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RESEARCH PAPER

Why tocotrienols work better: insights into the in vitro anti-cancer mechanism of vitamin E

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Abstract The selective constraint of liver uptake and the sustained metabolism of tocotrienols (T3) demonstrate the need for a prompt detoxification of this class of lipophilic vitamers, and thus the potential for cytotoxic effects in hepatic and extra-hepatic tissues. Hypomethylated (γ and δ) forms of T3 show the highest in vitro and in vivo metabolism and are also the most potent natural xenobiotics of the entire vitamin E family of compounds. These stimulate a stress response with the induction of detoxification and antioxidant genes. Depending on the intensity of this response, these genes may confer cell protection or alternatively they stimulate a senescence-like phenotype with cell cycle inhibition or even mitochondrial toxicity and apoptosis. In cancer cells, the uptake rate and thus the cell content of these vitamers is again higher for the hypomethylated forms, and it is the critical factor that drives the dichotomy between protection and toxicity responses to different T3 forms and doses. These aspects suggest the potential for marked biological activity of hypomethylated "highly metabolized" T3 that may result in cytoprotection and cancer prevention or even chemotherapeutic effects. Cytotoxicity and metabolism of hypomethylated T3 have been extensively investigated in vitro using different cell model systems that will be discussed in this review paper as regard molecular mechanisms and possible relevance in cancer therapy.

Keywords Tocotrienols · Vitamin E · Breast cancer · Antioxidants · Apoptosis · Cell signaling · Inflammation · Metabolism · Gene expression · Cell redox

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T3 structure–function and specificity of action

In the vitamin E family of molecules, tocotrienols (T3) are often considered of minor importance since these are less abundant than α and γ tocopherol (TOH) in the circulation and in solid tissues. This has contributed to hinder our knowledge on the biological functions of this group of vitamers that is now regaining great interest, thanks to a number of recent studies that suggested health-promoting functions related with the cholesterol-lowering, cytoprotective, and anticarcinogenic effects of T3 [reviewed in (Aggarwal et al. (2010)].

Structure–function studies demonstrate that many of these functions are more potent when the unsaturated (isoprenyl) side chain of T3 is combined with a hypomethylated (HM) chroman ring. The unsaturated chain characteristic of all the T3 forms confers well-defined physical and chemical characteristics that appear to include vitamer-specific interactions with other lipids and cell proteins (Atkinson

et al. 2008). The HM ring, other than bringing the hydroxyl group in position 6, i.e., the antioxidant moiety common to all the tocol derivatives, is responsible of signaling effects that differentiate these vitamers from the other vitamin E forms (Galli and Azzi 2010). The former and more characterized consequence of the combination of the hypomethylated chroman ring with the isoprenyl moiety is that of producing a well-defined response of bioavailability and transformation. For the vitamin E molecules introduced with the diet, these responses depend on specific pathways that are under the influence of the hepatic α-tocopherol transfer protein (α-TTP). As prototype vitamin E binding protein that regulates uptake and trafficking of all the vitamers in liver cells (Morley et al. 2008), this protein binds the fully methylated and saturated form α-TOH with at least 10-fold higher affinity than HM and all the T3 forms (Hosomi et al. 1997).

Specificity of action and health-promoting properties of T3, however, involve other and so far poorly understood players (Galli and Azzi 2010). These may include α-TTP-like and/or low affinity binding proteins and other protein-lipid and lipid-lipid interactions in cell membranes and in the cytosol (Atkinson et al. 2008), as well as intracellular receptors such as ERβ (Comitato et al. 2009), and orphan receptors such as SXR (Zhou et al. 2004) and PXR (Brigelius–Flohe 2005). As a consequence of a specific structure–function and in analogy with the behavior of α-TOH, T3 may co-localize and interact with PUFA and cholesterol molecules in the membrane to influence the composition and signaling function of membrane microdomains such as the "membrane rafts" (Atkinson et al. 2008, 2010), which are increasing their recognition as regions of the plasmalemma that may control cell signaling and redox responses through key elements such as the enzyme NADPH-oxidase and PKC (Galli 2007a, b). Specificity of T3 signaling includes the control of this latter protein kinase (Eitsuka et al. 2006) as well as of other kinases such as PI3K/Akt (Shah and Sylvester 2004; Shibata et al. 2008; Uto–Kondo et al. 2009) and ERK–MAPK (Sun et al. 2008) and cyclin-dependent kinases (Elangovan et al. 2008). Other discrete signals could be generated through the regulation of enzymes such as HMG–CoA reductase, the key step in cholesterol biosynthesis with a proposed role in inflammatory, endothelial, and cancer cell signaling (Theriault et al. 2002) that appears to be regulated by T3 at the post-translational level (Parker et al. 1993; Song and DeBose–Boyd 2006). Telomerase activity, a downstream component of the PKC signaling, may play a role in the T3-dependent control of cell proliferation and lifespan (Eitsuka et al. 2006).

Signaling routes and genes with marked responsiveness to T3, and particularly to HM forms of T3, also include drug metabolizing and stress-related genes (Traber 2007). Likewise to several other plant phenolics, T3 may trigger the expression of these genes behaving as cellular stressors and this property could be further enhanced by the lipophilicity of T3 molecules [reviewed in (Galli 2007a, b)]. Indeed, transcripts of T3 responsive genes include detoxification and antioxidant elements such as quinone reductase and heme-oxygenase (Das et al. 2008; Hsieh and Wu 2008), and glutathione (GSH)-related enzymes (Hsieh et al. 2010a), which are stimulated not only by other natural phenolics such as the isoflavon daidzein and its metabolite equol (Mann et al. 2009; Froyen and Steinberg 2010), but also by hormonal substances such as melatonin (Luchetti et al. 2010) and estrogens (Froyen and Steinberg 2010). Actually, these influence the expression of antioxidant responsive element (ARE)-dependent genes by means of transcription factors such as Nfr2 and NFκB, and receptors such as ERβ, to ultimately confer increased protection against a wide number of xenobiotics such as drugs, UV irradiation, and endobiotics which include endogenous ROS.

Metabolism and transformation of T3: detoxification or bioactivation processes?

DME induction: the detoxification response to T3

Metabolism and biological activities of T3 suggest that this group of vitamers is not likely to provide classical vitamin E activity as α-TOH does, but rather these appear to play other and distinct physiological functions that may extend to pharmacological effects of possible relevance in cancer therapy.

T3 represent the natural form of vitamin E with highest induction response of drug metabolizing enzyme (DME) genes that include the phase I PXR-dependent Cyt P-450 isoenzyme CYP3A4, the form with broader drug metabolizing activity in liver cells (Brigelius–Flohe 2005), and CYP4F2 that is the main isoenzyme so far demonstrated to catalyze the first step of vitamin E metabolism, i.e., the ϖ-oxidation of the side chain (Sontag and Parker 2007). The in vivo and in vitro supplementation of T3 also stimulates the expression of phase II detoxification enzymes such as sulfo and glucuronyl transferases (Freiser and Jiang 2009). γ-T3-induced inhibition of cell cycle regulatory proteins in estrogen receptor-positive MCF-7 human breast cancer cells is associated with redox changes and with the induction of the phase II detoxification enzyme quinone reductase NQO2, which is controlled through the activation of the redox-sensitive transcription complex Nrf2-Keap1 (Hsieh et al. 2010b). The transcription of other genes of relevance for the anti-inflammatory and anti-cancer activity of T3 can be regulated by means of the redox-sensitive response of

NFkB (Ahn et al. 2007). Other phase II and phase III genes involved in the coordinated response to α-TOH intake and metabolism are expected to be influenced by the highly metabolized HM-T3 such as glutathione-S-transferase, and biliary transporters that include for instance p-glycoprotein (Belli et al. 2009) and other functional homologs identified in rats fed with diets high in α-TOH (Mustacich et al. 2009).

At the same time, DME genes can be influenced by T3 forms through direct effects. GST P1-1 that has been reported to be inhibited with an allosteric mechanism by physiological concentrations of α-TOC and γ-T3 (van Haaften et al. 2002). DME induction may suggest a risk of interaction with the metabolism and therapeutic efficacy of drugs that could be particularly sustained when high doses of T3 are used as supplements to the diet (Brigelius–Flohe 2005). Phase II gene induction is a key event in drug resistance and may thus represent a problem in the application of T3 in cancer therapy.

Therefore, the effect on DME response can be considered the cipher of T3 cytotoxicity and also a putative mechanism for the anti-cancer and cell protection effects of these vitamers. Similarly to drugs, other natural bioactive compounds and endogenous factors with hormetic properties (Ristow et al. 2009), T3 show biphasic concentration-dependent effects that suggest overlapping or functional continuity between toxicity and therapeutic mechanisms. In vitro studies carried out in different cell model systems have contributed to elucidate this relationship between cytotoxicity mechanisms and possible therapeutic effects of T3. Studies on primary cultures of cerebellar neurons and astrocytes exposed to high concentrations of γ-T3 (100 μM or higher) have shown marked effects of toxicity that were absent in the case of α-TOH (Mazlan et al. 2006; Then et al. 2009). This toxicity was associated with the activation of stress-dependent p38 MAPK and p53 (Then et al. 2009). On the other hand, at lower concentrations γ-T3 was more potent than α-TOH in preventing H_2O_2 toxicity and apoptosis through the induction of the Bcl-2/Bax mitochondrial pathway.

Metabolite formation and activity

The induction of hepatic metabolism by T3 is responsible of the abundant in vivo transformation to metabolites with different chain length (Galli et al. 2007a, b). Tocotrienol and tocopherol metabolism involves essentially the same mechanism that consists in an initial CYP450-dependent ω-hydroxylation followed by β-oxidation and resulting in the rapid formation of carboxyhethyl hydroxychroman acid (CEHC). This is the main in vivo metabolite of vitamin E found in plasma and urine. As a consequence this is considered the most useful indicator of vitamin E metabolism in human and animal organisms (Galli et al. 2002, 2003). Side chain degradation starts with hydroxylation of the ω-methyl group which is followed by five cycles of β-oxidation. All intermediates have been identified by MS analysis; (Birringer 2010; Swanson et al. 1999; Schuelke et al. 2000; Birringer et al. 2002; Sontag and Parker 2002). Data from in vitro experiments performed with HepG2 cells have shown that in the case of γ-tocotrienol all possible carboxylic acid intermediates of degradation, including γ-carboxydimethyldecadienyl hydroxychroman (γ-CDMD(en)$_2$HC), γ-carboxydimethyl-decyl hydroxychroman (γ-CDMDHC), γ-carboxydimethyloctenyl hydroxychroman (γ-CDMOenHC), γ-carboxymethylhexenyl hydroxychroman (γ-CMHenHC), γ-carboxymethylbutyl hydroxychroman (CMBHC), and γ-CEHC are released, whereas for α-tocotrienol only the precursors, α-CEHC, α-CMBHC, α-CMHenHC, and α-CDMOenHC, but not α-CDMD(en)$_2$HC are formed (Birringer et al. 2002). The failure to detect CMBHC metabolites with the original double bond in the side chain from T3 metabolism α- and γ-CMHenHC, reveals that auxiliary enzymes can be involved, with a more complex mechanism compared to that of tocopherols suggesting that T3 side chain degradation follows the pathway which is used for branched-chain fatty acids (Birringer et al. 2002).

T3 responsive genes involved in vitamin E metabolism are also expressed in non-hepatic cancer cells since these have been demonstrated to produce these metabolites (Yang et al. 2010; Conte et al. 2004; You et al. 2005).

Vitamin E metabolites have been preliminary suggested to possess biological functions relevant to the anti-cancer activity of T3 and of the entire class of vitamin E compounds. Long chain metabolites of T3 are formed in A549 type II alveolar epithelial cells, but not in HepG2 hepatocytes, thus suggesting the existence of cancer-specific differences in T3 metabolism (You et al. 2005). A 9′-carboxychromanol was identified as main metabolite in this lung carcinoma cell line, whereas 3′- and 5′-carboxychromanols predominated in HepG2 cells, and this difference was proposed to depend on a cell-specific inefficient conversion to 7'-carboxychromanols in the peroxisomal compartment of A549 cells.

More recent work by Freiser et al. (Freiser and Jiang 2009) demonstrated the accumulation of sulfated derivatives of long-chain intermediates both in A549 type II alveolar epithelial cells and in vivo in rats supplemented with γ-T3 or γ-TOH. Sulfated 9′-, 11′-, and 13′-carboxychromanol and their unconjugated counterparts were identified after in vitro supplementation in A549 cells and derivatization to sulfated forms was two-time faster in the case of γ-T3 than γ-TOH. The rapid (within 6 h from the supplementation) in vivo metabolization of these vitamers was consistent with the appearance in plasma of

13′-carboxychromanol and sulfated 9′-, 11′-, 13′-carboxychromanol, with the sulfated 11′-carboxychromanol as the most abundant long-chain metabolite in γ-T3 supplemented rats.

The finding that CEHCs possess discrete biological activities has suggested the existence of a relationship between metabolism and bioactivity of vitamin E forms [reviewed in (Brigelius–Flohe 2006; Galli et al. 2007a, b)]. CEHC metabolite bioactivity has so far been reported to include direct antioxidant and anti-inflammatory effects, and mild natriuretic function and even anti-proliferative activity have been described for γ-CEHC. More recent work has suggested a role of CEHCs also in the control of PKC activity of neutrophils (Varga et al. 2008).

These appear to be the direct effects of the chroman structure of CEHC that, however, have not been reported to bind nuclear receptors, such as PXR and PPAR, nor vitamin E binding proteins [reviewed in (Traber 2004; Brigelius–Flohe 2005)], which may give limited relevance to short chain metabolites in the control of vitamin E sensitive genes.

The existence of bioactive derivatives of vitamin E has been confirmed in recent studies that have examined also long chain metabolites. These metabolites are rapidly processed by the metabolic machinery of liver cells to form the end product CEHC, and thus their levels in the circulation are extremely low, but their potency as in vitro anti-inflammatory and pro-apoptotic agents was proposed to be much stronger than that of CEHCs. Actually, long-chain metabolites, and particularly 13′-carboxychromanol, have been recently identified to possess inhibitory effects on cyclooxygenase activity (Jiang et al. 2008), which may be consistent with the proposed anti-inflammatory activity of T3 (Elangovan et al. 2008; Yam et al. 2009), and to promote oxidative stress and pro-apoptotic effects in HepG2 cells (Birringer et al. 2010).

Altogether, these pieces of evidence have suggested that metabolites may sustain and even expand the function of vitamin E, also providing further insights into molecular mechanisms and possible therapeutic applications of vitamin E in cancer therapy.

In vitro bioactivity as a function of uptake rate and cell content of T3 forms

Cell uptake rate and metabolic processing influence the in vitro bioactivity of different vitamin E forms. This is true for either cytoprotection or anti-cancer activity of T3 which show concentration-dependent functional regulation of different signaling routes and groups of genes (Nesaretnam et al. 2004; Aisa et al. 2006; Yap et al. 2008) (Fig. 1).

It has been recently observed that the difference between the in vitro cytoprotection function of α-T3 and α-TOH in glutamate toxicity originally shown in neuronal cells by Sen et al. (Sen et al. 2000, 2004) may depend on the faster cell uptake rate of α-T3 rather than on vitamer specific effects on cell signaling (Saito et al. 2010). Indeed, when T3 and TOH forms reached the same cell concentrations the same prevention activity was observed, suggesting higher potency of α-TOH in the prevention of glutamate cytotoxicity in vivo (Saito et al. 2010).

The same was demonstrated by our group comparing the anti-proliferative activity of TOH forms on C6 glioblastoma cells (Betti et al. 2006). The HM form γ-TOH shows the higher uptake rate, and thus the higher anti-proliferative activity, but once the same cell content is reached, all the forms show the same activity.

The higher potency of T3 as compared to TOH forms as inhibitors of cell growth in preneoplastic and even more in neoplastic mouse mammary epithelial cells (McIntyre et al. 2000), has been attributed to a preferential uptake of T3, which rapidly provides cell levels critical for the generation of a stress response and cytotoxic effects (Then et al. 2009). Recent data obtained in TUBO murine breast cancer cells, showed that the anti-cancer effect of δ-T3 follows the same cell uptake-dependent mechanism [(Viola et al. 2010), manuscript in preparation]. As preliminarily described in (Pierpaoli et al. 2010; Viola et al. 2010) and similarly to SKBr3 human breast carcinoma cells, TUBO cells showed higher cell viability inhibition by δ-T3 than α-tocopheryl-succinate (α-TOS), i.e., the synthetic and redox-silent derivative of vitamin E with one of the highest in vitro and in vivo anti-cancer efficacy so far reported in literature [reviewed in (Zhao et al. 2009)] (Table 1). The extent of uptake and the effect on cell viability of these vitamers changed in a concentration and time-dependent fashion. The δ-T3 was more potent inhibitor of cell viability than α-TOS after 18–24 h of incubation being more rapidly taken up by these breast cells. This difference in the anti-cancer activity of these two VE forms was lower after 48 h of treatment, when the cell content of α-TOS increased to levels comparable to δ-T3 [(Viola et al. 2010), manuscript in preparation].

The differential cell uptake of vitamin E forms could involve specific transporters such as the organic anion transport system (Negis et al. 2007), but also direct membrane transfer and intramembrane diffusion and interaction mechanisms that appear to favor the uptake and bioactivity of HM T3 forms (Palozza et al. 2006). The rapid and massive uptake of δ-T3 and in general of HM forms in cancer cells may influence the lipid structure and key functional domains of the plasmalemma (Atkinson et al. 2010).

These aspects suggest that selective uptake and membrane dislocation of T3 forms may contribute to the specificity of action of HM T3 in cancer tissues also suggesting

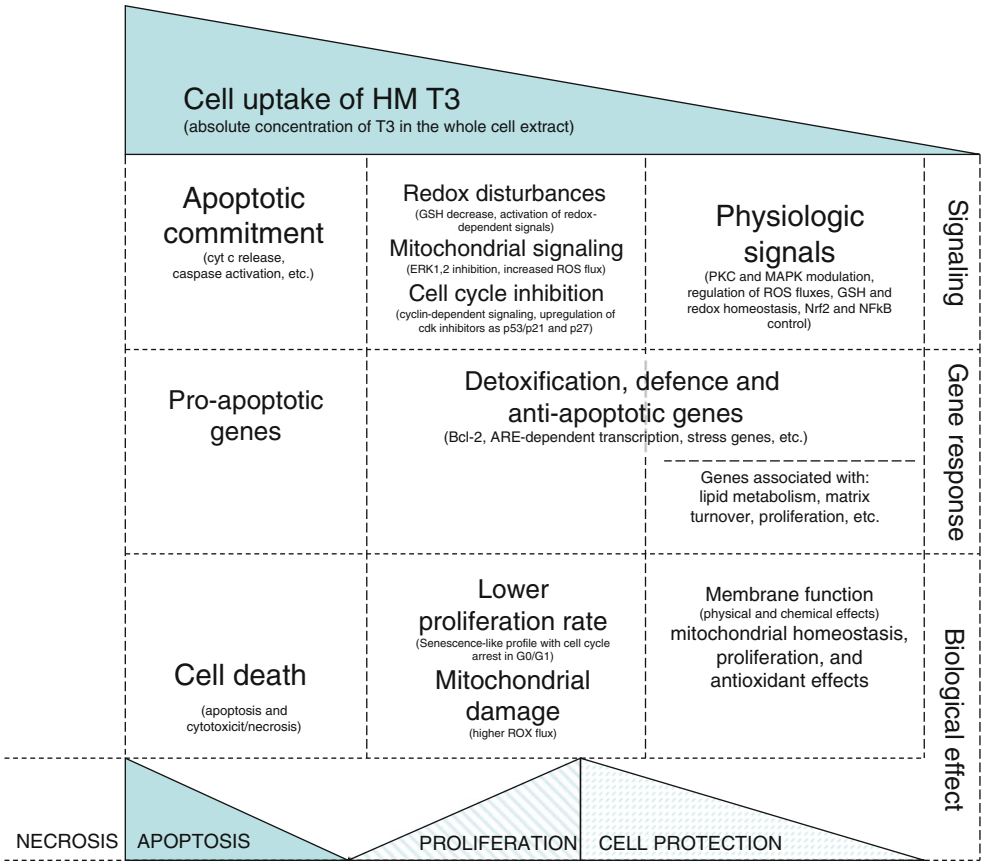

Fig. 1 Schematic representation of the proposed relationship between uptake (cell content) and biological responsiveness to T3 treatment in vitro. T3 recruit different responses in cancer and non-cancer cells that range from cytoprotection to anti-cancer pro-apoptotic effects. The cell content of T3 appears to drive these responses through the modulation of different signaling pathways and groups of genes that link in a functional continuity the concentration-dependent and time-dependent responses to T3. Further details are reported in the text

a strategy to overcome limited efficacy of therapeutic protocols based on oral administration of these vitamers. Bypassing the liver metabolism with local and tumor-targeted administration procedures may enhance the anti-cancer potency and specificity of action of T3, thus providing higher pharmacokinetics and therapeutic efficacy.

The anti-cancer mechanism of T3 forms: more toxic, more effective

In vivo and in vitro anti-cancer mechanisms of T3 so far described in literature include anti-proliferative effects by the inhibition of cell cycle, induction of apoptosis, antioxidant and anti-inflammatory effects, inhibition of angiogenesis, and suppression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity [reviewed in (Wada 2009)].

This literature is consistent with the evidence that hypomethylated configurations of T3 (HM-T3, i.e., delta and gamma-T3), i.e., the forms with highest metabolism and toxicity effects, represent the most potent in vitro anti-cancer forms of vitamin E [reviewed in (Constantinou et al. 2008)] (Tables 2, 3). When investigated in cell model systems of breast and prostate cancer, two hormone-dependent cancers with expected susceptibility to vitamin E interventions, HM-T3 show much stronger anti-proliferative and pro-apoptotic activity than TOH and α-T3. δ-T3 is also the most potent form tested in pancreatic adenocarcinoma cells (Hussein and Mo 2009) while γ-T3 was recently described to be the vitamer with anti-cancer activity in colon cancer cells (Kannappan, Ravindran et al.; Xu et al. 2009), which is another cancer form tentatively proposed as preventable by vitamin E therapy (Stone et al. 2004), and in melanoma cells (Chang et al. 2009).

In both estrogen sensitive (MCF-7 or ZR-75-1) and insensitive cells (MDA-MB 435 or 231), T3 and particularly γ-T3 and δ-T3, were demonstrated to inhibit cell proliferation (Nesaretnam et al. 1998) with an estrogen-independent effect and with synergistic activity with the anti-estrogen drug tamoxifen (Guthrie et al. 1997; Nesaretnam et al. 2000). More recent work has demonstrated that cell cycle

Table 1 Effect of δ-T3 and α-TOS treatments on cell viability of TUBO and SKBr3 mammary carcinoma cells

VE form	Breast cancer cells	IC50 [μM] (time of treatments)
δ-T3	TUBO[b]	>100 (6 h)
		35 (18 h)
		30 (24 h)
	SKBr3[c]	45.6 (24 h)[a]
		31.5 (48 h)[a]
α-TOS	TUBO[b]	>100 (6 h)
		55 (18 h)
		55 (24 h)
	SKBr3[c]	>100 (24 h)[a]
		68.4 (48 h)[a]

The cells were treated for the times shown in parentheses with increasing concentrations of the test compounds between 0.5 and 100 mM

IC50 data obtained from TUBO cell experiments have been presented in (Viola et al. 2010) (manuscript in preparation). In these cells, viability was assessed by MTT test as shown in (Mazzini et al. 2010)

[a] (Pierpaoli et al. 2010)

[b] *TUBO* mammary lobular carcinoma cells derived from BALB-neu T mice

[c] *SKBr3* Her-2/neu positive human mammary carcinoma cells (HTB-30; American Type Culture Collection (ATCC), Manassas, VA)

inhibition by T3 occurs through the control of the Rb/cdk4/cyclin D pathway (Elangovan et al. 2008) that is also affected by the HM forms of tocopherols, and particularly by γ-TOH (Betti et al. 2006). In the same cell models, T3 were demonstrated to induce apoptosis with δ-T3 as the most potent form (Yu et al. 1999) and with a mechanism that involves mitochondrial disruption and cyt c release (Takahashi and Loo 2004). As introduced above, recent studies by our group confirmed that δ-T3 is the most potent T3 form with pro-apoptotic activity higher than α-TOS in breast cancer cells (Table 1) (Pierpaoli et al. 2010). As described in the previous section, the activity of δ-T3 observed in this in vitro experimental model system, can be partially explained by a high uptake rate that rapidly brings the cell content of this vitamer to the levels required to produce its anti-cancer activity.

However, experimental evidence clearly suggests the existence of a marked cell and disease-related specificity for T3 effects that suggests other and more complex mechanisms than a simple increase in the uptake rate, and this appear to be true also for the other forms of vitamin E [reviewed in (Galli and Azzi 2010)]. Intrinsic characteristics of cancer cells may sustain the therapeutic potential and specificity of action of T3. Recent work has demonstrated for instance that the α-TTP like protein TAP/Sec14L2 normally expressed in non-neoplastic tissues as breast, prostate, and liver, is lost or markedly decreased as expression after cancerogenic differentiation (Wang et al. 2009). This protein may thus represent a tumor suppressor with a physiological role in the anti-proliferative activity of vitamin E molecules, which after down-regulation may produce an abnormal cell trafficking and metabolism of T3 in cancer cells, ultimately increasing T3 toxicity and leading to anti-proliferative and cell death effects. Interestingly enough, this downregulation effect is observed in tissues that develop hormone-sensitive cancers responsive to T3 treatments such as prostate and breast. As a further example, malignant proliferation is associated with an increased HMG CoA reductase activity (Mo and Elson 2004) and T3 suppress the activity of this enzyme by either inhibition of reductase synthesis and accelerated degradation (Song and DeBose–Boyd 2006). Synergistic effects between statins and δ-T3 or γ-T3 have been demonstrated in in vitro anti-proliferative activity tests carried out in diverse tumor cells (McAnally et al. 2007; Hussein and Mo 2009; Wali et al. 2009a, b).

The specificity of action of T3 in cancer cells may also involve other players. HM T3 forms are reported to produce the highest activation response of the caspase-dependent mitochondrial pathway of apoptosis (Pierpaoli et al. 2010; Sylvester 2007; Constantinou et al. 2009). In breast cancer cells, γ-T3-induced apoptosis has also been reported to be sustained by the endoplasmic reticulum stress signaling and caspase 12 activation (Wali et al. 2009a, b). These vitamers, however, are expected to activate also the caspase-independent pathway (Constantinou et al. 2009), and we recently demonstrated a differential effect of δ-T3 with respect to α-TOS and other vitamin E forms on the induction of senescence-like cell genes which further discloses the relationship between cell stress and anti-proliferative mechanism of T3 in breast cancer cells (Pierpaoli et al. 2010). These genes include cell cycle checkpoints such as the cyclin dependent kinase (cdk) inhibitor p21 that regulates the activity of cyclin E, and the oncogene p53. The same finding was obtained in estrogen receptor-positive MCF-7 human breast cancer cells by Heisen et al. (Hsieh et al. 2010b). Growth suppression of these cells by γ-T3 was accompanied by a time-dependent and dose-dependent modulation of cell cycle regulatory proteins such as the Rb/E2F complex, cyclin D1/cdk4, and cyclin B1/cdk1, which ultimately control Rb phosphorylation. Concomitantly, the Nrf2-dependent phase II enzyme quinone reductases 2 was induced thus confirming the hypothesis of a functional link between the T3 dependent control of drug metabolism and anti-cancer pathways (see above).

A lower cancer incidence by the induction of the p21 signaling pathway has been suggested to be the underlying mechanism of the 15% increased lifespan of mice supplemented lifelong with α-TOH (Banks et al. 2010).

Table 2 Effect of T3 molecules on cell viability of different breast cancer cell lines

T3 form	Breast cancer cells	IC50 [μM] (time of treatments)	References
δ-T3	MDA-MB-231[a]	22.6[h] (48 h)	(Guthrie et al. 1997)
	MCF7[b]	5.5[h] (48 h)	
	CL-S1[d]	7[h] (5 days)	(McIntyre et al. 2000)
	−SA[e]	4[h] (5 days)	
	+SA[f]	3[h] (5 days)	
	MDA-MB-231	10[h] (48 h)	(Elangovan et al. 2008)
	MDA-MB-231	20[h] (72 h)	
	SKBr3[g]	45.6[h] (24 h)	(Pierpaoli et al. 2010)
	SKBr3[g]	31.5[h] (48 h)	
γ-T3	MDA-MB-231[a]	73[h] (48 h)	(Guthrie et al. 1997)
	MCF7[b]	4.87[h] (48 h)	
	CL-S1[d]	8[h] (5 days)	(McIntyre et al. 2000)
	−SA[e]	5[h] (5 days)	
	+SA[f]	4[h] (5 days)	
	SKBr3[g]	55.2[h] (24 h)	(Pierpaoli et al. 2010)
	SKBr3[g]	34[h] (48 h)	
α-T3	MDA-MB-231[a]	>100[h] (24 h)	(Guthrie et al. 1997)
	MCF7[b]	14.2[a] (48 h)	
TRF	4T1[c]	8 ug/ml[h] (72 h)	(Hafid et al. 2009)

TRF standardized tocotrienol-rich fraction (TRF)

[a] *MDA-MB-231* Human epithelial breast cancer cell line, isolated by pleural effusion from mammary gland, low erbB-2 receptor-expressing (HTB-30; American type culture collection (ATCC), Manassas, VA)

[b] *MCF7* first cell line derived from a pleural effusion of a post menopausal 69 years patient with metastatic breast cancer. This is the most widely used and best characterized of all human breast cancer cell lines

[c] *4T1* mouse mammary tumor cell line; this is one of few breast cancer cells capable to metastasize efficiently to sites affected in human breast cancer

[d] *CL-S1* Preneoplastic non-malignant mouse mammary epithelial cells

[e] *−SA* murine mammary adenocarcinoma (HTB-30; American rype culture collection (ATCC), Manassas, VA)

[f] *+SA* highly malignant murine cells isolated from animals that spontaneously develop breast cancer (HTB-30; American Type Culture Collection (ATCC), Manassas, VA)

[g] *SKBr3* Her-2/neu positive human mammary carcinoma cell (HTB-30; American type culture collection (ATCC), Manassas, VA)

[h] Inhibition of viability assessed by MTT test or equivalent test

Hypomethylated forms, and particularly the gamma form of TOH, have been observed to activate cell cycle regulators such as p27/Rb both in vitro (Betti et al. 2006) and in vivo (Lee et al. 2009). Therefore, p21 and other genes directly or indirectly involved in cell cycle control, such as p27 and p53, may represent the real effectors in the anti-cancer signaling of T3, influencing the balance between signals that drive the cell into senescence pathways and cell cycle arrest or alternatively toward apoptotic cell death. Upstream elements in this signaling include PKC and the survival pathway ERK–MAPK that is known to influence the mitochondrial signaling in response to foreign stimuli and endobiotics such as hormones and redox-active metabolites [recently reviewed in (Luchetti et al. 2010)]. PKC was the former component in the cell signaling that was identified to be controlled by vitamin E (Galli and Azzi 2010) and is the earliest kinase that responds in a concentration and time-dependent manner to vitamin E leading to signal transduction from the plasmalemma to downstream elements. Membrane translocation and activity of PKC are directly influenced by chromanols (Varga et al. 2008), which may provide a mechanism to explain the close correlation between kinetics profiles of anti-proliferative signaling and uptake rate (i.e., relative concentration within the lipid bilayer) of vitamin E molecules (Betti et al. 2006).

Further anti-cancer mechanisms of T3 possibly associated with cell toxicity include the inhibition of angiogenesis that has been demonstrated both in vitro and in vivo (Miyazawa et al. 2004). This effect has been reported particularly in the case of δ-T3 with mechanisms that may involve the inhibition of the PI3K/PDK/Akt pathway and the inhibition of migration and adhesion of cancer cells onto endothelial cells, and also the induction of endothelial cell stress (Shibata et al. 2008).

Table 3 Effect of T3 molecules on cell viability of non-mammary cancer cell lines

T3 form	Non-mammary cancer cells	IC50[μM] (time of treatments)	References
δ-T3	MIA-PaCa[a]	28[h] (48 h)	(Hussein and Mo 2009)
	PANC-1[b]	35[h] (48 h)	
	BxPC3[c]	35[h] (48 h)	
	PC3[d]	32[h] (24 h)	(Yu et al. 1999)
	PC3[d]	25[h] (48 h)	(Srivastava and Gupta 2006)
	DU 145[e]	20[h] (24 h)	(Kumar et al. 2006)
	DU 145[e]	27[h] (48 h)	Recenty reviewed in (Constantinou et al. 2009)
	LNCap[g]	26[h] (24 h)	
	LNCap[g]	19[h] (48 h)	
γ-T3	SGC 7901[f]	27[h] (72 h)	(Sun et al. 2008)
	PC3[d]	33[h] (24 h)	(Yu et al. 1999)
	PC3[d]	24[h] (48 h)	(Srivastava and Gupta 2006)
	DU 145[e]	34[h] (24 h)	(Kumar et al. 2006)
	DU 145[e]	30[h] (48 h)	Recenty reviewed in (Constantinou et al. 2009)
	LNCap[g]	21[h] (24 h)	
	LNCap[g]	21[h] (48 h)	
α-T3	PC3[d]	>100[h] (24 h)	
	PC3[d]	>100[h] (48 h)	
	DU 145[e]	>100[h] (24 h)	
	DU 145[e]	>100[h] (48 h)	
	LNCap[g]	>100[h] (24 h)	
	LNCap[g]	>100[h] (48 h)	
β-T3	PC3[d]	59[h] (24 h)	
	PC3[d]	48[h] (48 h)	
	DU 145[e]	60[h] (24 h)	
	DU 145[e]	53[h] (48 h)	
	LNCap[g]	70[h] (24 h)	
	LNCap[g]	52[h] (48 h)	

[a] *MIA-PaCa* human pancreatic cancer cell line

[b] *PANC1* human pancreatic cancer cell line

[c] *BxPC3* human ductal pancreatic adenocarcinoma cell line, derived from a 61-year-old women with a primary adenocarcinoma of the pancreas. These cells produce mucin and the tumor produced in nude mice is moderately well to poorly differentiated, thus suggesting close similarity to primary adenocarcinoma cells

[d] *PC3* androgen-independent human prostate adenocarcinoma cells

[e] *DU145* epithelial-like human androgen-independent prostate carcinoma

[f] *SGC 7901* human gastric carcinoma cells

[g] *LNCap* androgen sensitive human prostate cancer cells

[h] Inhibition of viability assessed by MTT test or equivalent test

In search of a therapeutic window for the anti-cancer activity of T3

In vitro testing may also help to elucidate other key aspects of the therapeutic perspectives of T3 in cancer, and particularly the issues of efficacy and therapeutic window. The therapeutic window of a drug is the dose and blood concentration capable of maximizing positive therapeutic effects while excluding risks and side effects associated with toxicity and adverse reactions.

As far as toxicity and adverse reactions concern, oral vitamin E is considered to have very low toxicity in healthy humans (Hathcock et al. 2005; Galli and Azzi 2010); 1,000 mg/day is the upper safe limit and 17 mg/kg bw/day, assuming a 60 kg person, is the tolerable upper intake level of α-TOH for adults as proposed by the Food and Nutrition Board, Institute of Medicine (Food and Nutrition Board 2000). Although systematic investigation has not been performed, clinical studies on oral supplements and food rich in T3 are consistent with absence of toxicity in

humans. T3 is considered to be safe within the same window of dosage considered for α-TOH intake, i.e., with UL of 1,000 mg/day, and even T3-rich formulations with highest bioavailability (plasma levels of α-T3 that may increase up to ≈3 μM, i.e., up to threefold the levels obtained with normal T3 supplements) so far proposed for human supplementation have been demonstrated to be safe [reviewed in (Sen et al. 2007)].

Acute, subchronic, and chronic animal studies have confirmed low in vivo toxicity of T3 vitamers. Liver toxicity effects have been observed in mice, but only when these were feed with a diet very high in T3 (2% w/w, that is approx 0.5 g/animal with a direct proportion in humans of 1.5 kg/75 kg of body weight) (Tasaki et al. 2009).

As a consequence of this, palm oil-derived tocols, with T3 and α-TOH as the principal components, in all the ingredients and food intended for human use have recently obtained generally recognized as safe (GRAS) certification by the US FDA board (FDA 2010).

The fact that similar potential and mechanisms of cytotoxicity of T3 could be proposed for other natural compounds with lipophilic nature such as carotenoids and their oxidation metabolites (Siems et al. 2009) (both interfere with the cell redox and mitochondrial function) may suggest to consider co-administrations as potentially hazardous. In humans, diets very high in palm oil intake have been reported to produce signs of liver sufferance, while a moderate intake of this oil especially when used in the refined or red form (which is remarkably rich in T3 and carotenoids) has been proposed to be curative in different types of disorders [reviewed in (Edem 2002)].

Based on the available evidence, it is possible to assume that T3 toxicity is prevented by the same mechanisms that control α-TOH metabolism and toxicity. The α-TTP-mediated selective restriction of liver uptake and the sustained metabolism of T3, which are described above in details, provide the protection mechanism and on the other hand, confirm the need for a prompt detoxification and the potential for toxicity effects in human cells.

Thus, once toxicity of T3 is determined, to define a therapeutic window what remains to be established is the dose within the UL that will produce highest bioactivity and therapeutic effects.

Neuroprotection and anti-cancer effects are two biological functions of T3 that can be considered to discuss this aspect. The former was defined by the original work of Sen et al. (Sen et al. 2000) as the prevention activity that α-T3, but not α-TOH, has on glutamate-induced death of T4 hippocampal neuronal cells at nanomolar concentrations that are 4–10 times lower than in plasma. Indeed, α-T3 in human and animal plasma usually reaches maximum 1 μmol/l irrespective of the amount supplemented (Lodge et al. 2001; Mustad et al. 2002). Therefore, this cytoprotection effect is mediated by physiological T3 concentrations that produced in these neuronal cells c-Src-dependent stimulation of the survival pathway MAPK-ERK, and decreased 12-LOX and eicosanoid pathway activation through a lowered depletion of intracellular glutathione (Sen et al. 2004). If such a tiny T3 concentrations needed for neuronal cell protection can be reached in the human brain by feeding tocotrienols, remains to be conclusively demonstrated, but it is likely to occur (Khanna et al. 2005).

On the contrary, the well recognized in vitro anti-cancer activity of most potent T3 forms, i.e., HM forms, occurs at extracellular concentrations far from the physiological. Indeed, the majority of data obtained assessing the anti-proliferative and pro-apoptotic activity of HM T3 in different breast cancer cell lines showed IC50 values between 3 and 30 μM (Table 2) while in non-mammary cells IC50 values are between 20 and 70 μM (Table 3). The lowest IC50 levels in breast cancer cells (McIntyre et al. 2000) were obtained by means of days of in vitro exposure to concentrations of HM T3 that are close to, but lower than, the levels of γ- and δ-T3 found in human and animal plasma after supplementation (Lodge et al. 2001; Mustad et al. 2002). Furthermore, half life of all T3 investigated in humans (α, γ, δ) are 4.5–8.7 times shorter than those of α-TOH (Yap et al. 2001; Schaffer et al. 2005), thus suggesting unfavorable pharmacokinetics of oral administration protocols by sustained liver metabolism.

These aspects may lead to conclude that even at the highest dosages compatible with UL, T3 could not meet the extracellular levels required to generate main anti-cancer effects in vivo. Notwithstanding, levels and bioactivity of T3 could be higher and close to therapeutic efficacy thanks to local mechanisms, which could be further sustained by cancerogenic transformation (see above). Although tissue levels of T3 are very low (Podda et al. 1996), biodistribution studies in wild type and α-TTP null rodents have shown that supplemental T3 can be delivered to all organs (Khanna et al. 2005), thus suggesting the existence of α-TTP independent mechanisms of tissue distribution for T3. Tissue-specific differences between α-T3 and α-TOH levels after standard or supplemented diets have been reported, with preferential uptake and higher levels of α-T3 than α-TOH in some tissues such as skin and possibly adipose of these animals. Skin concentrations of α-T3 in the supplemented animals showed also a build up over time for regimens of prolonged supplementation, and tissue transport systems can be deactivated when animals are feed with diets deficient in T3 and rich in TOH, which suggest an interference of dietary α-TOH on T3 bioavailability and tissue distribution in animals supplemented with T3 [reviewed in (Sen et al. 2007)].

Preliminary evidence suggests that T3 levels are lowered in the adipose tissue of breast lumps obtained from

malignant breast cancer patients than in benign patients (Nesaretnam et al. 2007). This evidence supports the idea that tocotrienols may provide protection against breast cancer and that interventions aimed to increase T3 levels in the breast tissue may result in secondary prevention or even chemotherapic effects.

Based on this burden of knowledge, a therapeutic window for T3 might be proposed for the neuroprotective function that α-T3 can produce under conditions of glutamate-dependent cytotoxicity (this coincides with oral doses that maintain plasma levels of this form of T3 near to 1 μM), but not for the anti-cancer (chemoprevention) effect of HM T3 proposed by means of huge in vitro evidence.

In vivo studies so far performed in immunocompromised animals after ectopic implantation of breast cancer cells (Nesaretnam et al. 2004) as well as other studies that investigated tumor mass growth and vascularization in other models of ectopic implants (Nakagawa et al. 2007; Shibata et al. 2009; Weng–Yew et al. 2009), have not provided an affordable model system to clarify this point. These studies were based on the supplementation of high dosages (between 1 and 10 mg/day) of T3 under the form of palm oil derived T3-rich fraction (TRF) that provided different contributions to the observed anti-cancer effects by the individual forms of T3 contained in this type formulation. Moreover, information concerning bioavailability and tumor targeting of T3 in these studies was scant, and the models looks far from reflecting the situation of human therapy. To our knowledge, in vivo studies on the anti-cancer effect of T3 in humans are missing. This lack of knowledge is a gap to fill before the next generation of clinical trials on T3 therapy of cancer will be proposed. Distribution, metabolism, and local activity in human tissues are poorly understood and deserve further investigation to confirm mechanisms, specificity of action and therapeutic window of T3.

Conclusion and further perspectives

In conclusion, HM T3 are the more toxic forms of vitamin E behaving as cell stressors, mitocans, and pro-apoptotic agents. In vitro studies have demonstrated that all these effects are the key for the anti-cancer potential of T3 and may also provide a clue into the cell protection mechanisms of T3 that involves the induction of drug metabolizing and stress response genes.

Breast cancer appears to be the malignancy with highest responsiveness to T3 both in vitro and in vivo. Selective mechanisms of control of cell cycle and apoptosis in breast cancer cells have been identified. More research is needed to ascertain whether these mechanisms are supported by in vivo bioactivity and tissue targeting of T3 which is the key information to support future steps of translational research to bring T3 into chemoprevention and cancer therapy trials.

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