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Tocotrienols: The Emerging Face of Natural Vitamin E

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Natural vitamin E includes eight chemically distinct molecules: α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols. More than 95% of all studies on vitamin E are directed toward the specific study of α-tocopherol. The other forms of natural vitamin E remain poorly understood. The abundance of α-tocopherol in the human body and the comparable efficiency of all vitamin E molecules as antioxidants led

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biologists to neglect the non-tocopherol vitamin E molecules as topics for basic and clinical research. Recent developments warrant a serious reconsideration of this conventional wisdom. The tocotrienol subfamily of natural vitamin E possesses powerful neuroprotective, anticancer, and cholesterol-lowering properties that are often not exhibited by tocopherols. Current developments in vitamin E research clearly indicate that members of the vitamin E family are not redundant with respect to their biological functions. α-Tocotrienol, γ-tocopherol, and δ-tocotrienol have emerged as vitamin E molecules with functions in health and disease that are clearly distinct from that of α-tocopherol. At nanomolar concentration, α-tocotrienol, not α-tocopherol, prevents neurodegeneration. On a concentration basis, this finding represents the most potent of all biological functions exhibited by any natural vitamin E molecule. Recently, it has been suggested that the safe dose of various tocotrienols for human consumption is 200–1000/day. A rapidly expanding body of evidence supports that members of the vitamin E family are functionally unique. In recognition of this fact, title claims in publications should be limited to the specific form of vitamin E studied. For example, evidence for toxicity of a specific form of tocopherol in excess may not be used to conclude that high-dosage ''vitamin E'' supplementation may increase all-cause mortality. Such conclusion incorrectly implies that tocotrienols are toxic as well under conditions where tocotrienols were not even considered. The current state of knowledge warrants strategic investment into the lesser known forms of vitamin E. This will enable prudent selection of the appropriate vitamin E molecule for studies addressing a specific health need.

I. HISTORICAL DEVELOPMENTS AND THE VITAMIN E FAMILY

That certain foods are vital to maintaining healthy life was recognized long before the first vitamins were actually identified. In ancient times, the famous Greek physician Hippocrates not only described night blindness, a disease now known to be caused by a vitamin A deficiency, but recommended the eating of ''ox liver dipped in honey'' as a cure. In the centuries that followed, observers continued to report that certain diseases appeared to be nutritionally related. By and large, they attributed the problem to some unknown ''toxic substance'' in various foodstuffs. In 1747 when the Scottish physician James Lind proved he could cure scurvy by feeding citrus fruits to stricken sailors, his fellow physicians continued to ignore his work and to search for the ''toxin'' responsible for the illness. The unknown ''toxins'' were never found. In the last quarter of the nineteenth century, scientific thinking began to change. In 1886, Christiaan Eijkman, a physician working in the Dutch East Indies, began a serious

investigation into *Beriberi*, a thiamine deficiency disease. Eijkman's studies indicated that beriberi in animals was caused by diets excessively high in polished rice and that it could be cured by substituting unpolished rice. In 1901, a younger colleague, Gerrit Grijns, determined that polished rice lacked an essential "anti-beriberi" substance that could be found in rice hulls and a number of other foods. Contemporary Englishman William Fletcher determined that if special factors (vitamins) were removed from food disease ensued. Fletcher was researching the causes of the disease beriberi when he discovered that eating unpolished rice prevented *Beriberi* and eating polished rice did not. William Fletcher believed that there were special nutrients contained in the husk of the rice. Next year, English biochemist Sir Frederick Gowland Hopkins also discovered that certain "accessory food factors" were important to health. In 1912, Polish scientist Cashmir Funk named the special nutritional parts of food as a "vitamine" after "vita" meaning life and "amine" from compounds found in the thiamine he isolated from rice husks. Vitamine was later shortened to vitamin when it was discovered that not all of the vitamins contain nitrogen, and, therefore, not all are amines. Together, Hopkins and Funk formulated the vitamin hypothesis of deficiency disease—that a lack of vitamins could make people sick. At this point of time, the notion of fat-soluble vitamins was yet to be conceived.

Fat-soluble vitamins have their root in the 1913 discovery by Elmer V. McCollum, Thomas B. Osborne, and Lafayette B. Mendel who isolated a growth-producing substance from egg yolks. The *substance* appeared quite different from the water-soluble vitamins already discovered. In 1916, McCollum went on to show that at least two factors were responsible for the normal growth of rats, factors he named fat-soluble A and water-soluble B. McCollum therefore is credited with initiating the custom of labeling vitamins by letters. Vitamin E was discovered in 1922 in green leafy vegetables by University of California researchers, Herbert Evans and Katherine Bishop (Evans and Bishop, 1922). In 1924, Sure named it vitamin E. Because E supported fertility, it was scientifically named tocopherol. This comes from the Greek word *tokos* meaning childbirth, and *phero* meaning to bring forth, and the *ol* ending was added to indicate the alcohol properties of this molecule. In 1936, it was discovered that vitamin E was abundant in wheat germ oil. Two years later, it was chemically synthesized for the first time. The U.S. National Research Council sponsored studies on deficiencies of vitamin E, and based on the results E was designated an essential vitamin. Vitamin E emerged as an essential, fat-soluble nutrient that functions as an antioxidant in the human body. It is essential, because it is required to sustain life, and the body cannot manufacture its own vitamin E and foods and supplements must provide it. Since the elucidation of the chemical structure of vitamin E in 1938 by Fenholz and the synthesis of DL-α-tocopherol by Karrer in the same year, specific focus was directed on the chemical class of natural compounds that qualify to be vitamin E. Vitamin E was rediscovered as factor 2 antioxidant

in 1965 (Schwarz, 1965). α-Tocopherol drew most attention as the first natural form of vitamin E identified while its sisters remained under veil. At present, vitamin E represents a generic term for four tocopherols and four tocotrienols (Bruno and Traber, 2006). In nature, eight substances have been found to have vitamin E activity: α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols. Although it has been claimed that tocotrienol may be metabolized to tocopherol in the human tissue (Qureshi *et al.*, 2001c, 2002), the concept has not gained wide acceptance and the hypothesis remains open for additional considerations.

II. BIOSYNTHESIS OF TOCOPHEROLS AND TOCOTRIENOLS

Tocopherols consist of a chromanol ring and a 15-carbon tail derived from homogentisate (HGA) and phytyl diphosphate, respectively (Fig. 1). Condensation of HGA and phytyl diphosphate, the committed step in

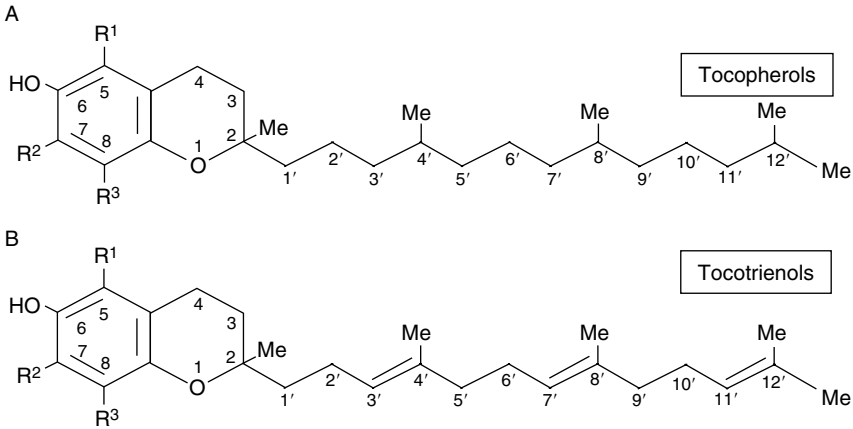

FIGURE 1. Vitamin E: variations and nomenclature. (A) R1 = R2 = R3 = Me, known as α-tocopherol, is designated α-tocopherol or 5,7,8-trimethyltocol; R1 = R3 = Me; R2 = H, known as, β-tocopherol, is designated, β-tocopherol or 5,8-dimethyltocol; R1 = H; R2 = R3 = Me, known as γ-tocopherol, is designated γ-tocopherol or 7,8-dimethyltocol; R1 = R2 = H; R3 = Me, known as δ-tocopherol, is designated δ-tocopherol or 8-methyltocol. (B) R1 = R2 = R3 = H, 2-methyl-2-(4,8,12-trimethyltrideca-3,7,11-trienyl)chroman-6-ol, is designated tocotrienol; R1 = R2 = R3 = Me, formerly known as ζ1- or ζ2-tocopherol, is designated 5,7, 8-trimethyltocotrienol or α-tocotrienol. The name tocochromanol-3 has also been used; R1 = R3 = Me; R2 = H, formerly known as ϵ-tocopherol, is designated 5,8-dimethyltocotrienol or β-tocotrienol; R1 = H; R2 = R3 = Me, formerly known as η-tocopherol, is designated 7,8-dimethyltocotrienol or γ-tocotrienol. The name plastochromanol-3 has also been used; R1 = R2 = H; R3 = Me is designated 8-methyltocotrienol or δ-tocotrienol.

tocopherol biosynthesis, is catalyzed by HGA phytyltransferase (HPT) (Venkatesh *et al.*, 2006). Tocopherol helps maintain optimal photosynthesis rate under high-light stress (Porfirova *et al.*, 2002). Tocotrienols differ structurally from tocopherols by the presence of three *trans* double bonds in the hydrocarbon tail. Because of these unsaturations in the isoprenoid side chain, tocotrienols are thought to assume a unique conformation (Atkinson, 2006). α-Tocotrienol seems to be very likely much more flexible in the side chain and that it puts a greater curvature stress on phospholipid membranes. This has been confirmed in scanning calorimetry data (Dr. Jeffrey Atkinson, unpublished personal communication).

Tocotrienols are the primary form of vitamin E in the seed endosperm of most monocots, including agronomically important cereal grains such as wheat, rice, and barley. Palm oil contains significant quantities of tocotrienol (Sundram *et al.*, 2003). Tocotrienols are also found in the seed endosperm of a limited number of dicots, including *Apiaceae* species and certain *Solanaeceae* species, such as tobacco. These molecules are found only rarely in vegetative tissues of plants. Crude palm oil extracted from the fruits of *Elaeis guineensis* particularly contains a high amount of tocotrienols (up to 800 mg/kg), mainly consisting of γ- and α-tocotrienols. Compared to tocopherols, tocotrienols are considerably less widespread in the plant kingdom (Horvath *et al.*, 2006). In 80 different plant species studied, 24 were found to contain significant amounts of tocotrienols. No taxonomic relation was apparent among the 16 dicotyledonous species that were found to contain tocotrienol. Monocotyledonous species (eight species) belonged either to the *Poaceae* (six species) or to the *Aracaceae* (two species). A more detailed analysis of tocotrienol accumulation revealed the presence of this natural vitamin E in several nonphotosynthetic tissues and organs, that is seeds, fruits, and latex. No tocotrienols could be detected in mature photosynthetic tissues. Transient accumulation of low levels of tocotrienols is found in the young coleoptiles of plant species whose seeds contained tocotrienols. No measurable tocotrienol biosynthesis was apparent in coleoptiles or in chloroplasts isolated from such coleoptiles. Tocotrienol accumulation in coleoptiles was not associated with chloroplasts. Tocotrienols seem to be transiently present in photosynthetically active tissues; however, it remains to be proven whether they are biosynthesized in such tissues or imported from elsewhere in the plant (Horvath *et al.*, 2006).

In contrast to tocotrienols, tocopherols occur ubiquitously in plant tissues and are the exclusive form of vitamin E in leaves of plants and seeds of most dicots. Transgenic expression of the barley homogentisic acid transferase (HGGT, which catalyzes the committed step of tocotrienol biosynthesis) in *Arabidopsis thaliana* leaves resulted in accumulation of tocotrienols, which were absent from leaves of nontransformed plants, and a 10- to 15-fold increase in total vitamin E antioxidants (tocotrienols plus tocopherols). Overexpression of the barley HGGT in corn seeds resulted in an increase in tocotrienol and tocopherol content of as much as sixfold. These results

provide insight into the genetic basis for tocotrienol biosynthesis in plants and demonstrate the ability to enhance the antioxidant content of crops by introduction of an enzyme that redirects metabolic flux (Cahoon *et al.*, 2003). Another strategy involving genetic engineering of metabolic pathways in plants has proved to be efficient in bolstering tocotrienol biosynthesis (Rippert *et al.*, 2004). In plants, phenylalanine is the precursor of a myriad of secondary compounds termed phenylpropanoids. In contrast, much less carbon is incorporated into tyrosine that provides *p*-hydroxyphenylpyruvate and HGA, the aromatic precursors of vitamin E. The flux of these two compounds has been upregulated by deriving their synthesis directly at the level of prephenate. This was achieved by the expression of the yeast prephenate dehydrogenase gene in tobacco plants that already overexpress the *Arabidopsis* *p*-hydroxyphenylpyruvate dioxygenase coding sequence. Massive accumulation of tocotrienols was observed in leaves. These molecules, which were undetectable in wild-type leaves, became the major forms of vitamin E in the leaves of the transgenic lines. An increased resistance of the transgenic plants toward the herbicidal *p*-hydroxyphenylpyruvate dioxygenase inhibitor diketonitril was also observed. Thus, the synthesis of *p*-hydroxyphenylpyruvate is a limiting step for the accumulation of vitamin E in plants (Rippert *et al.*, 2004).

III. CHANGING TRENDS IN VITAMIN E RESEARCH

A striking asymmetry in our understanding of the eight-member natural vitamin E tocol family has deprived us of the full complement of benefits offered by the natural vitamin E molecules (Fig. 2). Approximately, only 1% of the entire literature on vitamin E addresses tocotrienols. A review of the NIH CRISP database shows that funding for tocotrienol research represents less than 1% of all vitamin E research during the last 30+ years. Within the tocopherol literature, the non-α forms remain poorly studied (Dietrich *et al.*, 2006; Hensley *et al.*, 2004; O'Byrne *et al.*, 2000). This represents a major void in vitamin E research. It is important that conclusions drawn about the usefulness of vitamin E as a whole to human health be drawn in this light. At present, conclusions drawn about vitamin E as whole in light of results from α-tocopherol studies alone (Friedrich, 2004; Gorman, 2005; Greenberg, 2005; Hathcock *et al.*, 2005; Miller *et al.*, 2005) can be misleading. It is important to recognize the gaping voids in our understanding of the non-α-tocopherol forms of vitamin E and develop a more symmetrical understanding which would enable us to pick the right form of vitamin E for specific health indications. In this context we need to be cognizant of the fact the biological functions of the different homologues of natural vitamin E are not identical. Evidence supporting the unique biological significance of vitamin E family members is provided by current results derived from α-tocotrienol research.

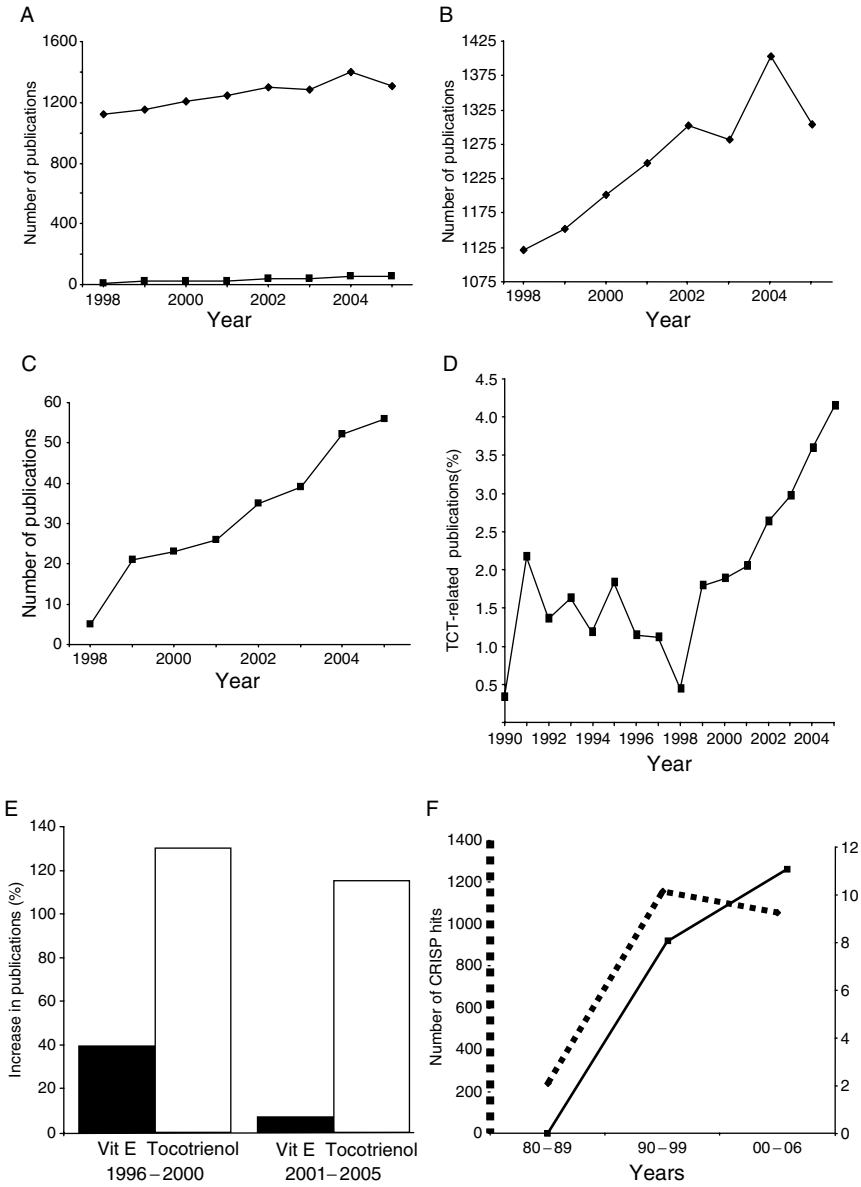

FIGURE 2. Trends in tocotrienol research and in vitamin E research as a whole. Publication data are based on PubMed entries. (A) Comparison of volume of all vitamin E (diamond) research and tocotrienol research (square), (B) time-dependent changes in the volume of vitamin E research as a whole, (C) time-dependent changes in the volume of tocotrienol research, (D) tocotrienol (TCT) publications as a percent of total vitamin E publications reported, (E) percent increase in tocotrienol publications and that of vitamin E as a whole over time, and (F) trends in NIH funding for tocotrienol research (solid line) and for vitamin E research as a whole (broken line). On the basis of hits in the Computer Retrieval of Information on Scientific Projects (CRISP) database.

During the last 5 years, tocotrienol research has gained substantial momentum (Fig. 2). More than two-thirds (189/280) of the entire PubMed literature on tocotrienols has been published on or after 2000. This represents a major swing in the direction of vitamin E research.

IV. UNIQUE BIOLOGICAL FUNCTIONS OF TOCOTRIENOLS

All eight tocols in the vitamin E family share close structural similarity (Fig. 1) and hence comparable antioxidant efficacy. Yet, current studies of the biological functions of vitamin E continue to indicate that members of the vitamin E family possess unique biological functions often not shared by other family members. One of the earliest observations suggesting that α-tocopherol may have functions independent of its antioxidant property came from the study of platelet adhesion. α-Tocopherol strongly inhibits platelet adhesion. Doses of 400 IU/day provide greater than 75% inhibition of platelet adhesion to a variety of adhesive proteins when tested at low shear rate in a laminar flow chamber? The antiadhesive effect of α-tocopherol appears to be related to a reduction in the number and size of pseudopodia on platelet activation and this finding led to the hypothesis that within the body vitamin E may exert functions beyond its antioxidant property (Steiner, 1993). That members of the tocopherol family may have functions independent of their antioxidant properties gained more prominence when vitamin E molecules with comparable antioxidant properties exhibited contrasting biological effects (Boscoboinik *et al.*, 1991). At the posttranslational level, α-tocopherol inhibits protein kinase C, 5-lipoxygenase (5-Lox), and phospholipase A2 and activates protein phosphatase 2A and diacylglycerol kinase. Some genes [e.g., scavenger receptors, α-tocopherol transfer protein (α-TTP), α-tropomyosin, matrix metalloproteinase-19, and collagenase] are specifically modulated by α-tocopherol at the transcriptional level. α-Tocopherol also inhibits cell proliferation, platelet aggregation, and monocyte adhesion. These effects have been characterized to be unrelated to the antioxidant activity of vitamin E and possibly reflect specific interactions of α-tocopherol with enzymes, structural proteins, lipids, and transcription factors (Zingg and Azzi, 2004). γ-Tocopherol represents the major form of vitamin E in the diet in the United States, but not in Europe. Desmethyl tocopherols, such as γ-tocopherol and specific tocopherol metabolites, most notably the carboxyethyl-hydroxychroman (CEHC) products, exhibit functions that are not shared by α-tocopherol. The activities of these other tocopherols do not map directly to their chemical antioxidant behavior but rather reflect anti-inflammatory, antineoplastic, and natriuretic functions possibly mediated through specific binding interactions (Hensley *et al.*, 2004). Metabolites of γ-tocopherol (2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman), but not that of α-tocopherol, provides natriuretic activity.

Moreover, a nascent body of epidemiological data suggests that γ-tocopherol is a better negative risk factor for certain types of cancer and myocardial infarction than is α-tocopherol (Wagner *et al.*, 2004).

α-Tocotrienol possesses numerous functions that are not shared by α-tocopherol (Sen *et al.*, 2006). For example, nanomolar concentrations of α-tocotrienol uniquely prevent inducible neurodegeneration by regulating specific mediators of cell death (Khanna *et al.*, 2003, 2006; Sen *et al.*, 2000). Oral supplementation of tocotrienol protects against stroke (Khanna *et al.*, 2005b). Micromolar amounts of tocotrienol suppress the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the hepatic enzyme responsible for cholesterol synthesis (Pearce *et al.*, 1992, 1994). Tocopherols do not share the cholesterol-lowering properties of tocotrienol (Qureshi *et al.*, 1986, 2002). Sterol-regulated ubiquitination marks HMG-CoA reductase for endoplasmic reticulum (ER)-associated degradation by 26S proteasomes. This degradation, which results from sterol-induced binding of reductase to ER membrane proteins called Insigs, contributes to the complex, multivalent feedback regulation of the enzyme. Recently, it has been demonstrated that δ-tocotrienol stimulates ubiquitination and degradation of reductase and blocks processing of sterol regulatory element-binding proteins (SREBPs), another sterol-mediated action of Insigs. The γ-tocotrienol analogue is more selective in enhancing reductase ubiquitination and degradation than blocking SREBP processing. Other forms of vitamin E neither accelerate reductase degradation nor block SREBP processing (Song and Debose-Boyd, 2006).

Tocotrienol, not tocopherol, administration reduces oxidative protein damage and extends the mean life span of *Caenorhabditis elegans* (Adachi and Ishii, 2000). Tocotrienols are thought to have more potent antioxidant properties than α-tocopherol (Serbinova and Packer, 1994; Serbinova *et al.*, 1991). The unsaturated side chain of tocotrienol allows for more efficient penetration into tissues that have saturated fatty layers such as the brain and liver (Suzuki *et al.*, 1993). Experimental research examining the antioxidant, free-radical-scavenging effects of tocopherol, and tocotrienols revealed that tocotrienols appear superior due to their better distribution in the fatty layers of the cell membrane (Suzuki *et al.*, 1993). Furthermore, tocotrienol but not tocopherol, suppresses growth of human breast cancer cells (Nesaretnam *et al.*, 1995).

In humans, tocotrienol supplementation results in peak blood plasma level of α-tocotrienol that is over an order of magnitude higher than that required to protect neurons against a range of neurotoxic insults (Khanna *et al.*, 2003, 2005a,b, 2006; Khosla *et al.*, 2006; Sen *et al.*, 2000). Despite such promising potential, tocotrienol research accounts for roughly 1% of all vitamin E research published in PubMed. The unique vitamin action of α-tocopherol, combined with its prevalence in the human body and the similar efficiency of tocopherols as chain-breaking antioxidants, led biologists to

almost completely discount the "minor" vitamin E molecules as topics for basic and clinical research. Recent discoveries warrant a serious reconsideration of this conventional wisdom.

V. NATURAL SOURCES OF TOCOTRIENOLS

Tocotrienol is synthesized in edible as well as inedible plant products. Rubber latex represents a major nonfood natural source of tocotrienols (Chow and Draper, 1970; Horvath *et al.*, 2006; Whittle *et al.*, 1966). Identification of α-tocotrienol as a cholesterogenesis inhibitory factor in barley (*Hordeum vulgare* L.) represents a landmark early discovery highlighting the unique significance of tocotrienols in health and disease (Qureshi *et al.*, 1986). Purification of an oily, nonpolar fraction of high-protein barley flour by high-pressure liquid chromatography yielded 10 major components. Two of these components were identified as potent inhibitors of cholesterogenesis both *in vivo* as well as *in vitro*. Addition of the purified inhibitor I (2.5–20 ppm) to chick diets significantly decreased hepatic cholesterogenesis and serum total and low-density lipoprotein (LDL) cholesterol and concomitantly increased lipogenic activity. The high-resolution mass spectrometric analysis and measurement of different peaks of inhibitor I gave a molecular ion at *m*/*e* 424 (C29H44O2) and main peaks at *m*/*e* 205, 203, and 165 corresponding to C13H17O2, C13H15O2, and C10H13O2 moieties, respectively. On the basis of these results, D-α-tocotrienol was identified as the active principle. This identification was confirmed against synthetic samples (Qureshi *et al.*, 1986).

Palm oil represents one of the most abundant natural sources of tocotrienols (Elson, 1992). The distribution of vitamin E in palm oil is 30% tocopherols and 70% tocotrienols (Sundram *et al.*, 2003). The oil palm (*E. guineensis*) is native to many West African countries, where local populations have used its oil for culinary and other purposes. Large-scale plantations, established principally in tropical regions of Asia, Africa, and Latin America are mostly aimed at the production of oil (Solomons and Orozco, 2003), which is extracted from the fleshy mesocarp of the palm fruit, and endosperm or kernel oil. Palm oil is different from other plant and animal oils in that it contains 50% saturated fatty acids, 40% unsaturated fatty acids, and 10% polyunsaturated fatty acids. Because of its high saturated fat content, palm oil has not been very popular in the United States. Hydrogenated fats contain high levels of *trans*-fatty acids which are now thought to have adverse health effects. The U.S. Food and Drug Administration's final ruling on *trans*-fatty acid labeling issued in 2003 has caused a rapid transformation in the fat and oil industries (Tarrago-Trani *et al.*, 2006). Palm oil is free of *trans*-fatty acid and is rapidly gaining wider acceptance by the food industry in the country. Primary applications include bakery products, breakfast cereals, wafers, and candies.

Rice bran oil (RBO), a by-product of the rice-milling industry, is a major natural source of γ-tocotrienol but a poor source of α-tocotrienol. In addition, RBO provides desmethyl tocotrienols. Two novel tocotrienols were isolated from stabilized and heated rice bran, apart from the known α-, β-, γ-, and δ-tocopherols and tocotrienols. These new tocotrienols are known as desmethyl tocotrienol [3,4-dihydro-2-methyl-2-(4,8,12-trimethyltrideca-3′(E),7′(E),11′-trienyl)-2H-1-benzopyran-6-ol] and didesmethy tocotrienol [3,4-dihydro-2-(4,8,12-trimethyltrideca-3′(E),7′(E),11′-trienyl)-2H-1-benzopyran-6-ol] (Qureshi *et al.*, 2000). Although scientific evidence is relatively limited, RBO is tenaciously believed to be a healthy vegetable oil in Asian countries (Sugano *et al.*, 1999).

Cereals such as oat, rye, and barley contain small amounts of tocotrienol in them. α-Tocotrienol is the predominant form of tocotrienol in oat (*Avena sativa* L.) and barley (56 and 40 mg/kg of dry weight, respectively). β-Tocotrienol is the major form of tocotrienol found in hulled and dehulled wheats (from 33 to 43 mg/kg of dry weight) (Panfili *et al.*, 2003). Steaming and flaking of dehulled oat groats results in moderate losses of tocotrienols but not of tocopherols (Bryngelsson *et al.*, 2002). Autoclaving of grains (including the hulls) increases the levels of all tocopherols and tocotrienols analyzed except β-tocotrienol, which was not affected. Drum drying of steamed rolled oats results in an almost complete loss of tocopherols and tocotrienols (Bryngelsson *et al.*, 2002). Although tocotrienols are present in edible natural products, it is questionable whether these dietary sources could provide sufficient amounts of tocotrienol to humans. Of note, processing of 1000 kg of crude palm oil is necessary to derive 1 kg of the commercial product Tocomin 50% (Carotech, New Jersy). Roughly, one would have to consume 100–200 g of palm/rice bran oil or 1.5–4 kg of wheat germ, barley or oat to achieve doses that have been published to be effective biologically. With this consideration in mind, appropriately configured dietary supplements seem to be a prudent choice.

VI. BIOAVAILABILITY OF ORAL TOCOTRIENOLS

During the last two decades, efforts to understand how dietary vitamin E is transported to the tissues have focused on α-tocopherol transport (Blatt *et al.*, 2001; Kaempf-Rotzoll *et al.*, 2003; Traber and Arai, 1999; Traber *et al.*, 2004). α-Tocopherol transfer protein (TTP) has been identified to mediate α-tocopherol secretion into the plasma while other tocopherol-binding proteins seem to play a less important role (Kaempf-Rotzoll *et al.*, 2003). Tocotrienols have been known for decades but why have they not been studied as well as α-tocopherol? Although there does not seem to be straightforward rational answer to this question, one contributing factor is whether

tocotrienol taken orally reaches vital organs of the body. This concern was primarily based on a 1997 finding that the transport system, α-tocopherol transfer protein (TTP), responsible to carry α-tocopherol to vital organs has a poorer efficiency to transport tocotrienols to tissues (Hosomi *et al.*, 1997). The lack of relative specific affinity of TTP for tocotrienols led to the notion that availability of dietary tocotrienol to vital organs is negligible.

TTP is a soluble 32-kDa protein expressed in liver that selectively binds and transports α-tocopherol. TTP maintains the concentration of serum α-tocopherol by facilitating α-tocopherol export from the liver. TTP is required to maintain normal α-tocopherol concentrations in plasma and extrahepatic tissues (Traber *et al.*, 2004). Although TTP is known to bind to α-tocotrienol with 8.5-fold lower affinity than that for α-tocopherol (Hosomi *et al.*, 1997), it has not been clear whether, or to what extent, the delivery of orally supplemented α-tocotrienol to vital organs is dependent on TTP. Previously, it has been reported that TTP-deficient females are infertile presumably because of vitamin E deficiency (Terasawa *et al.*, 2000). This important observation was confirmed in a lineage of TTP-deficient mice. Placenta of pregnant TTP-deficient females were severely impaired with marked reduction of labyrinthine trophoblasts, and the embryos died at mid-gestation even when fertilized eggs of TTP-containing wild-type mice were transferred into TTP-deficient recipients (Jishage *et al.*, 2001). Even in the presence of dietary α-tocopherol, TTP knockout mice are known to suffer from α-tocopherol deficiency (Jishage *et al.*, 2001; Terasawa *et al.*, 2000). It has been noted that oral supplementation of female mice with α-tocotrienol restored fertility of TTP knockout mice, suggesting that tocotrienol was successfully delivered to the relevant tissues and that tocotrienol supported reproductive function under conditions of α-tocopherol deficiency (Khanna *et al.*, 2005a). This observation was consistent with another line of evidence from rats where tocotrienol supplementation spared loss of fertility caused by long-term vitamin E deficiency in the diet (Khanna *et al.*, 2005a). TTP continues to be a key transport mechanism for the deliver of α-tocopherol to tissues. The significance of TTP in the transport of other forms of vitamin E remains unclear at present. It is clear, however, that natural isomers of vitamin E do get transported to vital organs even in the absence of TTP. Identification and characterization of TTP-independent vitamin E transport mechanisms *in vivo* is warranted.

Ten years ago in a study testing ligand specificity of vitamin E isomers for TTP concluded that the affinity of vitamin E analogues for TTP is one of the critical determinants of their biological activity (Hosomi *et al.*, 1997). This conclusion was based on the assumption that the biological function of vitamin E molecules is proportionate to their concentration and that vitamin E isomers have redundant function. Early postulates proposing that tissue concentration and relative biological function of tocopherol and tocotrienol are disparate and possibly unrelated (Hayes *et al.*, 1993). Developments during the last decade taught us that both assumptions are incorrect

warranting a revisit of the fundamental principles that guide vitamin E research (Azzi and Stocker, 2000; Azzi *et al.*, 1995; Sen *et al.*, 2004, 2006). Another contemporary study reported that tocotrienols, supplemented to laboratory chow, do not reach the brain (Podda *et al.*, 1996). Taken together, the case for *in vivo* efficacy of oral tocotrienol was seriously weakened by these reports (Hosomi *et al.*, 1997; Podda *et al.*, 1996). Today, however, the scenario has strikingly changed in light of new knowledge. For example, it is now clear that oral tocotrienol not only reaches the brain (Khanna *et al.*, 2005a,b; Roy *et al.*, 2002) but it does so in amounts sufficient to protect against stroke (Khanna *et al.*, 2005b). The standard laboratory chow contains excessive amounts of α-tocopherol (Khosla *et al.*, 2006; van der Worp *et al.*, 1998) but negligible amounts of tocotrienol. Long-term lack of tocotrienol in the diet may repress any putative tocotrienol transport mechanism *in vivo*. Thus, long-term supplementation studies are needed. In light of the knowledge that natural analogues of vitamin E may compete for specific transporting mechanisms (Hosomi *et al.*, 1997), it is important that tocotrienol supplementation be performed under conditions of minimized copresence of tocopherols. Another related consideration is that although incorporation of orally supplemented vitamin E into tissues is a slow and progressive process, rapid incorporation of the supplement into tissues of newborns may occur in response to gavaging of pregnant mother rats (Roy *et al.*, 2002). Thus, an experimental design incorporating long-term tocotrienol supplementation under conditions of minimal dietary copresence of tocopherols and breeding of the supplemented colony would be a valuable approach to generate proof of principle testing whether dietary α-tocotrienol is capable of being transported to vital organs *in vivo*. In a recent study, rats were maintained on vitamin E-deficient diet and gavaged with α-tocotrienol alone, α-tocopherol alone, or in combination. Five generations of rats were studied over 60 weeks (Khanna *et al.*, 2005a). Skin, adipose, heart, lungs, skeletal muscle brain, spinal cord, liver, and blood were studied. Oral tocotrienol was delivered to all vital organs. In some tissues, the level of tocotrienol exceeded that of tocopherols, indicating the presence of an efficient tocotrienol transport system *in vivo*. Baseline levels of α-tocotrienol in the skin of tocopherol-fed rats that never received any tocotrienol supplementation were negligible. Orally supplemented tocotrienol was rapidly taken up by the skin. Already in second generation rats, α-tocotrienol levels in the skin of tocotrienol supplemented rats exceeded twice the α-tocopherol levels in that organ. Of note, the α-tocotrienol level in the skin matched the α-tocotrienol level in the skin of rats fed with a comparable amount of tocopherol. When tocotrienol and tocopherol were cosupplemented, the uptake of α-tocotrienol by the skin was clearly blunted. In this group, α-tocotrienol levels were lower than α-tocotrienol levels in the skin, suggesting a direct competition between orally taken tocotrienol and tocopherol for delivery to the skin. Longer supplementation resulted in a marked increase in the α-tocotrienol

levels in the skin of tocotrienol-fed rats, indicating a buildup of α-tocotrienol over time. Interestingly, the levels of α-tocotrienol in the skin of these rats were folds higher than the α-tocopherol level in the skin of tocopherol-fed rats. This observation suggests the presence of an effective transport mechanism delivering α-tocotrienol to the skin and efficient retention of α-tocotrienol in the skin over time. Cosupplementation of tocotrienol and tocopherol demonstrated favorable uptake of α-tocopherol over α-tocotrienol. Adipose tissue serves as storage organ for vitamin E (Adachi *et al.*, 1990). Analysis of adipose tissue vitamin E content of fifth generation rats revealed substantially more accumulation of α-tocotrienol in that tissue than α-tocopherol.

In the case of tocotrienol as well as of tocopherol feeding, results from third and fifth generation rats indicate higher levels of vitamin E in the skin of female compared to that of male rats. This gender-specific effect suggesting better transport of tocotrienol in females than in males was noted as a general trend across all organs studied. Gender-based differences in the transport of dietary vitamins are known to exist in specific cases (Garry *et al.*, 1987). Although the effect of several physiological factors on vitamin E transport has been studied, the gender factor remains to be specifically addressed (Lodge *et al.*, 2004). It has been demonstrated that γ-tocopherol is more rapidly metabolized in women than in men (Leonard *et al.*, 2005). The level of α-tocotrienol in the ovary was over fivefold higher than that in the testes from the corresponding male rats (Khanna *et al.*, 2005a). In the ovary, tocopherol is known to accumulate via a lipoprotein receptor-dependent mechanism (Aten *et al.*, 1994). Whether tocotrienol shares that mechanism remain to be tested.

Vitamin E enters the circulation from the intestine in chylomicrons. The conversion of chylomicrons to remnant particles results in the distribution of newly absorbed vitamin E to all of the circulating lipoproteins and ultimately to tissues. This enrichment of lipoproteins with vitamin E is a key mechanism by which vitamin E is delivered to tissues (Traber *et al.*, 2004). In the liver, newly absorbed dietary lipids are incorporated into nascent very LDLs. The liver is responsible for the control and release of α-tocopherol into blood plasma. In the absence of TTP, α-tocopherol is not secreted back into the plasma. Excess vitamin E is not accumulated in the liver, but is excreted, mostly in bile (Traber *et al.*, 2004). It has been noted that α-tocotrienol levels in the liver of rats and of TTP-deficient mice were much lower than the levels of this vitamin E isoform in most peripheral tissues studied (Khanna *et al.*, 2005a). Such observation argues against a central role of the liver in delivering oral α-tocotrienol to peripheral tissues. TTP has the ability to bind to both α-tocopherol as well as α-tocotrienol. The affinity to bind α-tocopherol is severalfold higher than that for α-tocotrienol (Hosomi *et al.*, 1997). Thus, under conditions of coexistence, α-tocopherol is expected to out-compete α-tocotrienol for binding. Although studies with the TTP-deficient mice (Khanna *et al.*, 2005a) indicate the existence of a TTP-independent mechanisms for the tissue delivery of oral α-tocotrienol,

observations in the rat (Khanna *et al.*, 2005a) indicate that the mechanisms for transporting α-tocopherol and α-tocotrienol seem to compete such that transport of α-tocopherol is favored. Thus, cosupplementation of α-tocopherol and α-tocotrienol is likely to compromise tissue delivery of α-tocotrienol (Khanna *et al.*, 2005a).

Few studies have specifically looked at the fate of oral tocotrienol supplementation in humans. In a study investigating the pharmacokinetics and bioavailability of α-, γ-, and δ-tocotrienols under fed and fasted conditions in eight healthy volunteers, subjects were administered a single 300-mg oral dose of mixed tocotrienols under fed or fasted conditions. The peak concentration of α-tocotrienol in the blood plasma was just over 1 μM (Yap *et al.*, 2001). The fed state increased the onset as well as the extent of absorption of tocotrienols by more than twofolds. In addition, the mean apparent elimination half-life of α-, γ-, and δ-tocotrienols was estimated to be 4.4, 4.3, and 2.3 h, respectively, being between 4.5- and 8.7-fold shorter than that reported for α-tocopherol (Yap *et al.*, 2001). In another study, human subjects took tocotrienyl acetate supplements (250 mg/day) for eight weeks, while being on low-fat diet. In response to supplementation, the concentrations of tocotrienol in the mean blood plasma were as follows: α-tocotrienol, 0.98 μM; γ-tocotrienol, 0.54 μM; and δ-tocotrienol 0.09 μM (O'Byrne *et al.*, 2000). Thus, tocotrienyl acetate supplements were observed to be hydrolyzed, absorbed, and detectable in human plasma. A novel formulation for improved absorption of tocotrienols has been developed (Ho *et al.*, 2003). Emulsions are known to increase absorption of fat-soluble drugs. This invention is based on self-emulsifying drug delivery systems (SEDDS) technology (Araya *et al.*, 2006; Gao and Morozowich, 2006; Hong *et al.*, 2006). Soft gelatin capsules (Tocovid Suprabio^{TM}) containing tocotrienol have been produced. Once ingested, the tocotrienols form emulsion when the contents are released and mixed with human gastrointestinal fluid. In a recent study using Tocovid Suprabio^{TM}, the postabsorptive fate of tocotrienol isomers and their association with lipoprotein subfractions were examined in humans (Khosla *et al.*, 2006). The peak α-tocotrienol concentrations in supplemented individuals averaged ~3 μM in blood plasma, 1.7 μM in LDL, 0.9 μM in triglyceride-rich lipoprotein, and 0.5 μM in HDL. This peak plasma concentration of α-tocotrienol is two to three times more than the peak concentration reported in previous studies using generic supplements not based on SEDDS (O'Byrne *et al.*, 2000; Yap *et al.*, 2001).

VII. BIOLOGICAL FUNCTIONS

The biological functions of tocotrienol known so far have been listed in Table I. In this section, we discuss work that relate to the neuroprotective, anticancer, and cholesterol-lowering activities of tocotrienol.

TABLE I. Tocotrienols: The Emergent Face of Natural Vitamin E[a]

Neuroprotective	Mouse: At nanomolar concentrations, α-tocotrienol, in contrast with α-tocopherol, protects against glutamate-induced neuronal death by suppressing inducible pp60 c-src kinase activation. α-Tocotrienol provided the most potent neuroprotection among all vitamin E analogues. Reported effects of tocotrienol independent of antioxidant property (Sen *et al.*, 2000)	2000
	Rat: Oral tocotrienol crosses the blood–brain barrier to reach brain tissue; more so for fetal brain while pregnant mother is supplemented with tocotrienol (Roy *et al.*, 2002)	2002
	Mouse: At nanomolar concentrations, α-tocotrienol, in contrast with α-tocopherol, protects against glutamate-induced neuronal death by suppressing inducible 12-lipoxygenase activation (Khanna *et al.*, 2003). 12-Lipoxygenase-deficient mice are protected against stroke (Khanna *et al.*, 2005b)	2003
	Mouse: Injected α-tocotrienol decreased the size of the cerebral infarcts 1 day after stroke; γ- and δ-tocotrienols did not protect (Mishima *et al.*, 2003)	2003
	Human: Tocotrienols induced IKBKAP expression: a possible therapy for familial dysautonomia (Anderson *et al.*, 2003)	2003
	Rat: α-Tocotrienol provided the most potent neuroprotection among vitamin E analogues on cultured striatal neurons (Osakada *et al.*, 2004)	2004
	Human: Administration of tocotrienol to individuals with familial dysautonomia resulted in beneficial changes in their peripheral blood cells (Anderson and Rubin, 2005)	2005
	Rat: Attomole quantity of α-tocotrienol, not α-tocopherol, microinjected to primary neurons protects against glutamate cytotoxicity (Khanna *et al.*, 2005b)	2005
	SHR: α-Tocotrienol protects against stroke *in vivo* (Khanna *et al.*, 2005b)	2005
	Rat: α- and γ-tocotrienols have comparable protective effects on H_2O_2-induced death of astrocytes (Mazlan *et al.*, 2006)	2006
	Rat: At nanomolar concentration, α-tocotrienol protects neurons. Vitamin E analogues play an essential role in neuronal maintenance and survival in the CNS (Numakawa *et al.*, 2006)	2006
	Mouse: The neuroprotective property of α-tocotrienol is antioxidant-independent at nanomolar but antioxidant-dependent a micromolar concentrations (Khanna *et al.*, 2006)	2006
	Mouse: At nanomolar concentration, α-tocotrienol protects against homocysteic acid-induced neurotoxicity (Khanna *et al.*, 2006)	2006

Hypocholesterolemic	Chicken: Three double bonds in the isoprenoid chain essential for the inhibition of cholesterogenesis; tocopherols do not share this property (Qureshi *et al.*, 1986)	1986
	Human: Lowered serum cholesterol in hypercholesterolemics (Qureshi *et al.*, 1991b); lowered both serum total cholesterol (TC) and low-density-lipoprotein cholesterol (Tan *et al.*, 1991)	1991
	Pigs: Reduced plasma cholesterol, apolipoprotein B, thromboxane B2, and platelet factor 4 in pigs with inherited hyperlipidemias (Qureshi *et al.*, 1991a)	
	In vitro: Posttranscriptional suppression of HMG-CoA reductase by a process distinct from other known inhibitors of cholesterol biosynthesis (Pearce *et al.*, 1992)	1992
	Regulate cholesterol production in mammalian cells by posttranscriptional suppression of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (Parker *et al.*, 1993)	1993
	HepG2: The farnesyl side chain and the methyl/hydroxy substitution pattern of γ-tocotrienol responsible for HMG-CoA reductase suppression (Pearce *et al.*, 1994)	1994
	Isoprenoid-mediated suppression of mevalonate synthesis depletes tumor tissues of two intermediate products, farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which are incorporated posttranslationally into growth control-associated proteins (Elson and Qureshi, 1995)	1995
	Human: Lowered plasma cholesterol level in hypercholesterolemic subjects (Qureshi *et al.*, 1995)	1995
	Chicken: The effects of a tocotrienol–lovastatin combination were no greater than that of tocotrienol alone, indicating that tocotrienol produced a maximum cholesterol lowering effect (Qureshi and Peterson, 2001)	2001
	Swine: Tocotrienols suppress cholesterogenesis in hereditary hypercholesterolemic swine (Qureshi *et al.*, 2001a)	2001
	Human: Tocotrienol, not tocopherol, hypocholesterolemic in humans; claimed that tocotrienol is converted to tocopherol *in vivo* (Qureshi *et al.*, 2001c)	2001
	Human: Dose-dependent suppression of serum cholesterol by tocotrienol-rich fraction of rice bran in hypercholesterolemic humans (Qureshi *et al.*, 2002)	2002
	Hamster: Tocotrienols lower total cholesterol and low-density lipoprotein plasma levels (Raederstorff *et al.*, 2002)	2002
	Rat: Suppression of hypercholesterolemia in rats by tocotrienol-rich fraction isolated from rice bran oil (Iqbal *et al.*, 2003)	2003

(*Continues*)

TABLE I. (*Continued*)

	Rat: TRF lowered HMG-CoA reductase activity in hyperlipidemics (Minhajuddin *et al.*, 2005)	2005
	Rat: Tocotrienol-rich rice bran oil-containing diet can significantly suppress hyperlipidemic and hyperinsulinemic responses in diabetics (Chen and Cheng, 2006)	2006
	δ- and γ-tocotrienols, but not other forms of vitamin E, cause HMG Co-A reductase ubiquitination and degradation. Results explain hypocholesterolemic effects of tocotrienol noted in humans and animals (Song and Debose-Boyd, 2006)	2006
ApoB level reduction in hypercholesterolemic subjects	Human: In HepG2 cells, tocotrienol (not tocopherol) stimulates apoB degradation possibly as the result of decreased apoB translocation into the endoplasmic reticulum lumen (Theriault *et al.*, 1999)	1999
Antihypertensive	Rat: Depressed (better than α-tocopherol) age-related increase in the systolic blood pressure of spontaneously hypertensive rats (Koba *et al.*, 1992)	1992
Hypocholesterolemic and antioxidant	Rat: Spares plasma tocopherol (Watkins *et al.*, 1993)	1993
Lowering blood pressure; antioxidant	SHR: Supplement of γ-tocotrienol may prevent increased blood pressure, reduce lipid peroxides in plasma and blood vessels and enhance total antioxidant status (Newaz and Nawal, 1999)	1999
Cardioprotective	Rat: TRF protected against ischemia-reperfusion in isolated heart by c-Src inhibition (Das *et al.*, 2005)	2005
Antioxidant	*In vitro*: Better than α-tocopherol (Serbinova *et al.*, 1991)	1991
	In vitro: Facilitates antioxidant recycling (Kagan *et al.*, 1992)	1992
	In vitro: Tocotrienol is better than tocopherol; tocotrienol is located closer to the cell membrane surface (Suzuki *et al.*, 1993)	1993
	Human: Dietary tocotrienols become incorporated into circulating human lipoproteins where they react with peroxyl radicals as efficiently as the corresponding tocopherol isomers (Suarna *et al.*, 1993)	1993
	Rat: Protects brain against oxidative damage (Kamat and Devasagayam, 1995)	1995
	Human: Controls the course of carotid atherosclerosis (Tomeo *et al.*, 1995)	1995
	Human: α-Tocotrienol is more potent than α-tocopherol in protecting against free radical-induced impairment of erythrocyte deformability (Begum and Terao, 2002)	2002

	Rat: Comparable effects of a tocotrienol-rich fraction and tocopherol in aspirin-induced lipid peroxidation mediated gastric lesions (Nafeeza *et al.*, 2002)	2002
	Rat: Antioxidant effects of γ-tocotrienol in spontaneously hypertensive rats (Newaz *et al.*, 2003)	2003
	Tocopherols and tocotrienols have comparable antioxidant properties. Some of the vitamin E formulations tested showed antioxidant activities superior to D-α-tocopherol (Naguib *et al.*, 2003)	2003
	The corresponding tocopherols and tocotrienols exert comparable antioxidant activity; tocotrienols are more readily transferred between the membranes and incorporated into the membranes than tocopherols (Yoshida *et al.*, 2003)	2003
	Human: Topical α-tocotrienol supplementation inhibits lipid peroxidation in human skin (Weber *et al.*, 2003)	2003
	Human: Lack of oxidative stress in a selenium-deficient area in Ivory Coast potential nutritional antioxidant role of crude palm oil (Tiahou *et al.*, 2004)	2004
	Rat: Palm oil tocotrienol mixture better than α-tocopherol acetate in protecting bones against free-radical induced elevation of bone-resorbing cytokines (Soelaiman *et al.*, 2004)	2004
	Mouse: Rice–trienol exerted a protective effect against oxidative damage in diabetes mellitus (Kanaya *et al.*, 2004)	2004
	Antioxidant property of tocols: $\alpha > \beta = \gamma > \delta$; not influenced by the nature of the isoprenoid tail (Sonnen *et al.*, 2005)	2005
	α- and α-tocopherols have comparable antioxidant efficacy (Yamasaki *et al.*, 2005)	2005
	Mouse: Both γ-tocopherol as well as γ-tocotrienol has antioxidant properties *in vivo* (Yoshida *et al.*, 2005)	2005
	Polyunsaturated isoprenoid side chain in tocotrienols has antioxidant properties (Yu *et al.*, 2005)	2006
	Individual tocotrienols display different antioxidant potencies: $\delta > \gamma > \alpha$ (Palozza *et al.*, 2006)	2006
	γ-Tocotrienol > α-tocotrienol > α-tocopherol as antioxidant. Tocotrienol regenerated oxidized carotenes demonstrating synergistic action (Schroeder *et al.*, 2006)	2006
Antiaging/ antioxidant	*Caenorhabditis elegans*: Tocotrienol, not tocopherol, administration reduced the accumulation of protein carbonyl and consequently extended the mean life span but not the maximum life span (Adachi and Ishii, 2000; Collins *et al.*, 2006)	2000

(*Continues*)

TABLE I. (*Continued*)

Anticancer	Mouse: Intraperitoneally injected tocotrienol prevented transplanted tumors (Komiyama *et al.*, 1989)	1989
	Rat: Tocotrienol-rich palm oil prevented chemically induced mammary tumorigenesis (Sundram *et al.*, 1989)	1989
	Rat: Tocotrienol, but not tocopherol, increased tumor latency in mammary tumor model (Gould *et al.*, 1991)	1991
	Rat: Tocotrienol chemopreventive in hepatic tumor model (Ngah *et al.*, 1991)	1991
	Rat: Tocotrienol chemopreventive in hepatic tumor model (Rahmat *et al.*, 1993)	1993
	Human: Suppresses activation of Epstein–Barr virus early antigen expression in PMA-activated lymphoblastoid Raji cells (Goh *et al.*, 1994)	1994
	Human: Tocotrienol, not tocopherol, suppresses growth of a human breast cancer cell line in culture (Nesaretnam *et al.*, 1995)	1995
	Human: Inhibited proliferation of estrogen receptor negative MDA-MB-435 and estrogen receptor positive MCF-7 breast cancer cells (Guthrie *et al.*, 1997) Mouse: Isoprenoids suppress the growth of murine B16 melanomas *in vitro* and *in vivo* (He *et al.*, 1997)	1997
	Human: Inhibit the growth of human breast cancer cells irrespective of estrogen receptor status (Nesaretnam *et al.*, 1998)	1998
	Human: Apoptosis and cell cycle arrest in human and murine tumor cells are initiated by isoprenoids (Mo and Elson, 1999)	1999
	Human: Naturally occurring tocotrienols and RRR-δ-tocopherol are effective apoptotic inducers for human breast cancer cells (Yu *et al.*, 1999)	1999
	Human: Tocotrienols inhibit growth of ZR-75–1 breast cancer cells (Nesaretnam *et al.*, 2000)	2000
	Mouse: Highly potent γ- and δ-tocotrienol isoforms may play a physiological role in modulating normal mammary gland growth, function, and remodeling (McIntyre *et al.*, 2000b)	2000
	Mouse: Highly malignant breast cancer cells were the most sensitive, whereas the preneoplastic cells were the least sensitive to the antiproliferative and apoptotic effects of tocotrienols (McIntyre *et al.*, 2000a)	2000
	Mouse: Tocotrienols are significantly more potent than tocopherols in suppressing EGF-dependent normal mammary epithelial cell growth. The inhibitory effects of specific tocopherol and tocotrienol isoforms on EGF-dependent normal mammary epithelial cell mitogenesis occurs downstream from the EGF receptor and appears to be mediated, at least in part, by a reduction in PKCα activation (Sylvester *et al.*, 2001)	2001

Mouse: Antiproliferative effects of tocotrienols in preneoplastic mammary epithelial cells do not reflect a reduction in EGF-receptor mitogenic responsiveness, but rather, result from an inhibition in early postreceptor events involved in cAMP production upstream from EGF-dependent MAPK and phosphoinositide 3-kinase/Akt mitogenic signaling (Sylvester *et al.*, 2002)	2002
Rat: Suppression of 7,12-dimethylbenz[α]anthracene-induced carcinogenesis by tocotrienol-rich fraction isolated from rice bran oil (Iqbal *et al.*, 2003)	2003
Mouse: Tocotrienol-induced apoptosis in mammary cancer cells is mediated through activation of the caspase-8 signaling pathway and is independent of caspase-9 activation (Shah *et al.*, 2003)	2003
Mouse: Tocotrienol induces caspase-8 activation, unrelated to death receptor apoptotic signaling, in neoplastic mammary epithelial cells (Shah and Sylvester, 2004)	2004
Rat: Tocotrienol induces apoptosis in dRLh-84 hepatoma cells (Sakai *et al.*, 2004)	2004
Rat: Tocotrienol-rich fraction isolated from rice bran oil suppressed diethylnitrosamine and 2-acetylaminofluorene-induced hepatocarcinogenesis (Iqbal *et al.*, 2004)	2004
Human: Tocotrienol disrupts mitochondrial function and causes apoptosis of breast cancer cells (Takahashi and Loo, 2004)	2004
Human: Proapoptotic properties of δ-tocotrienol in breast cancer cells (Shun *et al.*, 2004)	2004
Human: Supplementation of tocotrienol-rich fraction of palm oil significantly and specifically affected MCF-7 cell response after tumor formation *in vivo* by an antioxidant-independent mechanism (Nesaretnam *et al.*, 2004)	2004
Human: Tocotrienol-rich fraction of palm oil activated p53, modulated Bax/Bcl-2 ratio, and induced apoptosis independent of cell cycle association in colorectal cancer RKO cells (Agarwal *et al.*, 2004)	2004
Mouse: Tocotrienol kills liver cancer cells (Har and Keong, 2005)	2005
Human: γ-Tocotrienol induces apoptosis of hepatoma Hep3B cells (Sakai *et al.*, 2005)	2005
Human: A redox-silent analogue of α-tocotrienol, 6-*O*-carboxypropyl-α-tocotrienol, possesses anticancer effects against lung adenocarcinoma showing poor prognosis based on the mutation of ras genes (Yano *et al.*, 2005)	2005
Mouse: γ-Tocotrienol is antineoplastic in mammary epithelial cells (Shah and Sylvester, 2005a,b; Sylvester and Shah, 2005a,b; Sylvester *et al.*, 2005)	2005
Mouse: Tocotrienols have anticancer properties *in vitro* and *in vivo* (Wada *et al.*, 2005)	2005

(*Continues*)

TABLE I. (*Continued*)

	Isoprenoid side chain of tocotrienol, not found in tocopherols, may prevent E2 epoxide induced breast cancer carcinogenesis at the initiation (Yu *et al.*, 2005)	2005
	Mouse: Preferential radiation sensitization of prostate cancer by γ-tocotrienol (Kumar *et al.*, 2006)	2006
	Tocotrienols targeted both pol lambda and angiogenesis as anticancer agents (Mizushina *et al.*, 2006)	2006
	Human: TRF of palm oil inhibited cellular proliferation and accelerated apoptosis (Srivastava and Gupta, 2006)	2006
	Human: The vitamin E succinate selenium-conjugated γ-tocotrienyl-2-phenylselenyl succinate decreased prostate cancer cell viability by stimulating caspase-3-dependent apoptosis (Vraka *et al.*, 2006)	2006
	Human: In contrast to tocopherols, tocotrienol potently inhibited telomerase activity in colorectal adenocarcinoma cells (Eitsuka *et al.*, 2006)	2006
Modulating normal mammary gland growth, function, and remodeling	Mouse: Mammary epithelial cells more easily or preferentially took up tocotrienols as compared to tocopherols (McIntyre *et al.*, 2000b)	2000
Antiangiogenic	Bovine: Tocotrienol, but not tocopherol, inhibited both the proliferation and tube formation of aortic endothelial cells (Inokuchi *et al.*, 2003)	2003
	Human/Chicken: Tocotrienol, not tocopherol, inhibited angiogenesis and telomerase activity (Nakagawa *et al.*, 2004)	2004
	Bovine: Tocotrienol, not tocopherol, limited angiogenic responses *in vitro* (Miyazawa *et al.*, 2004)	2004
	Bovine: Tocotrienols inhibited the proliferation of and formation of tubes by aortic endothelial cells, with δ-tocotrienol having the greatest effect. Tocotrienols targeted both pol lambda and angiogenesis as anticancer agents (Mizushina *et al.*, 2006).	2006
Antiproliferative and apoptotic	Mouse: Preneoplastic and neoplastic mammary epithelial cells α- and γ-tocopherols had no effect on cell proliferation (McIntyre *et al.*, 2000a)	2000
	Cancer cell lines: Not α-tocotrienol but γ-tocotrienol was apoptogenic, and more so when succinylated. Shortening the aliphatic side chain of γ-tocotrienol by one isoprenyl unit increased its activity (Birringer *et al.*, 2003)	2003

Hypocholesterolemic, antioxidant and antitumor	Chicken: The number and position of methyl substituents in tocotrienols affect their hypocholesterolemic, antioxidant, and antitumor properties; tocotrienol better than α-tocopherol (Qureshi *et al.*, 2000)	2000
Antiatherogenic	Mouse: Palm tocotrienols protect ApoE+/− mice from diet-induced atheroma formation (Black *et al.*, 2000)	2001
	Mouse: Tocotrienols inhibit atherosclerotic lesions in ApoE-deficient mice (Qureshi *et al.*, 2001b)	2001
	Rat: TRF supplementation decreased the lipid parameters in a dose-dependent manner in rats fed atherogenic diet (Minhajuddin *et al.*, 2005)	2005
	Human: Daily intake of dietary TRF by type 2 diabetics was beneficial against atherogenesis (Baliarsingh *et al.*, 2005)	2005
Serum lipoproteins; platelet function	Human: In men at risk for cardiovascular disease tocotrienol supplements used had no marked favorable effects (Mensink *et al.*, 1999)	1999
Anti-inflammatory	Human: Tocotrienols inhibit monocyte endothelial cell adhesion (Chao *et al.*, 2002)	2002
	Human: Tocotrienol is the most effective vitamin E for reducing endothelial expression of adhesion molecules and adhesion to monocytes (Theriault *et al.*, 2002)	2002
	Human: The efficacy of tocotrienol for reduction of VCAM-1 expression and adhesion of THP-1 cells to HUVECs was tenfold higher than that of tocopherol (Noguchi *et al.*, 2003)	2003
	Human: Compared to α-tocopherol, tocotrienols more potent displayed a more profound inhibitory effect on adhesion molecule expression and monocytic cell adherence (Naito *et al.*, 2005)	2005
Antifibrotic	Human: α-Tocotrienol, not tocopherol, restricted proliferation of human Tenon's capsule fibroblast (Meyenberg *et al.*, 2005)	2005
Hypolipidemic	Rat: Serum triglycerides lower in tocotrienol fed; higher IgM productivity of spleen lymphocytes and IgA, IgG, and higher IgM productivity mesenteric lymph node lymphocytes (Kaku *et al.*, 1999)	1999
	Human: Daily intake of dietary TRF by type 2 diabetics was beneficial against hyperlipidemia (Baliarsingh *et al.*, 2005)	2005
Immune function	Rats: Feeding affects proliferation and function of spleen and mesenetric lymph node lymphocytes (Gu *et al.*, 1999)	1999
Lymphatic transport	Rat: Preferential absorption of α-tocotrienol compared to γ- and δ-tocotrienols and α-tocopherol (Ikeda *et al.*, 1996)	1996

(*Continues*)

TABLE I. (*Continued*)

Drug metabolism	Tocotrienols inhibit human glutathione *S*-transferase P1–1 (van Haaften *et al.*, 2002)	2002
	Human: Vitamin E is able to activate gene expression via the pregnane X receptor (PXR), a nuclear receptor regulating a variety of drug-metabolizing enzymes. Tocotrienols more potent than tocopherols (Landes *et al.*, 2003)	2003
	Human: Tocotrienols, not tocopherols, activate the steroid and xenobiotic receptor (SXR) and selectively regulate expression of its target genes (Zhou *et al.*, 2004)	2004
	Mouse: Tocopherol, but not tocotrienol, may induce CYP3A11 and interfere with drug metabolism (Kluth *et al.*, 2005)	2005
Eye	Rat: Preferential uptake of topically applied tocotrienol, over tocopherol, by ocular tissues (Tanito *et al.*, 2004)	2004
Bone	Rat: Tocotrienols are needed for normal bone calcification in growing female rats (Norazlina *et al.*, 2002)	2002
	Rat: Tocotrienol offers better protection than tocopherol from free radical-induced damage of bone (Ahmad *et al.*, 2005)	2005
Obesity and osteoporosis	Rat: Tocotrienol, not tocopherol, has the potential to be utilized as a prophylactic agent in preventing side effects of long-term glucocorticoid use (Ima-Nirwana and Suhaniza, 2004)	2004
Diabetes	Rat: Tocotrienols-rich diet decreased advanced glycosylation end products in nondiabetic rats and improved glycemic control in streptozotocin-induced diabetic rats (Wan Nazaimoon and Khalid, 2002)	2002
Gastric lesion	Rat: Tocopherol, not alone, but in combination with tocotrienol and ubiquinone decreased gastric lesion (Nafeeza and Kang, 2005)	2005
	Rat: Tocotrienol, not tocopherol, prevents stress-induced adverse changes in the gastric acidity and gastrin level (Azlina *et al.*, 2005)	2005
Natriuretic function	Rat: An oral administration of γ-tocotrienol increases plasma concentration of 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxy chroman (LLU-α, γ-CEHC), a natriuretic compound (Hattori *et al.*, 2000)	2000
	Rat: γ-Tocotrienol is a natriuretic hormone precursor (Saito *et al.*, 2003)	2003
Bioavailability	Mouse: Supplemented tocotrienol not detected in the brain (Podda *et al.*, 1996). See 2002* below	1996
	Human: Following supplementation, ~1μM tocotrienol detected in human plasma (O'Byrne *et al.*, 2000)	2000
	Rat: The skin is a unique tissue in respect to its ability to discriminate between various vitamin E analogs; it preferentially uptakes dietary tocotrienols (Ikeda *et al.*, 2000)	2000

Human: Increased absorption of the tocotrienols in the fed versus fasted state; ~1 μM tocotrienol detected in human plasma (Yap *et al.*, 2001)	2001
Human: Tocotrienols, like tocopherols, are metabolized to CEHC; however, the quantities excreted in human urine are small in relation to dose size (Lodge *et al.*, 2001)	2001
Rat: Dietary sesame seeds elevate the tissue concentrations of orally taken tocopherols and tocotrienols (Ikeda *et al.*, 2001)	2001
Rat: Oral tocotrienol crosses the blood–brain barrier to reach brain tissue; more so for fetal brain while pregnant mother is supplemented with tocotrienol (Roy *et al.*, 2002)	2002*
Human: In HepG2 cells, tocotrienols are metabolized essentially like tocopherols, that is, by ω-oxidation followed by β-oxidation of the side chain. Quantitatively, tocotrienols are degraded to a larger extent than tocopherols (Birringer *et al.*, 2002)	2002
Rat: Sesame lignans added to diet increased plasma and tissue concentrations of supplemented tocotrienols (Yamashita *et al.*, 2002)	2002
Rat: In epididymal adipose, renal adipose, subcutaneous adipose, and brown adipose tissues and in the heart, the tocotrienol levels were maintained or increased for 24 h after intragastric administration. In the serum, liver, mesenteric lymph node, spleen, and lungs, the tocotrienol levels were highest 8 h after the administration (Okabe *et al.*, 2002)	2002
Human: Novel formulation of tocotrienol developed to improve bioavailability in humans (Ho *et al.*, 2003)	2003
Rat: Dietary α-tocopherol decreases α-tocotrienol but not γ-tocotrienol concentration in rats (Ikeda *et al.*, 2003)	2003
Tocotrienols are more readily transferred between the membranes and incorporated into the membranes than tocopherols (Yoshida *et al.*, 2003)	2003
Human: α-Tocotrienol accumulate in endothelial cells to levels approximately tenfold greater than that of α-tocopherol (Noguchi *et al.*, 2003)	2003
Rat: Of the three tocotrienols, α-tocotrienol had the highest oral bioavailability, at about $27.7 \pm 9.2\%$, compared with γ- and δ-tocotrienols, which had values of $9.1 \pm 2.4\%$ and $8.5 \pm 3.5\%$, respectively. Tocotrienols were found to be negligibly absorbed when administered intraperitoneally and intramuscularly (Yap *et al.*, 2003)	2003
Human: The $t_{1/2}$ of tocotrienols is short, ranging from 3.8 to 4.4 h for γ- and α-tocotrienols (Schwedhelm *et al.*, 2003)	2003

(*Continues*)

TABLE I. (*Continued*)

Human: Following the intervention with palm vitamin E, tocotrienols are detected in total blood plasma, TRP, LDL and HDL. Tocotrienols appeared in the blood stream at 2 h interval and disappeared within 24 h. Tocotrienols concentration in total plasma plasma, TRP and LDL peaked between 4 and 6 h; in HDL, tocotrienol concentrations peaked at 8 h after supplementation. α-Tocopherol was the major vitamin E detected in plasma. Tocotrienols have a very short duration of absorption and distribution in circulating blood (Fairus *et al.*, 2004).	2004
Rat: Following topical application of small amounts, the concentration of α-tocotrienol increased markedly in ocular tissues (e.g., crystalline lens, neural retina, and eye cup); however, no significant increase was observed in the case of α-tocopherol (Tanito *et al.*, 2004)	2004
Human: Tocotrienol uptake by aortic endothelial cells ~25- to 95-fold greater than that of α-tocopherol (Naito *et al.*, 2005)	2005
Rat: Orally taken tocotrienol reaches all vital organs *in vivo* (Khanna *et al.*, 2005a)	2005
Mouse: Orally fed tocotrienol can be delivered to vital organs *in vivo* even in TTP-deficient mice (Khanna *et al.*, 2005a). There are mechanisms other than TTP to transport tocotrienol to tissues	2005
Chicken: Estimated that the safe dose of various tocotrienols for human consumption might be 200–1000 mg/day (Yu *et al.*, 2006)	2006
Human: Single dose of tocotrienol supplementation results in 3-μM peak plasma concentration; 1.7 μM in LDL, 0.9 μM in triglyceride-rich lipoprotein, and 0.5 μM in HDL. The peak plasma level corresponds to 12- to 30-fold more than the concentration of α-tocotrienol required to completely prevent neurodegeneration. Tocotrienols were detected in the blood plasma and all lipoprotein subfractions studied postprandial (Khosla *et al.*, 2006)	2006

[a]CEHC, carboxyethyl-hydroxychromans; EGF, epidermal growth factor; HDL, high-density lipoprotein; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; HUVEC, human umbilical vein (derived) endothelial cells; IKBKAP, gene-encoding IκB kinase complex-associated protein; LDL, low-density lipoprotein; SHR, spontaneously hypertensive rats; TRF, tocotrienol-rich fraction; TRP, triglyceride-rich particles, TTP, tocopherol transfer protein.

A. NEUROPROTECTION

Glutamate toxicity is a major contributor to neurodegeneration. It includes excitotoxicity and an oxidative stress component also known as oxytosis (Schubert and Piasecki, 2001; Tan *et al.*, 2001). Murine HT hippocampal neuronal cells, lacking intrinsic excitotoxicity pathway, have been used as a standard model to characterize the oxidant-dependent component of glutamate toxicity. In 1999, we conducted a side by side comparison of all eight forms of natural vitamin E in a model of glutamate-induced neurodegeneration of HT neural cells. In subsequent experiments, it was observed that the neuroprotective property of tocotrienol applies not only to neural cell lines but also to primary cortical neurons. This line of experimentation led to an observation that eventually turned out to be the most potent function of any natural form of vitamin E on a concentration basis reported. Until then, all biological functions of vitamin E studied *in vitro* were observed at micromolar concentration. Our studies led to the first evidence that α-tocotrienol was the most potent neuroprotective form of vitamin E in glutamate-induced degeneration of HT4 hippocampal neurons (Sen *et al.*, 2000). What was striking in this study was the observation that nanomolar concentrations of α-tocotrienol, not α-tocopherol, provide complete neuroprotection. At such low dose, tocotrienol was not protective against direct oxidant insult, suggesting that the observed neuroprotective effects of nanomolar tocotrienol was not dependent on the widely known antioxidant property of vitamin E. That tocotrienol-dependent neuroprotection includes a significant antioxidant-independent mechanism has been now established (Khanna *et al.*, 2006). The neuroprotective property of tocotrienol holds good not only in response to glutamate challenge but also in response to other insults such as homocysteic acid, glutathione deficiency, and linoleic acid-induced oxidative stress (Khanna *et al.*, 2006; Sen *et al.*, 2000). It is now evident that at micromolar concentrations tocotrienol protects neural cells by virtue of its antioxidant property. At nanomolar concentrations, however, tocotrienol regulates specific neurodegenerative signaling processes.

The major tocotrienol-sensitive signaling pathways which are known to be involved in glutamate-induced neurodegeneration include c-Src and 12-lipoxygenase (12-Lox) (Khanna *et al.*, 2003, 2005b, 2006; Sen *et al.*, 2000, 2004). In our initial search for signaling pathways that are sensitive to tocotrienol and play a decisive role in neurodegeneration we were led to c-Src kinase (Sen *et al.*, 2000). c-Src and the structurally related members of the Src family are nonreceptor tyrosine kinases that reside within the cell associated with cell membranes and appear to transduce signals from transmembrane receptors to the cell interior. SH2 and SH3 domains are known to play a central role in regulating the catalytic activity of Src protein tyrosine kinase. High-resolution crystal structures of human Src, in their repressed state, have provided a structural explanation for how intramolecular interactions of the SH3 and

SH2 domains stabilize the inactive conformation of Src (Thomas and Brugge, 1997).

Our hypothesis that tocotrienol prevents neurodegeneration by regulating specific signaling processes involved in neurotoxicity led to screening for potential tocotrienol-sensitive candidate death pathways in HT4 cells. During such screening studies, inhibitors of the protein tyrosine kinase activity completely prevented glutamate-induced cell death. Herbimycin and geldanamycin potently inhibit c-Src tyrosine kinase activity (Hall *et al.*, 1994; Yoneda *et al.*, 1993), whereas lavendustin A is an inhibitor of extracellular growth factor receptor protein tyrosine kinase activity (Hsu *et al.*, 1991). The observation that herbimycin and geldanamycin, but not lavendustin A prevented glutamate-induced death of HT4 neuronal cells hinted the involvement of c-Src kinase activity in the death pathway. Immunoprecipitation of tyrosine phosphorylated protein from cellular extracts confirmed that protein tyrosine phosphorylation reactions were indeed triggered by exposure of cells to elevated levels of glutamate and that such reactions were inhibited by nanomolar concentrations of α-tocotrienol (Sen *et al.*, 2000). These results, however, did not provide any information regarding the specific kinases involved. The involvement of c-Src kinase activity in the death pathway was verified by experiments involving the overexpression of catalytically active or inactive Src kinase. Indeed, overexpression of catalytically active Src kinase markedly sensitized the cells to HT4-induced death. Tocotrienol treatment completely prevented glutamate-induced death even in active c-Src kinase overexpressing cells, indicating that it either inhibited c-Src kinase activity or regulated one or more events upstream of c-Src kinase activation. Further evidence supporting this contention was provided by results obtained from the determination of c-Src kinase activity in HT4 cells. Glutamate treatment resulted in marked enhancement of c-Src kinase activity, and this change was completely blocked in cells treated with nanomolar amounts of α-tocotrienol. Further evidence establishing that signal transduction processes related to the cell death pathway are involved in glutamate-induced cytotoxicity was obtained from the study of ERK1 and ERK2 activation. Mitogen-activated/extracellular response kinase kinase (MEK) kinase (MEKK) is a serine–threonine kinase that regulates sequential protein phosphorylation pathways, leading to the activation of mitogen-activated protein kinases (MAPKs), including members of the extracellular signal-regulated kinases (ERKs). MEKK selectively regulates signal transduction pathways that contribute to the apoptotic response (Johnson *et al.*, 1996). When activated, p44 and p42 MAPKs (ERK1 and ERK2) are phosphorylated at specific threonine and tyrosine residues. ERK has been implicated in mediating the signaling events that precede apoptosis. ERK2 plays an active role in mediating anti-IgM-induced apoptosis of B cells (Lee and Koretzky, 1998). It has also been shown that H_2O_2 induces the activation of multiple MAPKs in oligodendrocyte progenitors and that the activation of ERK is

associated with oxidant-mediated cytotoxicity (Bhat and Zhang, 1999). Our studies showed that ERK1 and ERK2 are sensitive to elevated levels of extracellular glutamate. Rapid activation of ERK, particularly ERK2, was observed in response to glutamate treatment. Such response of ERK was completely inhibited in cells treated with α-tocotrienol, suggesting that α-tocotrienol influences an early event in the glutamate-induced death pathway (Sen *et al.*, 2000). In some cases, Src kinase activity is known to be required for the activation of ERK (Aikawa *et al.*, 1997). Thus, it is likely that tocotrienol inhibits inducible ERK activation by downregulating Src kinase activity (Sen *et al.*, 2000).

c-Src is heavily expressed in the brain (Soriano *et al.*, 1991) and in human neural tissues (Pyper and Bolen, 1989). Differentiating rodent neurons are known to express high levels of c-Src. In neurons and astrocytes, c-Src is present at 15–20 times higher levels than that found in fibroblasts. The specific activity of the c-Src protein from neuronal cultures is 6–12 times higher than that from the astrocyte cultures, suggesting a key function of this protein in neurons (Brugge *et al.*, 1985). Initially, c-Src was identified as being important in growth cone-mediated neurite extension and synaptic plasticity (Maness *et al.*, 1988) and in neuronal differentiation (Ingraham *et al.*, 1989). Targeted disruption of c-Src, however, did not cause any abnormality in the brain (Soriano *et al.*, 1991). Our pursuit for the neuroprotective mechanisms of tocotrienols led to the first evidence demonstrating that rapid c-Src activation (Khanna *et al.*, 2002; Sen *et al.*, 2000) plays a central role in executing neurodegeneration. Consistently, it was demonstrated in a subsequent report that Src deficiency or blockade of Src activity in mice provides cerebral protection following stroke (Paul *et al.*, 2001). Further support of our claim that c-Src is a key player in neurodegeneration is provided by observation that the Src family kinase inhibitor PP2 reduces focal ischemic brain injury (Lennmyr *et al.*, 2004). Our observation that tocotrienol-dependent inhibition of c-Src is beneficial for neuroprotection has now been extended to the heart. A recent study showed that c-Src mediates postischemic cardiac injury and dysfunction. Tocotrienol supplementation inhibited c-Src activation and protected the heart (Das *et al.*, 2005). Many intracellular pathways can be stimulated on Src activation, and a variety of cellular consequences can result. High c-Src is tightly associated with carcinogenesis. c-Src inhibitors are being actively studied for cancer therapy (Alper and Bowden, 2005; Ishizawar and Parsons, 2004; Lau, 2005; Shupnik, 2004). On the basis of the inducible c-Src inhibitory properties of tocotrienol, one may postulate that tocotrienol has anticancer properties. The anticancer properties of tocotrienol have been discussed in a separate section below.

GSH is the major cellular thiol present in mammalian cells and is critical for maintenance of redox homeostasis (Sun *et al.*, 2006). GSH is a key survival factor in cells of the nervous system and lowered [GSH]i is one of the early markers of neurotoxicity induced by a variety of agonists

(Bains and Shaw, 1997; Dringen *et al.*, 2000). We observed that α-tocotrienol clearly protects primary cortical neurons against a number of GSH-lowering neurotoxins (Khanna *et al.*, 2003). Of interest, the neurons survived even in the face of GSH loss. These observations led to the hypothesis that loss of [GSH]i alone is not lethal (Khanna *et al.*, 2003). Given that pro-GSH agents are known to be neuroprotective in a variety of scenarios (Bains and Shaw, 1997; Han *et al.*, 1997; Schulz *et al.*, 2000), it becomes reasonable to hypothesize that glutamate-induced lowering of [GSH]i triggers downstream responses that execute cell death. Our works led to the identification of 12-Lox as a key tocotrienol-sensitive mediator of neurodegeneration (Khanna *et al.*, 2003). Specific inhibition of 12-Lox by BL15 protected neurons from glutamate-induced degeneration, although [GSH]i is compromised by 80%. Similar protective effects of BL15 were noted when BSO, a specific inhibitor of GSH synthesis, was used as the agonist. Importantly, neurons isolated from mice lacking the 12-Lox gene were observed to be resistant to glutamate-induced loss of viability (Khanna *et al.*, 2003). This key piece of evidence established that indeed 12-Lox represents a critical checkpoint in glutamate-induced neurodegeneration.

Understanding of the intracellular regulation of 12-Lox requires knowledge of the distribution of both enzyme protein and its activity. For example, in human erythroleukemia cells, the membrane fraction contains about 90% of the total cellular 12-Lox activity, whereas only 10% of 12-Lox activity resides in the cytosol. However, the majority of cellular 12-Lox protein is found in the cytosol (Hagmann *et al.*, 1993). On activation, 12-Lox may translocate to the membrane (Hagmann *et al.*, 1993). Consistently, we have observed the decreased presence of 12-Lox in the cytosol and increased presence in the membrane of glutamate-treated cells. For 5-Lox, both catalytic function and translocation of the enzyme from the cytosol to the membrane are known to be regulated by tyrosine kinases (Lepley *et al.*, 1996). Recently, we have noted that 12-Lox is subject to rapid tyrosine phosphorylation in neuronal cells challenged with glutamate or GSH-lowering agents. Such rapid phosphorylation coincides with the timeline of c-Src activation (Khanna *et al.*, 2005b). Inhibitors of c-Src abrogated inducible 12-Lox tyrosine phosphorylation, supporting the notion that c-Src may directly phosphorylate 12-Lox in challenged neurons. To test this hypothesis, we utilized genetic approaches of overexpressing kinase-active, kinase-dead, or dominant negative c-Src in neuronal cells. Findings from cell biology studies as well as from the study of c-Src and 12-Lox in cell-free systems indicate that in response to challenge by glutamate or GSH-lowering agents, c-Src is rapidly activated and phosphorylates 12-Lox (Khanna *et al.*, 2005b).

Neurons and the brain are rich in arachidonic acid (AA; 20:4ω-6). Massive amounts of AA are released from the membranes in response to brain ischemia or trauma (Bazan, 1970, 1971a,b, 1976; Bazan and Rakowski, 1970). Subsequent work has established that AA and its metabolites may

be neurotoxic. There are three major pathways of AA metabolism: Loxs, cycloxygenases, and cytochrome P450. The cycloxygenase pathway has been preliminarily ruled out from being a contributor to neurodegeneration (Kwon *et al.*, 2005). In the Lox pathway, metabolites of 12-Lox seem to be the major metabolite of arachidonic acid in the brain (Adesuyi *et al.*, 1985; Carlen *et al.*, 1994) as well as in cultured cortical neurons (Ishizaki and Murota, 1991; Miyamoto *et al.*, 1987a,b). Lipoxygenases, mainly 5-, 12-, and 15-Lox, are named for their ability to insert molecular oxygen at the 5-, 12-, or 15-carbon atom of arachidonic acid forming a distinct hydroperoxy-eicosatetraenoic (HPETE) acid (Yamamoto, 1992). 12-Lox produces 12(*S*)-HPETE which is further metabolized into four distinct products: an alcohol [12(*S*)-HETE], a ketone (12-keto-eicosatetraenoic acid), or two epoxy alcohols (hepoxilins A3 and B3). Immunohistochemical studies revealed the occurrence of 12-Lox in neurons; particularly in hippocampus, striatum, olivary nucleus, as well as in glial and in cerebral endothelial cells (Nishiyama *et al.*, 1992, 1993). Using immature cortical neurons and HT cells, it has been shown that a decrease in [GSH]i triggers the activation of neuronal 12-Lox, which leads to the production of peroxides, the influx of Ca^{2+}, and ultimately to cell death (Li *et al.*, 1997; Tan *et al.*, 2001). The 12-Lox metabolite 12-HPETE proved to be capable of causing cell death (Gu *et al.*, 2001). Inhibition of 12-Lox protected cortical neurons from β-amyloid-induced toxicity (Lebeau *et al.*, 2001). Intracellular calcium chelation delayed cell death by Lox-mediated free radicals in mouse cortical cultures (Wie *et al.*, 2001). In sum, 12-Lox poses clear threat to neuronal survival especially under GSH-deficient conditions.

Lipoxygenase activity is sensitive to vitamin E. α-Tocopherol strongly inhibits purified 5-Lox with an IC50 of 5 μM. The inhibition is independent of the antioxidant property of tocopherol. Tryptic digestion and peptide mapping of the 5-Lox–tocopherol complex indicated that tocopherol binds strongly to a single peptide (Reddanna *et al.*, 1985). Another study reported inhibition of 15-Lox by tocopherol *via* specific interaction with the enzyme protein (Grossman and Waksman, 1984). Of interest, inhibitors specific for cycloxygenase or 5-Lox are not effective in protecting neuronal cells against glutamate-induced death, suggesting a specific role of 12-Lox in glutamate-induced death (Khanna *et al.*, 2003, 2005b). Our studies addressing the effect of α-tocotrienol on pure 12-Lox indicate that α-tocotrienol directly interacts with the enzyme to suppress arachidonic acid metabolism. *In silico* studies, examining possible docking sites of α-tocotrienol to 12-Lox supports the presence of a α-tocotrienol-binding solvent cavity close to the active site. Previously, it has been demonstrated in 15-Lox that COOH terminal of arachidonic acid enters this solvent cavity while accessing the catalytic site (Borngraber *et al.*, 1999). It is therefore plausible that the binding position of α-tocotrienol prevents access of the natural substrate arachidonic acid to the active site of 12-Lox (Khanna *et al.*, 2003). Does 12-Lox have a tangible

impact on neurodegenerative processes *in vivo*? In 1992, it was reported that a mixed Lox/cyclooxygenase inhibitor SK&F 105809 reduced cerebral edema after closed head injury in rat (Shohami *et al.*, 1992). We noted that 12-Lox, but not 5-Lox (Kitagawa *et al.*, 2004), deficient mice were significantly protected against stroke-related injury of the brain (Khanna *et al.*, 2005b). The case for 12-Lox as an important mediator of neurodegeneration *in vivo* is gaining additional support from independent studies (Musiek *et al.*, 2006). 12-Lox has been also implicated in the pathogenesis of Alzheimer's (Yao *et al.*, 2005). α-Tocotrienol is capable of resisting neurodegeneration *in vivo* by opposing the c-Src and 12-Lox pathways.

B. ANTICANCER

Pure and mixed isoprenoids have potent anticancer activity (Mo and Elson, 1999). As discussed earlier in this work, tocotrienols are isoprenoids but tocopherols are not. Unlike in the case of neuroprotection where α-tocotrienol has emerged to be the most potent isoform (Khanna *et al.*, 2005b, 2006; Sen *et al.*, 2004, 2006), there seems to somewhat of a consensus that γ- and δ-tocotrienols are the most potent anticancer isoform of all natural existing tocotrienols. One of the first studies addressing the role of tocotrienols in neoplastic disorders was reported in 1989 (Komiyama *et al.*, 1989). The effects of intraperitoneally injected α- and γ-tocotrienols, as well as that of α-tocopherol, have been examined. Both tocotrienols were effective against sarcoma 180, Ehrlich carcinoma, and invasive mammary carcinoma. γ-Tocotrienol showed a slight life-prolonging effect in mice with Meth A fibrosarcoma, but the tocotrienols had no antitumor activity against P388 leukemia at doses of 5–40 mg/kg/day (Komiyama *et al.*, 1989). In contrast to tocotrienols, α-tocopherol was not as effective. The antitumor activity of γ-tocotrienol was higher than that of α-tocotrienol. In contrast to α-tocopherol, tocotrienols showed growth inhibition of human and mouse tumor cells when the cells were exposed to these agents for 72 h *in vitro* (Komiyama *et al.*, 1989). In an independent study published in the same year, the anticarcinogenic properties of palm oil, a rich source of tocotrienols, was reported (Sundram *et al.*, 1989). In this study, young female Sprague-Dawley rats were treated with a single dose of 5 mg of 7,12-dimethylbenz[α]anthracene (DMBA) intragastrically. Three days after carcinogen treatment, the rats were put on semisynthetic diets containing 20% by weight of corn oil, soybean oil, crude palm oil (CPO), refined, bleached, deodorized palm oil (RBD PO) and metabisulfite-treated palm oil (MCPO) for 5 months. During the course of experiments, rats fed on different dietary fats had similar rate of growth. Rats fed 20% corn oil or soybean oil diet had marginally higher tumor incidence than rats fed on palm oil diets. At autopsy, rats fed on high corn oil or soybean oil diets had significantly more tumors than rats fed on the three palm oil diets. Palm oil is different from corn oil and soybean oil in

many ways. In addition to possessing higher levels of tocotrienol, palm oil has a contrasting fatty acid profile and also much higher levels of tocopherol and carotenes. As such, the favorable anticarcinogenic effects noted in this study cannot be directly associated with tocotrienols (Sundram *et al.*, 1989). The antioxidant or redox property of tocotrienol is not responsible for its anticancer property. Results in support of this hypothesis show that a redox-silent analogue of α-tocotrienol, 6-*O*-carboxypropyl-α-tocotrienol is cytotoxic against A549 cells, a human lung adenocarcinoma cell line (Yano *et al.*, 2005). Although the phenolic antioxidant group in tocotrienol may not be implicated in its anticancer property, it is apparent that the phytyl side chain has some antioxidant property which prevents against carcinogenesis (Yu *et al.*, 2005).

1. Breast Cancer

Among the various forms of cancer, breast cancer has been most extensively studied in cell culture and rodent *in vivo* models for the efficacy of tocotrienols. Tocopherol and tocotrienol have been tested side-by-side for chemopreventive activity in a chemically induced rat mammary tumor model. When mammary tumors were induced by DMBA, only the tocotrienol group showed enhanced tumor latency (Gould *et al.*, 1991). The tocotrienol-rich fraction (TRF) of palm oil is not only rich in tocotrienols but also contains some α-tocopherol. The effects of TRF and α-tocopherol on the proliferation, growth, and plating efficiency of the MDA-MB-435 estrogen receptor negative human breast cancer cells have been examined (Nesaretnam *et al.*, 1995). TRF inhibited the proliferation of these cells with a concentration required to inhibit cell proliferation by 50% of 180 μg/ml, whereas α-tocopherol had no effect at concentrations up to 1000 μg/ml. The effects of TRF and α-tocopherol were also tested in longer-term experiments, using concentrations of 180 and 500 μg/ml. TRF, but not α-tocopherol, inhibited the growth as well as plating efficiency of the cells. These findings point toward the hypothesis that α-tocopherol contained in the TRF does not account for its beneficial effects and that tocotrienols may have been the active principle responsible for the observed effects of TRF (Nesaretnam *et al.*, 1995). It is now known that TRF, α-, γ- and δ-tocotrienols inhibited proliferation of estrogen receptor negative MDA-MB-435 human breast cancer cells with 50% inhibitory concentrations (IC50) of 180, 90, 30, and 90 μg/ml, respectively, whereas α-tocopherol is not effective at concentrations up to 500 μg/ml. Tocotrienols inhibit the proliferation of estrogen receptor positive MCF-7 cells. The IC50s for TRF, α-, γ-, and δ-tocotrienols have been estimated to be 4, 6, 2, and 2 μg/ml, respectively. In sharp contrast, the efficiency of α-tocopherol under comparable conditions is 20–50 times lower with an IC50 of 125 μg/ml (Guthrie *et al.*, 1997). Tamoxifen, a widely used synthetic anti-estrogen, inhibits the growth of MCF-7 cells with an IC50 of 0.04 μg/ml. In the MCF-7 cells, only 1:1 combinations of γ- or δ-tocotrienol

with tamoxifen showed a synergistic inhibitory effect on the proliferative rate and growth of the cells. α-Tocopherol did not exhibit this beneficial synergistic effect with tamoxifen (Guthrie *et al.*, 1997). The inhibition by tocotrienols was not overcome by addition of excess estradiol to the culture medium, suggesting that tocotrienols are effective inhibitors of both estrogen receptor negative and positive cells and that combinations with tamoxifen may be useful for breast cancer therapy (Guthrie *et al.*, 1997). Studies to come would strengthen support for the case that tocotrienols are effective against breast cancer *in vitro*. TRF inhibits growth of MCF-7 cells in both the presence and absence of estradiol such that complete suppression of growth is achieved at 8 μg/ml. MDA-MB-231 cells are also inhibited by TRF such that 20-μg/ml TRF is needed for complete growth suppression. The study of the individual component tocotrienols in TRF revealed that all fractions inhibit growth of both estrogen-responsive as well as estrogen-nonresponsive cells and of estrogen-responsive cells in both the presence and absence of estradiol. This estradiol-independent effect of tocotrienols is of clinical interest (Nesaretnam *et al.*, 1998, 2000). γ- and δ-Tocotrienol fractions were most potent inhibitors of breast cancer cell growth. Complete inhibition of MCF-7 cell growth was achieved at 6 μg/ml of γ/δ-tocotrienol in the absence of estradiol and 10 μg/ml of δ-tocotrienol in the presence of estradiol. In contrast, complete suppression of MDA-MB-231 cell growth was not achieved even at concentrations of 10 μg/ml of δ-tocotrienol. Of note, unlike tocotrienols α-tocopherol does not inhibit MCF-7, MDA-MB-231, or ZR-75–1 cell growth in either the presence or the absence of estradiol (Mo and Elson, 1999; Nesaretnam *et al.*, 1998, 2000). Studies examining the mechanisms by which tocotrienols check the growth of breast cancer cells have identified that tocotrienols do not act via an estrogen receptor-mediated pathway and must therefore act differently from estrogen antagonists. Furthermore, tocotrienols did not increase levels of growth inhibitory insulin-like growth factor-binding proteins in MCF-7 cells, implying also a different mechanism from that proposed for retinoic acid inhibition of estrogen-responsive breast cancer cell growth (Nesaretnam *et al.*, 1998).

Unlike α-tocopherol, δ-tocopherol seems to be more promising albeit much less so than the tocotrienols. The apoptosis-inducing properties of RRR-α-, β-, γ-, and δ-tocopherols and α-, γ-, and δ-tocotrienols have been compared in estrogen-responsive MCF-7 and estrogen-nonresponsive MDA-MB-435 human breast cancer cell lines. Vitamin E succinate, a known inducer of apoptosis in several cell lines, including human breast cancer cells, served as a positive control. The estrogen-responsive MCF-7 cells were found to be more susceptible than the estrogen-nonresponsive MDA-MB-435 cells, with concentrations for half-maximal response for tocotrienols (α, γ, and δ) and RRR-δ-tocopherol of 14, 15, 7, and 97 μg/ml, respectively. The tocotrienols (α, γ, and δ) and RRR-δ-tocopherol induced MDA-MB-435 cells to undergo apoptosis, with concentrations for half-maximal response of 176, 28, 13, and

145 μg/ml, respectively. With the exception of RRR-δ-tocopherol, the tocopherols (α, β, and γ) and the acetate derivative of RRR-α-tocopherol (RRR-α-tocopheryl acetate) were ineffective in induction of apoptosis in both cell lines when tested within the range of their solubility, that is 10–200 μg/ml (Yu *et al.*, 1999).

Mammary tissue homeostasis depends on dynamic interactions between the epithelial cells, their microenvironment (including the basement membrane and the stroma), and the tissue architecture, which influence each other reciprocally to regulate growth, death, and differentiation in the gland. The study of normal mammary epithelial cells isolated from midpregnant mice grown in collagen gels and maintained on serum-free media showed that treatment with 0- to 120-μM α- or γ-tocopherol had no effect, whereas 12.5- to 100-μM TRF, 100- to 120-μM δ-tocopherol, 50- to 60-μM α-tocotrienol, and 8- to 14-μM γ- or δ-tocotrienol significantly inhibited cell growth in a dose-responsive manner. In acute studies, 24-h exposure to 0- to 250-μM α-, γ-, and δ-tocopherols had no effect, whereas similar treatment with 100- to 250-μM TRF, 140- to 250-μM α-tocotrienol, 25- to 100-μM γ- or δ-tocotrienol significantly reduced cell viability. The observed growth inhibitory doses of TRF, δ-tocopherol, and α-, γ-, and δ-tocotrienols induced apoptosis in these cells. Mammary epithelial cells preferentially took up tocotrienols as compared to tocopherols, suggesting that at least part of the reason tocotrienols display greater potency than tocopherols is because of greater cellular uptake. These observations suggest that the highly biopotent γ- and δ-tocotrienol isoforms may play a physiological role in modulating normal mammary gland growth, function, and remodeling (McIntyre *et al.*, 2000b). A later study identified that highly malignant cells are specifically more sensitive, whereas the preneoplastic cells are least sensitive to the antiproliferative and apoptotic effects of tocotrienols (McIntyre *et al.*, 2000a). The comparative effects of tocopherols and tocotrienols were examined using preneoplastic (CL-S1), neoplastic (−SA), and highly malignant (+SA) mouse mammary epithelial cells. Over a 5-day culture period, treatment with 0- to 120-μM α- and γ-tocopherols had no effect on cell proliferation, whereas cell growth was inhibited 50% (IC50) as compared with controls by treatment with the following: 13-, 7-, and 6-μM tocotrienol-rich fraction of palm oil (TRF); 55-, 47-, and 23-μM δ-tocopherol; 12-, 7-, and 5-μM α-tocotrienol; 8-, 5-, and 4-μM γ-tocotrienol; or 7-, 4-, and 3-μM δ-tocotrienol in CL-S1, −SA, and +SA cells, respectively. Acute 24-h exposure to 0- to 250-μM α- or γ-tocopherol (CL-S1, −SA, and +SA) or 0- to 250-μM δ-tocopherol (CL-S1) had no effect on cell viability, whereas cell viability was reduced 50% (LD50) as compared with controls by treatment with 166- or 125-μM δ-tocopherol in −SA and +SA cells, respectively. Additional LD50 doses were determined as the following: 50-, 43-, and 38-μM TRF; 27-, 28-, and 23-μM α-tocotrienol; 19-, 17-, and 14-μM γ-tocotrienol; or 16-, 15-, or 12-μM δ-tocotrienol in CL-S1, −SA, and +SA cells, respectively. Treatment-induced

cell death resulted from activation of apoptosis. Consistent with previous observations, CL-S1, −SA, and +SA cells preferentially accumulated tocotrienols as compared with tocopherols. Highly malignant +SA cells were the most sensitive, whereas the preneoplastic CL-S1 cells were the least sensitive to the antiproliferative and apoptotic effects of tocotrienols (McIntyre *et al.*, 2000a).

How do tocotrienols induce apoptosis in breast cancer cells? δ-Tocotrienol induces TGF-β receptor II expression and activates TGF-β-, Fas-, and JNK signaling pathways (Shun *et al.*, 2004). Are the caspase-3, -8, -9 pathways involved in tocotrienol-induced death of cancer cells? To respond to this question, highly malignant +SA mouse mammary epithelial cells were grown in culture and maintained on serum-free media. Treatment with TRF or γ-tocotrienol, but not α-tocopherol, induced a dose-dependent decrease in +SA cell viability (Shah *et al.*, 2003). TRF- and γ-tocotrienol-induced cell death resulted from apoptosis. Treatment of cells with TRF or γ-tocotrienol increased intracellular activity and levels of processed caspase-8 and -3 but not caspase-9. Furthermore, treatment with specific caspase-8 or -3 inhibitors, but not caspase-9 inhibitor, completely blocked tocotrienol-induced apoptosis in +SA cells, suggesting that tocotrienol-induced apoptosis in +SA mammary cancer cells is mediated through activation of the caspase-8 signaling pathway and is independent of caspase-9 activation (Shah *et al.*, 2003). Tocotrienol-induced caspase-8 activation is not associated with death receptor apoptotic signaling (Shah and Sylvester, 2004). γ-Tocotrienol significantly decreases the relative intracellular levels of phospho-phosphatidylinositol 3-kinase (PI3K)-dependent kinase 1 (phospho-PDK-1 active), phospho-Akt (active), and phospho-glycogen synthase kinase 3. It also decreases the intracellular levels of FLICE-inhibitory protein (FLIP), an antiapoptotic protein that inhibits caspase-8 activation. Because stimulation of the PI3K/PDK/Akt mitogenic pathway is associated with increased FLIP expression, enhanced cellular proliferation, and survival, these observations suggest that tocotrienol-induced caspase-8 activation and apoptosis in malignant +SA mammary epithelial cells is associated with a suppression in PI3K/PDK-1/Akt mitogenic signaling and subsequent reduction in intracellular FLIP levels (Shah and Sylvester, 2004). It has been reported that the antiproliferative effects of γ-tocotrienol results, at least in part, from a reduction in Akt and NF-κB activity in neoplastic +SA mammary epithelial cells (Shah and Sylvester, 2005a).

α-Tocotrienol (20 μM) seems to share some of the cytotoxic effects on cancer cells by inducing caspase-8 and -3 activity (Sylvester and Shah, 2005a). Combined treatment with specific caspase-8 or -3 inhibitors completely blocked α-tocotrienol-induced apoptosis and caspase-8 or -3 activity, respectively. In contrast, α-tocotrienol treatment had no effect on caspase-9 activation, and combined treatment with a specific caspase-9 inhibitor did not block α-tocotrienol-induced apoptosis in +SA cells. α-Tocotrienol-induced

caspase-8 activation and apoptosis is not mediated through death receptor activation in malignant +SA mammary epithelial cells. Tocotrienol-induced caspase-8 activation and apoptosis in malignant +SA mammary epithelial cells is not mediated through the activation of death receptors, but appears to result from the suppression of the PI3K/PDK/Akt mitogenic signaling pathway, and subsequent reduction in intracellular FLIP expression (Sylvester and Shah, 2005a).

Bcl-2 family proteins tightly control apoptosis by regulating the permeabilization of the mitochondrial outer membrane and, hence, the release of cytochrome *c* and other proapoptotic factors. Is tocotrienol-induced apoptosis of cancer cells dependent on mitochondrial pathways? Incubation of MDA-MB-231 cells with γ-tocotrienol causes membrane blebbing, formation of apoptotic bodies, chromatin condensation/fragmentation, and phosphatidylserine externalization (Takahashi and Loo, 2004). These are all hallmarks of apoptosis. In γ-tocotrienol-treated cells, mitochondria were disrupted. Collapse of the mitochondrial membrane potential was followed by the release of mitochondrial cytochrome *c*. However, the expression of Bax and Bcl-2 mRNA and protein did not change. In contrast to other studies reporting that tocotrienol-induced cell death is caspase dependent (Shah and Sylvester, 2004; Shah *et al.*, 2003), it was noted that in this model caspases were not involved in γ-tocotrienol-induced apoptosis (Takahashi and Loo, 2004). In a study of +SA cells, it was noted that although γ-tocotrienol induced apoptosis, it did not disrupt mitochondrial membrane potential or cause the release of mitochondrial cytochrome *c* into the cytoplasm. Tocotrienol-treated apoptotic +SA cells showed a paradoxical decrease in mitochondrial levels of proapoptotic proteins Bid, Bax, and Bad, and a corresponding increase in mitochondrial levels of antiapoptotic proteins, Bcl-2 and Bcl-xL, suggesting that mitochondrial membrane stability and integrity might actually be enhanced for a limited period of time following acute tocotrienol exposure. This significance of this unusual finding remains obscure (Shah and Sylvester, 2005b).

Over the past 30 years, a relatively simple growth factor and its cognate receptor have provided seminal insights into the understanding of the genetic basis of cancer, as well as growth factor signaling. The epidermal growth factor (EGF), its cognate receptor (EGFR), and related family members have been shown to be important in normal as well as the malignant growth of many cell types including breast cancer. EGF is a potent mitogen for normal and neoplastic mammary epithelial cells. Initial events in EGFR mitogenic signaling are G-protein activation, stimulation of adenylyl cyclase and cyclic AMP (cAMP) production. Do the antiproliferative effects of tocotrienols associate with reduced EGF-induced G-protein and cAMP-dependent mitogenic signaling? To answer this question, preneoplastic CL-S1 mouse mammary epithelial cells were grown in culture and maintained on serum-free media, containing 0- to 25-μmol/liter tocotrienol-rich fraction of

palm oil and/or different doses of pharmacological agents that alter intracellular cAMP levels. Tocotrienol-induced effects on EGF-receptor levels of tyrosine kinase activity, as well as EGF-dependent MAPK and Akt activation, were examined. It was noted that the antiproliferative effects of tocotrienols in preneoplastic mammary epithelial cells do not reflect a reduction in EGF-receptor mitogenic responsiveness, but rather, result from an inhibition in early postreceptor events involved in cAMP production upstream from EGF-dependent MAPK and phosphoinositide 3-kinase/Akt mitogenic signaling (Sylvester *et al.*, 2002).

DMBA is a potent inducer breast cancer in rats. The antitumor and anticholesterol impacts have been examined in rats treated with the chemical carcinogen DMBA, which is known to induce mammary carcinogenesis and hypercholesterolemia. DMBA induced multiple tumors on mammary glands after 6 months. Feeding of TRF (10 mg/kg body weight/day) for 6 months, isolated from RBO, to DMBA-administered rats, attenuated the severity and extent of neoplastic transformation in the mammary glands. Consistently, plasma and mammary alkaline phosphatase activities increased during carcinogenesis were significantly decreased in TRF-treated rats. TRF treatment to rats maintained low levels of glutathione *S*-transferase activities in liver and mammary glands, which is consistent with the anticarcinogenic properties of TRF (Iqbal *et al.*, 2003). Administration of DMBA also caused a significant increase of 30% in plasma total cholesterol and 111% in LDL cholesterol levels compared with normal control levels. Feeding of TRF to rats caused a significant decline of 30% in total cholesterol and 67% in LDL cholesterol levels compared with the DMBA-administered rats. The experimental hypercholesterolemia caused a significant increase in enzymatic activity (23%) and protein mass (28%) of hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Consistent with TRF-mediated reduction in plasma lipid levels, enzymatic activity and protein mass of HMG-CoA reductase was significantly reduced. These observations support that TRF has potent anticancer and anticholesterol effects in rats (Iqbal *et al.*, 2003).

Tocotrienols act on cell proliferation in a dose-dependent manner and can induce programed cell death in breast cancer cells. To elucidate the molecular basis of the effect of tocotrienols, MCF-7 breast cancer cells were injected into athymic nude mice. Feeding quite large amounts (1 mg/day) of TRF for 20 weeks delayed the onset, incidence, and size of tumors. At autopsy, the tumor tissue was excised and cDNA array analysis was performed. Thirty out of 1176 genes were significantly affected by TRF. Ten genes were downregulated and 20 genes upregulated with respect to untreated animals. The expression of the interferon-inducible transmembrane protein-1 gene was significantly upregulated in tumors excised from TRF-treated animals compared with control mice. Within the group of genes related to the immune system, CD59 glycoprotein precursor gene was upregulated.

Among the functional class of intracellular transducers/effectors/modulators, the c-myc gene was significantly downregulated in tumors in response to TRF treatment. This work on the survey of TRF-sensitive genes in the tumor *in vivo* presented useful insight (Nesaretnam *et al.*, 2004).

2. Prostate

Unlike the literature on breast cancer cells, work on prostate cancer cells investigating the effect of tocotrienol is scant. In a model where prostate cancer was induced by injecting PC-3 cells into nude BALB/c mice, it has been noted that the radiotherapy efficacy of prostate cancer can be increased with γ-tocotrienol and a prooxidant if the kidneys can be shielded (Kumar *et al.*, 2006). When the tumors were about 5 mm in diameter, mice were injected subcutaneously with 400-mg/kg γ-tocotrienol and irradiated 24 h later at the site of the tumor with a dose of 12 Gy (60) Cobalt. The size of the tumors was reduced by almost 40%, but only in tocotrienol-treated and irradiated mice (Kumar *et al.*, 2006). The growth inhibitory and apoptotic effects of TRF have been tested on normal human prostate epithelial cells (PrECs), virally transformed normal human prostate epithelial cells (PZ-HPV-7), and human prostate cancer cells (LNCaP, DU145, and PC-3) (Srivastava and Gupta, 2006). TRF selectively resulted in potent growth inhibition in cancer cells but not normal cells. In response to TRF, cancer cells underwent G0/G1 phase arrest and sub G1 accumulation. Colony formation by all three prostate cancer cell lines studied was clearly arrested by TRF. The IC(50) after 24-h TRF treatment in LNCaP, PC-3, and DU145 cells were in the order 16.5, 17.5, and 22.0 μg/ml. TRF treatment resulted in significant apoptosis of cancer cells but not of normal cells (Srivastava and Gupta, 2006).

3. Immune System

Inhibition of tumor promotion by tocopherols and tocotrienols was examined by an *in vitro* assay utilizing the activation of Epstein–Barr virus early antigen expression in Epstein–Barr virus genome-carrying human lymphoblastoid cells. γ- and δ-tocotrienols derived from palm oil exhibited a strong activity against tumor promotion by inhibiting Epstein–Barr virus early antigen expression in Raji cells induced by 12-*O*-tetradecanoylphorbol-13-acetate. In contrast, the corresponding tocopherols lacked this activity (Goh *et al.*, 1994).

4. Liver

Tocotrienol inhibits the growth of hepatoma cells but not that of hepatocytes from healthy rat liver (Sakai *et al.*, 2004). Consistently, tocotrienol killed murine liver cancer cells but not normal cells (Har and Keong, 2005). Of note, this interesting function of tocotrienol is not shared by tocopherol. Tocotrienol-induced apoptosis of hepatoma cells is mediated by caspase-3 activation.

In addition, tocotrienol induced caspase-8 activity. An inhibitor of caspase-8 suppressed the induction of apoptosis in hepatoma by tocotrienol. Compared to tocopherol, tocotrienol was more quickly taken up by the cancer cells suggesting that this could be one reason why tocotrienol was so effective in killing the hepatoma cells (Har and Keong, 2005; Sakai *et al.*, 2004). γ-Tocotrienol inhibits the proliferation of human hepatoma Hep3B cells at lower concentrations and shorter treatment times than α-tocotrienol. γ-Tocotrienol induces poly(ADP-ribose) polymerase (PARP) cleavage activating caspase-3. In addition, γ-tocotrienol activates caspase-8 and -9 and upregulates Bax and fragments of Bid (Sakai *et al.*, 2005). In human hepatocellular carcinoma HepG2 cells, δ-tocotrienol exerts more significant antiproliferative effect than α-, β-, and γ-tocotrienols. δ-Tocotrienol induced apoptosis, and also tended to induce S phase arrest. The phase I enzyme CYP1A1 was induced by δ-tocotrienol (Wada *et al.*, 2005).

2-Acetylaminofluorene is a potent hepatocarcinogen. Prolonged feeding of rats with 2-acetylaminofluorene causes hepatocellular damage. Such damage is prevented by tocotrienol supplementation (Ngah *et al.*, 1991). 2-Acetylaminofluorene significantly increased the activities of both plasma and liver microsomal γ-glutamyltranspeptidase (GGT) and liver microsomal UDP-glucuronyltransferase (UDP-GT). Tocotrienols administered together with AAF significantly decrease the activities of plasma GGT after 12 and 20 weeks and liver microsomal UDP-GT after 20 weeks, when compared with matched controls (Ngah *et al.*, 1991). In a scenario of stronger chemical carcinogen insult caused by 2-acetylaminofluorene in conjunction with diethylnitrosamine (DEN), the effects of tocotrienol turned out to be more encouraging. In response to challenge by the chemical carcinogens, all ten rats in the group showed the presence of two grayish white nodules in the liver. Rats subjected to long-term administration of tocotrienol were protected. Only one out of six rats studied in this group had the hepatocarcinoma (Rahmat *et al.*, 1993).

The anticancer efficacy of TRF has been evaluated during DEN/2-acetylaminofluorene (AAF)-induced hepatocarcinogenesis in male Sprague-Dawley rats. TRF treatment was carried out for 6 months and was started 2 weeks before the initiation phase of hepatocarcinogenesis. Morphological examination of the livers from DEN/AAF rats showed numerous off-white patches and few small nodules, which were significantly reduced by TRF treatment. DEN/AAF caused a twofold increase in the activity of alkaline phosphatase in the plasma as compared with normal control rats. This increase of the tissue damage marker was prevented significantly by TRF treatment. Hepatic activity of glutathione *S*-transferase was also increased (3.5-fold) during the induction of hepatic carcinogenesis. Lipid peroxidation and LDL oxidation increased threefold following initiation by DEN/AAF as compared with normal control rats. TRF treatment to DEN/AAF-treated rats substantially decreased (62–66%) the above parameters and thus limited

the action of DEN/AAF. Thus, TRF exhibited clear protective properties in this model of chemical carcinogenesis (Iqbal *et al.*, 2004).

5. Gastrointestinal Tract

RKO, a poorly differentiated colon carcinoma cell line, represents a commonly used *in vitro* model for human colon carcinoma. RKO cells contain wild-type p53 but lack endogenous human thyroid receptor nuclear receptor (h-TRbeta1). In a dose- and time-dependent manner, TRF inhibited the growth and colony formation of RKO. In addition, TRF induced WAF1/p21 which appeared to be independent of cell cycle regulation and was transcriptionally upregulated in p53 dependent fashion. TRF treatment also resulted in alteration in Bax/Bcl-2 ratio in favor of apoptosis, which was associated with the release of cytochrome *c* and induction of apoptotic protease-activating factor-1. This altered expression of Bcl-2 family members triggered the activation of initiator caspase-9 followed by activation of effector caspase-3. Thus, in RKO cells the pathways involved in TRF-induced apoptosis are fairly well characterized (Agarwal *et al.*, 2004). Since the discovery that telomerase is repressed in most normal human somatic cells but strongly expressed in most human tumors, telomerase emerged as an attractive target for diagnostic, prognostic, and therapeutic purposes to combat human cancer (Shay and Wright, 2006). Tocotrienol has been noted to inhibit telomerase activity of DLD-1 human colorectal adenocarcinoma cells in a time- and dose-dependent manner. δ-Tocotrienol demonstrated the highest inhibitory activity. Tocotrienol inhibited protein kinase C activity, resulting in downregulation of c-myc and human telomerase reverse transcriptase (hTERT) expression, thereby reducing telomerase activity. Of note, tocopherol does not share the potent activity of tocotrienol in this regard (Shay and Wright, 2006).

6. Skin

How much tocotrienol is needed to inhibit the increase in population of murine B16(F10) melanoma cells during a 48-h incubation by 50% (IC50 value)? The IC50 value estimated for farnesol, the side chain analogue of the tocotrienols (50 μmol/liter) falls midway between that of α-tocotrienol (110 μmol/liter) and those estimated for γ- (20 μmol/liter) and δ- (10 μmol/liter) tocotrienol. Experimental diets were fed to weanling C57BL female mice for 10 days prior to and 28 days following the implantation of the aggressively growing and highly metastatic B16(F10) melanoma. The isomolar (116 μmol/kg diet) and the vitamin E equivalent (928 μmol/kg diet) substitution of D-γ-tocotrienol for DL-α-tocopherol in the AIN-76A diet produced 36 and 50% retardations, respectively, in tumor growth. Thus, in this skin melanoma model, both tocotrienol as well as tocopherol were significantly effective (He *et al.*, 1997). The growth suppressive effects of γ-tocotrienol on murine B16(F10) melanoma cells have been independently reproduced (Mo and Elson, 1999).

Recent works have led to the identification of antiangiogenic properties of tocotrienol (Table I). This novel development warrants further research testing the anticancer effects of tocotrienol *in vivo*.

C. CHOLESTEROL LOWERING

That the α-tocotrienol form of natural vitamin E, not tocopherol, may have significant cholesterol-lowering properties represents one of the early findings describing the unique biological properties of tocotrienol that was reported two decades ago (Qureshi *et al.*, 1986). The ER enzyme 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase produces mevalonate, which is converted to sterols and other products. It is proposed that tocotrienols are effective in lowering serum total and LDL cholesterol levels by inhibiting the hepatic enzymic activity of HMG-CoA reductase through a posttranscriptional mechanism. α-Tocopherol, however, has an opposite effect (induces) on this enzyme activity (Qureshi *et al.*, 2002). This contrast is of outstanding significance and requires further characterization. α-Tocotrienol, contained in the oily nonpolar fraction of high protein barley (*H. vulgare* L.) flour, decreased hepatic cholesterogenesis and serum total and LDL cholesterol and concomitantly increased lipogenic activity when added to chick diet. It was suspected that the isoprenoid side chain of tocotrienol was responsible for the observed inhibition of cholesterogenesis (Qureshi *et al.*, 1986). Evidence that TRF may indeed lower plasma cholesterol in mammals came from a study of normolipemic and genetically hypercholesterolemic pigs of defined lipoprotein genotype (Qureshi *et al.*, 1991a). The pigs were fed a standard diet supplemented with 50-μg/g TRF isolated from palm oil. Hypercholesterolemic pigs fed the TRF supplement showed a 44% decrease in total serum cholesterol, a 60% decrease in LDL cholesterol, and significant decreases in levels of apolipoprotein B (26%), thromboxane B2 (41%), and platelet factor 4 (PF4; 29%). It was thus noted that TRF had a marked protective effect on the endothelium and platelet aggregation. The effect of the lipid-lowering diet persisted only in the hypercholesterolemic swine after 8 week feeding of the control diet (Qureshi *et al.*, 1991a). These interesting observations were quickly put to test in humans by means of a double-blind, crossover, 8-week study (Qureshi *et al.*, 1991b). The goal was to compare effects of the tocotrienol-enriched fraction of palm oil (200-mg palmvitee capsules/day) with those of 300-mg corn oil/day on serum lipids of hypercholesterolemic human subjects (serum cholesterol 6.21–8.02 mmol/liter). Concentrations of serum total cholesterol (−15%), LDL cholesterol (−8%), Apo B (−10%), thromboxane (−25%), PF4 (−16%), and glucose (−12%) decreased significantly only in the 15 subjects given palmvitee during the initial 4 weeks. Results from the crossover study established that the noted beneficial effects were indeed caused by palmvitee. A carry over effect of palmvitee was reported. Serum cholesterol concentrations of seven hypercholesterolemic

subjects (>7.84 mmol/liter) decreased 31% during a 4-week period in which they were given 200-mg γ-tocotrienol/day. These results suggested that γ-tocotrienol could be the active principle cholesterol inhibitor in palmvitee capsules (Qureshi *et al.*, 1991b). Experimental data from the study of hamsters are in agreement (Raederstorff *et al.*, 2002). What added to the interest in tocotrienol as a cholesterol-lowering nutrient in humans was a concurrent independent study reporting the hypocholesterolemic effects of palmvitee (Tan *et al.*, 1991). Each palmvitee capsule contained ~18, 42, and 240 mg of tocopherols, tocotrienols, and palm olein, respectively. All volunteers took one palmvitee capsule per day for 30 consecutive days. Overnight fasting blood was recorded from each volunteer before and after the experiment. Palmvitee lowered both serum total cholesterol and LDL cholesterol concentrations in all subjects. The magnitude of reduction of serum total cholesterol ranged from 5.0 to 35.9%, whereas the reduction of LDL cholesterol values ranged from 0.9 to 37.0% when compared with their respective baseline values (Tan *et al.*, 1991). In another study, the cholesterol-lowering effects of palmvitee and γ-tocotrienol were examined in hypercholesterolemic subjects after acclimation to the American Heart Association Step 1 dietary regimen for 4–8 weeks, respectively (Qureshi *et al.*, 1995). The 4-week dietary regimen alone elicited a 5% significant decrease in the cholesterol level of the 36 subjects. Subjects continuing on the dietary regimen for a second 4-week period benefited from an additional 2% decrease in their cholesterol levels. The subjects experienced significant palmvitee- and γ-tocotrienol-mediated decreases in plasma cholesterol. The group of subjects acclimated to the dietary regimen for 4 weeks responded to palmvitee with a 10% statistically significant decrease in cholesterol. Of interest, α-tocopherol attenuated the cholesterol-suppressive action of the tocotrienols. This antagonism between tocopherol and tocotrienol warrants further research. The second group of subjects acclimated to the dietary regimen for 8 weeks received 200-mg γ-tocotrienol/day for 4 weeks. The cholesterol-suppressive potency of this α-tocopherol-free preparation was calculated to be equivalent to that of the mixture of tocotrienols (220 mg) used in the prior study. Cholesterol levels of the 16 subjects in the second group were significantly decreased by 13% during the 4-week trial. Plasma apolipoprotein B and *ex vivo* generation of thromboxane B2 were similarly responsive to the tocotrienol preparations, whereas neither preparation had an impact on high-density lipoprotein (HDL) cholesterol and apolipoprotein A1 levels (Qureshi *et al.*, 1995).

Tocotrienol not only of palm oil origin but also isolated from rice bran shows cholesterol-lowering properties (Chen and Cheng, 2006; Qureshi *et al.*, 2001a). A human study with 28 hypercholesterolemic subjects has been executed in five phases of 35 days each. The goal was to check the efficacy of a TRF preparation from rice bran alone and in combination with lovastatin. After placing subjects on the American Heart Association (AHA)

Step-1 diet (phase II), the subjects were divided into two groups, A and B. The AHA Step-1 diet was continued in combination with other treatments during phases III–V. Group A subjects were given 10-mg lovastatin, 10-mg lovastatin plus 50-mg TRF, 10-mg lovastatin plus 50-mg α-tocopherol per day, in the third, fourth, and fifth phases, respectively. Group B subjects were treated exactly according to the same protocol except that in the third phase, they were given 50-mg TRF instead of lovastatin. The TRF or lovastatin plus AHA Step-1 diet effectively lowered serum total cholesterol (14%, 13%) and LDL cholesterol (18%, 15%), respectively. The combination of TRF and lovastatin plus AHA Step-1 diet significantly reduced the lipid parameters by 20–25%. Especially significant were the increase in the HDL/LDL ratio to 46% in group A and 53% in group B. None of the subjects reported any side-effects throughout the study of 25 weeks (Qureshi *et al.*, 2001c). Consistent results were obtained using rice bran derived TRF in another human study (Qureshi *et al.*, 2002). A dose of 100 mg/day of TRF decreased the level of serum total cholesterol, LDL cholesterol, apolipoprotein B and triglycerides compared with the baseline values. The work led to the suggestion that a dose of 100 mg/day TRF plus AHA Step-1 diet could control the risk of coronary heart disease in hypercholesterolemic humans (Qureshi *et al.*, 2002).

Mechanistic evidence supporting the cholesterol-lowering properties of tocotrienol is considerable. Tocotrienols cause posttranscriptional suppression of HMG-CoA reductase by a process distinct from other known inhibitors of cholesterol biosynthesis (Pearce *et al.*, 1992). In addition, γ-tocotrienol may stimulate cholesterol catabolism (Chen and Cheng, 2006). *In vitro*, γ-tocotrienol possesses 30-fold greater activity toward cholesterol biosynthesis inhibition compared to α-tocotrienol. The synthetic (racemic) and natural (chiral) tocotrienols exhibited nearly identical cholesterol biosynthesis inhibition and HMG-CoA reductase suppression properties (Pearce *et al.*, 1992). Incubation of several cell types with γ-tocotrienol inhibits the rate of [14C] acetate but not [3H] mevalonate incorporation into cholesterol in a concentration- and time-dependent manner, with 50% inhibition at $\sim$2 μM and maximum $\sim$80% inhibition observed within 6 h in HepG2 cells (Parker *et al.*, 1993). Both HMG-CoA reductase activity and protein expression are sensitive to tocotrienol. *In vivo* studies lend support to that *in vitro* observation (Iqbal *et al.*, 2003). Tocotrienols influence the mevalonate pathway in mammalian cells by posttranscriptional suppression of HMG-CoA reductase, and specifically modulate the intracellular mechanism for controlled degradation of the reductase protein, an activity that mirrors the actions of the putative nonsterol isoprenoid regulators derived from mevalonate (Parker *et al.*, 1993). It is suggested that the farnesyl side chain and the methyl/hydroxy substitution pattern of γ-tocotrienol deliver a high level of HMG-CoA reductase suppression, unsurpassed by synthetic analogues studied (Pearce *et al.*, 1994). HMG-CoA reductase activity in tumor tissues differs from that of liver in being resistant to sterol feedback regulation. Tumor reductase activity retains

sensitivity to the posttranscriptional regulation. As a consequence, tocotrienol is effective in suppressing mevalonate synthesis. By doing so, tocotrienol can deplete tumor tissues of two intermediate products, farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which are incorporated posttranslationally into growth control-associated proteins (Elson and Qureshi, 1995).

Ubiquitination followed by rapid degradation by 26S proteasomes represents a key mechanism to silence HMG-CoA reductase. This pathway is activated when sterols and nonsterol end products of mevalonate metabolism accumulate in cells. Sterol-accelerated ubiquitination of HMG-CoA reductase requires Insig-1 and Insig-2, membrane-bound proteins of the ER (Sever *et al.*, 2003). Recently, it has been elegantly demonstrated that δ-tocotrienol stimulates the ubiquitination and degradation of HMG-CoA reductase and blocks processing of SREBPs, another sterol-mediated action of Insigs. The γ-tocotrienol analogue was noted to be more selective in enhancing reductase ubiquitination and degradation than blocking the processing of SREBPs. Interestingly, other forms of vitamin E neither accelerate reductase degradation nor block the processing of SREBPs. δ- and γ-tocotrienols trigger reductase ubiquitination directly and do not require further metabolism for their activity (Song and Debose-Boyd, 2006).

VIII. CONCLUSION

Often, the term vitamin E is synonymously used with α-tocopherol. While the expression is correct, it is incomplete and may be often misleading. D-α-Tocopherol (RRR-α-tocopherol) has the highest bioavailability and is the standard against which all the others must be compared. However, it is only one out of eight natural forms of vitamin E. The rapidly expanding body of evidence indicating that members of the vitamin E family are functionally unique calls for a revisit of the current practices in vitamin E research and consumption. Research claims should be limited to the specific form of vitamin E studied. For example, evidence for toxicity of a specific form of tocopherol in excess may not be used to conclude that high-dosage vitamin E supplementation may increase all-cause mortality (Miller *et al.*, 2005). Along these lines, it may not be prudent to express frustrations about the net yield of vitamin E research as a whole (Greenberg, 2005) when all that has been tested for efficacy on a limited basis in clinical trials is α-tocopherol—just one out of eight forms. It has been suggested that the safe dose of various tocotrienols for human consumption is 200–1000 mg/day (Yu *et al.*, 2006). Vitamin E represents one of the most fascinating natural resources that have the potential to influence a broad range of mechanisms underlying human health and disease. Yet, clinical outcomes studies have failed to meet expectations (Friedrich, 2004; Greenberg, 2005). The current state of knowledge warrants strategic investment into the lesser known forms of vitamin E with

emphasis on uncovering the specific conditions that govern the function of vitamin E molecules *in vivo*. Outcome studies designed in light of such information would yield lucrative returns.

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