularly γ- and δ-TT) oxazine derivatives could be obtained (Ananthula et al., 2014a; Ananthula et al., 2014b; Behery et al., 2013). These compounds were reported to decrease the growth of breast cancer cells, both *in vitro* and *in vivo* (Behery et al., 2013). In mammary cancer cells, a semisynthetic δ-TT oxazine derivative was shown to counteract the overexpression of HIF-1α consequent to artificially-induced hypoxic conditions; a corresponding decrease of the Akt/mTOR signaling pathway, the major regulator of HIF-1α synthesis, was also observed (Ananthula et al., 2014b). Interestingly, oxazine derivatives of γ- and δ-TT were also prepared as lipid nanoemulsions. After intratumor injection in breast cancer xenografts, these TT formulations were found to significantly reduce tumor growth and this antitumor activity was associated with alterations of the expression of different cell cycle-regulatory proteins (Ananthula et al., 2014a).

To improve the aqueous solubility of TTs, Abu-Fayyad and Nazzal chemically conjugated these compounds with terminally methylated poly ethylene glycols (mPEG) with molecular weights of approximately 350 (mPEG350) and 1000 (mPEG1000), using a succinate molecule as the linker to the 6-OH group on the chroman ring (domain III). Among these ester conjugates, γ-TTPGS1000 and δ-TTPGS1000 were shown to be the most effective in exerting a cytotoxic activity on breast and pancreatic cancer cells, being less toxic on non-tumorigenic cells (Abu-Fayyad and Nazzal, 2017b). On the other hand, the presence of the mPEG molecule reduced the antitumor effects of TTs, possibly due to the conjugation of mPEG to the 6-OH group, known to be crucial for their activity. Based on this observation, these authors developed novel

conjugates in which the mPEG moiety is linked to carbon-5 of the chroman ring via an amide bond or via hydrazone linkage. They reported that the amide derivative exerts a greater cytotoxic activity than the hydrazone conjugate on breast and pancreatic cancer cells. More importantly, the γ-TT amide conjugate was significantly more active than the ester conjugates (Abu-Fayyad and Nazzal, 2017c), supporting the hypothesis that a free OH group is crucial for TTs to exert their antitumor activity. Promising results were also reported in pancreatic cancer cells with a novel gemcitabine-γ-TT conjugate entrapped into nanoemulsions (Abu-Fayyad and Nazzal, 2017a).

To increase the systemic bioavailability of TTs, different formulations were developed. Nano-emulsified TT formulations were found to possess a significant anticancer activity *in vitro* and were proposed as a potential topical application of TTs against skin carcinomas (Pham et al., 2016); these formulations were also reported to exert a radioprotective effect *in vivo* after oral administration in mice exposed to total body gamma radiation (Ledet et al., 2016). A potentiation of the antitumor activity of both TTs and simvaSTATin against mammary cancer cells was observed after concurrent delivery of the two drugs by lipid nanoemulsions (Alayoubi et al., 2013). Enhanced solubility and oral bioavailability of TTs (specifically γ- and δ-TT) were also observed when using a self-emulsifying drug delivery system (Alqahtani et al., 2014).

Recently, tumor-targeted (transferrin-bearing) vesicles entrapping either the TRF extracted from palm oil or the individual TTs were developed. The rationale of this formulation is that transferrin receptors are frequently expressed in cancer cells (Calzolari et al., 2007) and can thus represent an effective target for the delivery of therapeutic drugs into cancer cells. It was reported that transferring-bearing vesicles entrapping α-TT are highly effective in reducing the growth of human epidermoid carcinoma cancer cells and of murine melanoma cells; when intravenously administered in nude mice, these vesicles induced a significant suppression of both tumor xenografts, without signs of toxicity (Karim et al., 2017).

Based on these promising results, the development of novel TT derivatives/formulations will likely improve the biological activity of these compounds further supporting their potential role as novel chemopreventive/treatment strategies against cancer.

7 | CLINICAL STUDIES OF THE ANTICANCER ACTIVITY OF TOCOTRIENOLS

Despite the high number of *in vitro* and *in vivo* (preclinical) studies supporting a significant effect of TTs in counteracting cancer development and progression, the clinical data so far available are still scanty. The first clinical trial was performed by Nesaretnam and coworkers (Nesaretnam et al., 2010) in breast cancer patients. A double-blinded, placebo-controlled pilot trial to test the effectiveness of adjuvant tocotrienol therapy in combination with tamoxifen was conducted for 5 years in women with early breast cancer. Breast cancer patients with either Stage I or II estrogen receptor positive breast cancer were assigned to two groups: placebo plus tamoxifen (control group)

or tocotrienol rich fraction (TRF, 400 mg/day) plus tamoxifen (intervention group), for 5 years. The five-year breast cancer-specific survival was 98.3% in the intervention group and 95% in the control group; moreover, the five-year disease-free survival was 86.7% and 83.3% in the two groups, respectively. The mortality risk was 60% lower in the TRF group *versus* controls; however, this finding was not statistically significant and the authors concluded that no association seems to exist between adjuvant TT therapy and breast cancer-specific survival in women with early breast cancer.

It is generally believed that this unexpected negative result was possibly due to the small sample size of the study.

On the other hand, 25 patients with pancreatic ductal neoplasia were enrolled in a phase I dose escalation study and treated with different doses of δ-TT (from 200 to 3200 mg/day) for two weeks prior to surgery. The treatment was well tolerated at all the doses of δ-TT. At the end of the treatment, apoptosis markers (active caspase-3) was assessed in neoplastic cells. It was found that, in cancer tissues from treated patients, the levels of the active form (i.e., cleaved) of caspase-3 were significantly increased with respect to tumor control tissues. The higher percentage of caspase-3 positive cells was found in tissues of patients treated with dose levels of 200-600 mg of δ-TT. In these patients, δ-TT was well tolerated and reached bioactive levels in blood. Thus, this vitamin E isoform significantly induces apoptosis in pancreatic ductal neoplasia tissues (Springett et al., 2015).

Based on the promising results from *in vitro* and *in vivo* studies, clinical trials investigating the chemopreventive/antitumor efficacy of TTs in cancer patients to further assess the efficacy of these compounds as novel treatment strategies are currently ongoing (see ClinicalTrials.gov).

8 | CONCLUSION AND FUTURE DIRECTION

Vitamin E derived TTs (but not TPs) were reported to be associated with significant health benefits in different chronic diseases, such as neurodegenerative and cardiovascular diseases. TTs (specifically γ- and δ-TT) were also shown to possess antitumor activity by suppressing cancer cell proliferation, and this was initially attributed to their antioxidant and antiinflammatory properties.

On the other hand, *in vitro* and *in vivo* studies clearly pointed out that TTs exert their anticancer (antiproliferative/proapoptotic, antimetastatic and antiangiogenic) activity also by targeting different intracellular pathways, such as: cell cycle- and apoptosis-related proteins, growth factor receptors signaling cascades, the ER stress-autophagy pathway, EMT transition, VEGF secretion

from tumor cells and activity on endothelial cells, etc. Interestingly, TTs were also reported to reduce cancer cell growth by interfering with the post-translational modification and metabolic degradation of HMG-CoA reductase, the key enzyme involved in cholesterol synthesis. The intermediates of this pathway are responsible for the post-translational modifications (i.e., activation) of proteins, such as Ras and αβγ-G protein subunits, thus allowing them to anchor to the membrane and to trigger the intracellular mechanisms leading to cell proliferation. Downregulation of HMG-CoA reductase hamper these pathways, leading to cell death.

On the other hand, despite these consistent observations, the precise origin of the antitumor activity of TTs is still unclear. In addition to their ability to directly bind to and inactivate specific molecular targets (such as HMG-CoA reductase and Src), it has been suggested that these compounds, based on their hydrophobic structure, might accumulate within membrane lipid rafts microdomains, leading to the disruption of their integrity, thus interfering with growth factor HER2 receptor dimerization, phosphorylation and downstream signaling.

In addition to their anticancer effects, TTs were widely reported to exert a synergistic/additive activity with both standard anticancer drugs and natural compounds with antitumor activity, *in vitro* and *in vivo*. For instance, TTs were shown to potentiate the anticancer activity of statins in different cancer cell lines.

Taken together, these promising results recently led to the design and synthesis of different novel TT derivatives/formulations with the aim to improve the solubility and bioavailability, and therefore the biological activity, of these compounds.

In conlusion, results from several *in vitro* and *in vivo* studies strongly support the notion that vitamin E derived TTs (but not TPs) exert a significant anticancer activity in a wide range of human cancer cell lines. However, the clinical observations so far available are still scanty; thus, clinical trials investigating the potential effectiveness of these compounds as novel chemopreventive/treatment strategies (i.e., in combination with standard therapies) in tumors are urgently needed.

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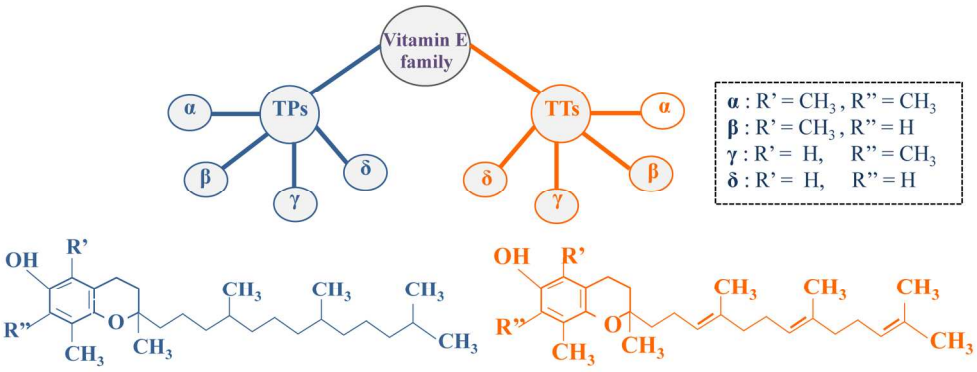

FIGURE 1 Chemical structure of the vitamin E derived tocopherols (TPs) and tocotrienols (TTs).

163x64mm (300 x 300 DPI)

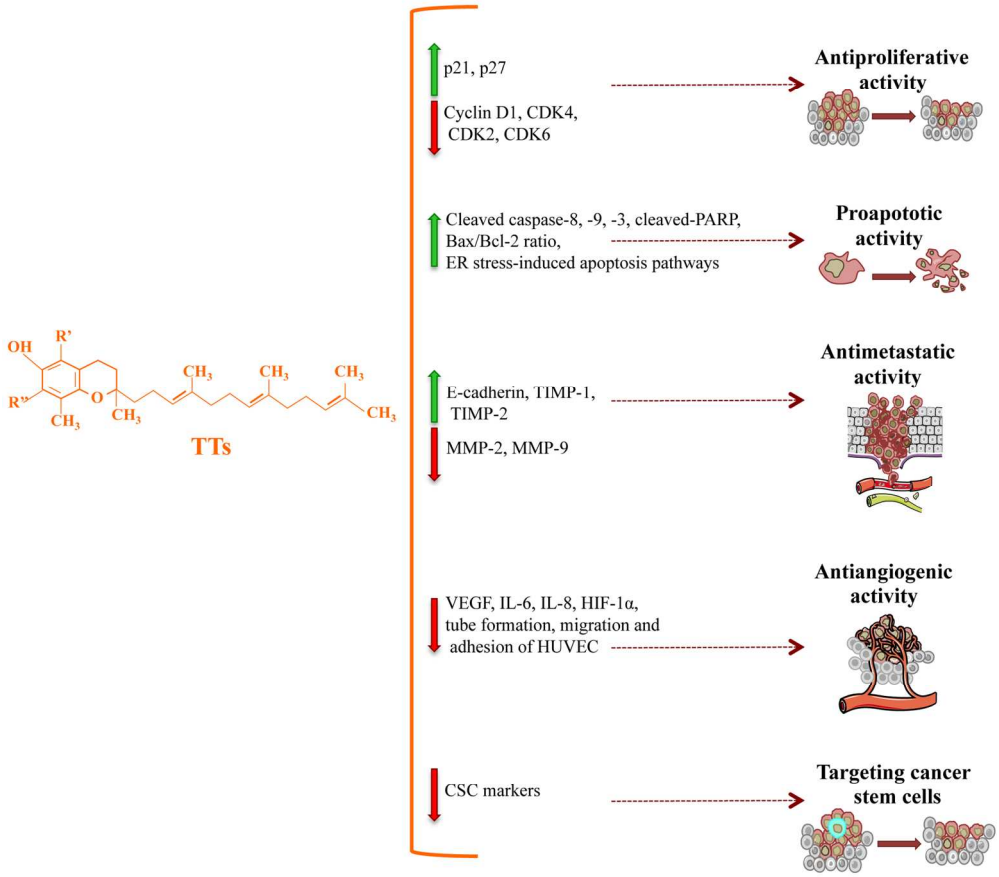

FIGURE 2 Molecular mechanisms of the antitumor activity of TTs. In a wide range of cancer cells, TTs were reported to: counteract cell proliferation while triggering apoptosis, exert an antimetastatic and antiangiogenic activity, specifically target the aggressive cancer stem cell subpopulation. Bax: bcl-2-like protein, Bcl-2: B cell lymphoma-2, CDK: cyclin dependent kinase, CSC: cancer stem cells, ER: endoplasmic reticulum, HIF-1α: hypoxia inducible factor-1α, IL: interleukin, MMP: matrix metalloproteinase, PARP: poly (ADP-ribose) polymerase, TIMP: tissue inhibitor of metalloproteinase, VEGF: vascular-endothelial growth factor.

160x142mm (300 x 300 DPI)

TABLE 1 Presence of tocotrienols in different plant sources

Source	TRF	α-TT	γ-TT	δ-TT
Palm oil	738 mg/l	28%	59%	13%
Annatto seeds	160 mg/100 gr	-	10%	90%
Rice bran	585 mg/l	41%	59%	-
Wheat germ	26 mg/l	100%	-	-

TRF: tocotrienol rich fraction; TT: tocotrienol.

Adapted from Ahsan et al., 2015.

TABLE 2 Effects of tocotrienol combination treatments on different types of cancer cells

	Compound	Tumor cell types	Effects	References
Anticancer drugs				
	Cisplatin	Non-small lung cancer cells	↓ Cell viability, migration and invasiveness	Ji et al.(2012)
	Capecitabine	Gastric cancer cells, colorectal cancer cells	↓ Cell proliferation, cell cycle-related proteins, NFκB, VEGF and MMP-9 expression	Manu et al. (2012), and Prasad et al. (2016)
	Erlotinib, Gefitinib	Malignant mammary epithelial cells	↓ ErbB2-4 expression, Akt and STAT pathways. Overcoming resistance to TKIs, apoptosis induction	Bachawal et al. (2010)
	Celecoxib	Malignant mammary epithelial cells	↓ Tumor cell growth, Akt/NFκB pathway, COX-2 ErbB2-4 and PGE2 expression	Shirode et al. (2011)
Natural compounds				
	Sesamin	Mammary tumor cells	↓ Cell proliferation, cell cycle progression, ErbB receptor activation, MAPK, PI3K/Akt, JAK/STAT, NFκB pathways. ↑ TT bioavailability	Sontag et al. (2002), and Akl et al. (2012, 2013)

EGCG	Breast cancer cells	↓Cell proliferation. ↑Nrf2, NQO1 expression (antioxidant activity)	Hsieh et al. (2008), and Dinkova-Kostova et al. (2010)
Resveratrol	Breast cancer cells	↓Cell proliferation, HMG-CoA reductase expression	Cho et al. (2008), and Wong et al. (2011)
Ferulic acid	Prostate cancer cells, breast cancer cells, pancreatic cancer cells	↓Telomerase activity, TERT expression. ↑TT intracellular levels	Eitsuka et al. (2014, 2016)
6-gingerol	Colorectal cancer cells	↑Apoptosis	Yusof et al. (2015)
Oridonin	Mammary cancer cells	↑Autophagy	Tiwari et al. (2015)

COX-2 = cyclooxygenase 2, HMG-CoA = 5-hydroxy-3-methylglutaryl-coenzyme A, JNK = c-Jun N-terminal kinase, MAPK = mitogen activated protein kinase, MMP-9 = matrix metalloproteinase-9, NF-κB = nuclear factor-kappa B, NQO1 = NAD(P)H quinone dehydrogenase 1, Nrf2 = nuclear factor E2-related factor 2, PGE2 = prostaglandin 2, PI3K = phosphoinositide-3 kinase, STAT = signal transducer and activator of transcription, TERT = telomerase reverse transcriptase, TKIs = tyrosine kinase inhibitors.