Oxazine Derivatives of γ- and δ-Tocotrienol Display Enhanced Anticancer Activity In Vivo

SURYATHEJA ANANTHULA, PARASH PARAJULI, FATHY A. BEHERY, ALAADIN Y. ALAYOUBI, KHALID A. EL SAYED, SAMI NAZZAL and PAUL W. SYLVESTER

School of Pharmacy, University of Louisiana at Monroe, Monroe, LA, U.S.A.

Abstract. Background: Oxazine derivatives of tocotrienols display enhanced anticancer activity. Studies were conducted to further characterize these effects in vivo. Materials and Methods: Tetrazolium assay was used to determine the inhibitory effects of oxazine derivatives of γ-tocotrienol and δ-tocotrienol in vitro. These compounds were further formulated as lipid nanoemulsions and intraläsional administration was used to examine their anticancer activity in vivo. Results: Tocotrienol oxazine derivatives significantly inhibited +SA mammary tumor growth in syngeneic mice as compared to their respective parent compound, and these effects were associated with a reduction in cell proliferation and survival (phosphorylated protein kinase B (AKT) and nuclear factor κappa-light-chain-enhancer of activated B cells (NFκB), and cyclooxygenase-2 (COX2) and cell-cycle progression (cyclin D1, cyclin-dependent kinase 2 (CDK2), CDK4 and CDK6) markers, and increase in cell-cycle arrest proteins (p21 and p27). Conclusion: Tocotrienol oxazine derivatives may provide benefit as therapeutic agents against breast cancer.

The vitamin E family of compounds consists of eight naturally-occurring compounds that are further divided into two sub-groups called tocopherols and tocotrienols (1, 2). Tocopherols and tocotrienols have the same basic chemical structure characterized by a long aliphatic chain attached at the 2-position of a chromane ring structure. However, tocopherols have a saturated, while tocotrienols have an unsaturated phytyl chain (Figure 1). Individual congeners (α, β-, γ-, and δ) in each sub-group differ from each other based on the number and position of methyl groups attached to their chromane ring (Figure 1). It is becoming increasingly evident that these subtle differences in chemical structure between tocopherols and tocotrienols are reflected in large differences in biological activity and protective effects against various diseases (3). Tocotrienols, in contrast to tocopherols, display potent anticancer activity at treatment doses that have little or no effect on normal cell viability or function (4, 5). Furthermore, structural modifications of tocopherols and tocotrienols have been shown to result in derivatives that display increased (or decreased) biological activity compared to their respective parent vitamin E congener (6-8).

Recent studies have shown that electrophilic substitution resulting from Mannich and Lederer-Manasse reactions, can produce a novel carbon–carbon bond within the chromane ring of γ- and δ-tocotrienol to produced oxazine derivatives that displayed potent anticancer activity against different breast cancer cell lines (8). In brief, these reactions involve the condensation of the phenolic group of γ- and δ-tocotrienols with a primary amine and formaldehyde to produce a variety of oxazine derivatives. Using this methodology, multiple synthetic derivatives can be produced depending on the type of primary amine used and the ratio of individual reactants (8).

Since tocotrienols are highly lipophilic compounds and are poorly soluble in aqueous solutions, natural limitations exist in tocotrienol absorption, transport and tissue distribution that are characterized by low bioavailability following oral administration (9-11). In order to circumvent these natural limitations, tocotrienols and their oxazine derivatives were formulated in a lipid nanoemulsion in order to enhance solubility and stability in biological fluids when administered in vivo. As previously described in detail, emulsions of tocotrienol and tocotrienol derivatives consisted of a blend of phospholipids with hydrophilic co-emulsifiers that produced a high hydrophilic-lipophilic balance and ensured a highly stable drug-delivery system (12, 13).

In the present animal model study, tocotrienol nanoemulsions were administered by intraläsional injection. Previous studies have shown that intraläsional administration provides an effective means for concentrating drugs in breast...
carcinoma tissue and reducing systemic toxicity (14). Additional studies were also conducted to further characterize the anticancer activity and intracellular mechanism of action of specific natural tocotrienols and their oxazine derivatives.

Materials and Methods

Reagents and antibodies. All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Purified γ-tocotrienol (γT3) was generously provided by First Tech International Ltd. (Hong Kong). Antibodies for cyclin D1, cyclin-dependent kinase 2 (CDK2), CDK4, CDK6, protein kinase B (AKT), phosphorylated-AKT (activated), phosphatidylinositol 3-kinase (PI3K), cyclooxygenase-2 (COX2), phosphorylated-NFκB (activated), p21, p27, phosphorylated-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (p-IkBα) (inactivated) and α-tubulin were purchased from Cell Signaling Technology (Beverly, MA, USA). Goat anti-rabbit and anti-mouse secondary antibodies were purchased from PerkinElmer Biosciences (Boston, MA, USA). Polyoxyethylene sorbitan monooleate (Tween® 80) was provided by Uniqema (Paterson, NJ, USA). Phospholipids (Lipoid® E80S) isolated from soybean oil (64-79% phosphatidyl choline and 12-18% phosphatidyl ethanolamine) was a generous gift from Lipoid® GmbH (Ludwigshafen, Germany). Medium-chain triglyceride (MCT, Miglyol® 812) was provided by Sasol (Witten/Ruhr, Germany). PEG2000-DSPE [1,2-distearyl-sn-glycero-3-phosphoethanolamine-N-(amino-(polyethylene glycol) 2000)] ammonium salt was purchased from Avanti Polar Lipids (Pelham, AL, USA).

Cell culture. Estrogen receptor-independent +SA mouse mammary epithelial cells were derived from an adenocarcinoma that developed spontaneously in a BALB/c female mouse. These cells can be cultured on plastic and display anchorage-independent growth in soft agarose gels (15-17). +SA cells were cultured in serum-free defined media, as described previously (4, 18, 19). Briefly, cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium, supplemented with 5 mg/ml bovine serum albumin (BSA), 10 μg/ml transferrin, 100 U/ml soybean trypsin inhibitor, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 μg/ml insulin at 37°C, in an environment of 95% air and 5% CO2 in a humidified incubator. For sub-culturing, cells were washed twice with sterile Ca2+ and Mg2+-free phosphate-buffered solution (PBS) and incubated with 0.05% trypsin containing 0.025% EDTA in PBS for 5 min at 37°C. Released cells were centrifuged and re-suspended in serum-containing media and counted using a hemocytometer.

Preparation of oxazines. Preparation, structural verification and classification of oxazine derivatives of γ- and δ-tocotrienol have been previously described in detail (8). Based on results obtained in this previous investigation, oxazine derivatives of γ-tocotrienol (compounds 39, 26, and 31) and δ-tocotrienol (compounds 40 and 44) displayed enhanced anticancer activity compared to their respective natural parent compounds, and were therefore selected for further characterization of their in vivo anticancer activity and mechanism of action. Structures of natural and synthesized γ- and δ-tocotrienol compounds are shown in Figure 1.

Experimental treatments. In order to dissolve highly lipophilic tocotrienols in aqueous culture media, they must first be suspended in a sterile 10% bovine serum albumin (BSA) stock solution, as previously described (4, 18, 19). Briefly, a known amount of tocotrienol was first dissolved in 100 μl of absolute ethanol and then added to a small volume of sterile solution of 10% BSA in water to produce a final 10 mM tocotrienol/BSA stock solution. This stock solution was then used to prepare treatment media containing different concentrations of tocotrienols. Similarly, stock solutions of tocotrienol oxazine derivatives were prepared in DMSO, and then an appropriate amount of this stock solution was added directly to treatment media. Appropriate amounts of ethanol and DMSO were then added to all media so that exposure to these agents was the same for all cells in a particular experiment. The final concentration of ethanol and DMSO in any given experiment was always less than 0.1%.

Growth studies. Dose–response studies were conducted to examine the anti-proliferative effects of the treatments. +SA cells at a density of 5×103 suspended in 100 μl control serum-free defined media were seeded in each well of 96-well culture plates, and then returned to the incubator to allow cells to adhere to the bottom of the plate. On the following day, cells were divided into different treatment groups (6 wells/group), the original media were removed, and all wells received 100 μl of their respective treatment media containing 0.5 μM α-tocopherol, γ-tocotrienol, δ-tocotrienol, or γ-tocotrienol oxazine derivatives (compounds 26, 31, or 39), or δ-tocotrienol oxazine derivatives (compounds 40 or 44). Cells in all treatment groups were provided fresh media every other day throughout the experiment. After a 4-day treatment period, the viable cell number was determined in all treatment groups.

Measurement of viable cell number. The viable cell number was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay, as described previously (4, 18, 19). Briefly, media in all treatment groups were replaced with fresh medium (without compound) containing 0.5 mg/ml MTT. After a 3-h incubation period, the medium was removed, MTT crystals were dissolved in DMSO (100 μl/well), and the optical density of each sample was measured at 570 nm on a microplate reader (SpectraCount; Packard BioScience Company, Meriden, CN, USA). The number of cells/well was calculated against a standard curve prepared by plating known densities of cells, as determined by hemocytometer, at the beginning of each experiment.

Preparation of nanoemulsions. Nanoemulsions were prepared using high-pressure homogenization techniques as described previously (12, 13, 20). Briefly, an individual vitamin E isoform or its derivative was mixed at a 1:1 (w/w) ratio with medium-chain triglycerides, and then the mixture was dissolved in chloroform to ensure homogeneity of the oil phase. Samples were then placed in a vacuum oven overnight to remove the chloroform by evaporation. In a separate vial, primary and secondary emulsifiers (0.12% Lipoid® E80S and 0.05% Tween® 80) were dispersed in deionized water to which 0.25% PEG2000-DSPE was added to form the aqueous phase of the nanoemulsion. Glycero1 (2.25%) was then added to adjust tonicity. The two phases were then placed in a vacuum oven overnight to remove the chloroform by evaporation.
Figure 1. Chemical structures of α-tocopherol, γ-tocotrienol, δ-tocotrienol and their respective oxazine derivatives.
**Physical characterization of the nanoemulsions.** The intensity-weighted mean droplet size and population distribution (polydispersity index, PI) of the nanoemulsions were measured by photon correlation spectroscopy at 23°C and a fixed angle of 90° using Nicomp™ 380 ZLS submicron particle size analyzer (PSS Inc., Santa Barbara, CA, USA). Samples for size analysis were further diluted with 0.2 ml of filtered deionized water in order to minimize multiple-particle scattering and to achieve an optimal scattering intensity of 300 kHz. The size was recorded for 3 min, with viscosity and dielectric constant of the medium set to 1.33 and 78.5, respectively. Analyses were performed in triplicates unless otherwise specified.

**In vivo tumor model and study design.** Female BALB/c mice, 4-6 weeks of age, were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA) and housed in plastic cages in a temperature-regulated (24±0.5°C) and light-controlled (12 h light/12 h dark) room and allowed standard laboratory mouse chow and water *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee (Animal Welfare Assurance Number, A3641-01). At the time of tumor cell inoculation, animals were anesthetized with an *i.p.* injection of ketamine/xylazine (10 mg ketamine: 1 mg xylazine/ml saline; Henry Schein, Inc, Melville, NY) at a dose of 0.1 ml/10 g body weight. A small incision was made in the skin along the midline of the abdomen, and 1x10⁶ +SA mammary cells suspended in 100 µl 0.05 M PBS was injected into the abdominal fat pad of the number 4 left mammary gland and the incision was then closed. Animals were allowed to recover, and then returned to their cage. Mice developed palpable mammary tumors within 4-6 weeks after implantation. When tumors reached 4-5 mm in diameter, mice were randomly divided into nine experimental treatment groups (eight mice/group) that included: a) untreated control; b) α-tocopherol; c) γ-tocotrienol; d) δ-tocotrienol; e) compound 26; f) compound 31; g) compound 39; h) compound 40; and i) compound 44. Nanoemulsion treatments were administered by intralesional injection at a concentration of 120 µg/20 µl. Mice received treatment injections every other day for 11 days (total of six intralesional injections). Tumor size was determined daily for each tumor by measuring the two largest orthogonal diameters, and tumors were measured for 11 days (total of six intralesional injections). Tumor size was recorded for 3 min, with viscosity and dielectric constant of the medium set to 1.33 and 78.5, respectively. Analyses were performed in triplicates unless otherwise specified.

**Western blot analysis.** A small portion (5-10 mg) of each tumor from all treatment groups was homogenized in Laemmli buffer that consisted of 0.5 M Tris base, 10% sodium dodecyl sulfate (SDS), 2-β-mercaptoethanol, and glycerol containing 100 µM sodium orthovanadate, a protease inhibitor (22). The tissue lysates were incubated at 4°C for 30 min and mixed intermittently using a vortex, then centrifuged at 12,000 x g for 15 min at 4°C. Supernatant was collected and protein concentration in each sample was determined using Bio-Rad protein assay kit (Bio Rad, Hercules, CA, USA). Equal amounts of protein (30-40 µg) of each sample were then subjected to electrophoresis through 10-15% SDS polyacrylamide mini-gels. Each gel was then equilibrated in transfer buffer and transblotted at 30 V for 12-16 h at 4°C to a polyvinylidene fluoride membrane (PerkinElmer Lifesciences, Wellesley, MA, USA) in a Trans-Blot Cell (Bio-Rad Laboratories) according to the methods of Towbin et al. (23). Nonspecific antibody binding sites were blocked by incubating transblotted membranes in 2% BSA in 10 mM Tris-HCl containing 50 mM NaCl and 0.1% Tween 20, pH 7.4 (TBST) for 2 h. Afterwards, membranes were washed 5 times with TBST followed by incubation with specific primary antibodies raised against cyclin D1, CDK2, CDK4, CDK6, p21, p27, AKT, phosphorylated-AKT, PI3K, phosphorylated-NFκB, phosphorylated-IRkBα, COX2 and α-tubulin, diluted to 1:3000 to 1:15000 in 2% BSA in TBST for overnight at 4°C. Membranes were then washed five times in TBST and incubated with respective horseradish peroxide-conjugated secondary antibody diluted 1:3000 to 1:5000 in 2% BSA in TBST for 1 h at room temperature, and then washed three times with TBST. Specific target protein bands on each membrane were then visualized by chemiluminescence according to the manufacturer’s instructions (Pierce, Rockford, IL, USA). Images of protein bands from all treatment groups were acquired using the Syngene Imaging System (Beacon House, Nuffield Road, Cambridge, UK). The visualization of α-tubulin was used to ensure equal sample loading in each lane. Scanning densitometric analysis was performed with Kodak molecular imaging software version 4.5 (Carestream Health Inc, Rochester, NY, USA). All experiments were repeated at least three times and a representative western blot image from each experiment is shown in the Figures.

**Results.**

**Antiproliferative effects of natural tocotrienols and their synthetic oxazine derivatives in vitro.** Effects of 0-5 µM α-tocopherol, γ-tocotrienol, γ-tocotrienol oxazine derivatives (compounds 26, 31, 39), and δ-tocotrienol and δ-tocotrienol oxazine derivatives (compounds 40 and 44) on growth of +SA mammary tumor cells in culture after 4 days are shown in Figure 2. Treatment with 4-5 µM γ-tocotrienol or γ-tocotrienol oxazine derivative 39 significantly inhibited +SA tumor cell growth, compared to cells in the vehicle-treated control group (Figure 2). However, γ-tocotrienol oxazine derivatives 26 and 31 were found to be more potent than their natural γ-tocotrienol parent compound and significantly inhibited +SA mammary tumor cell growth at treatment doses equal to or greater than 1 µM and 2 µM, respectively (Figure 2). Treatment with 2-5 µM δ-tocotrienol significantly inhibited +SA mammary tumor cell growth compared to cells in the vehicle-treated control group. However, δ-tocotrienol oxazine derivative 40 was found to be less potent, while δ-tocotrienol derivative 44 was found to be more...
Figure 2. Anti-proliferative effects of natural tocotrienols and their respective oxazine derivatives on the highly malignant mouse +SA mammary tumor cell growth in serum-free defined culture media. +SA cells were initially plated at a density of 5 x 10^3 cells/well in 96-well culture plates (six replicates/group) and exposed to treatment media for a 4-day culture period. Afterward, viable cell number was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. Vertical bars indicate mean viable cell number±SEM in each treatment group. *p<0.05 compared to the respective vehicle-treated control group.
potent than their natural δ-tocotrienol parent compound in suppressing +SA tumor cell growth. Treatment with 0.5 μM or greater compound 44 significantly inhibited +SA cell growth as compared to cells in the vehicle-treated control group, whereas treatment with more than 4 μM compound 40 was required to achieve significant inhibition (Figure 2). In contrast, treatment with up to 5 μM α-tocopherol was found to have no significant effect on +SA mammary tumor cell growth (Figure 2).

Table I summarizes the relative anti-proliferative potencies of specific natural tocotrienols and their oxazine derivatives on +SA mammary tumor cell growth. Results showed that IC50 values for the γ-tocotrienol derivatives 26 and 31, and the δ-tocotrienol derivative 44 were lower than their respective natural tocotrienol parent compounds (Table I). In contrast, γ-tocotrienol derivative 30, and the δ-tocotrienol derivative 40 each had higher IC50 values than their respective natural tocotrienol parent compound (Table I). Treatment with up to 5 μM α-tocopherol had no inhibitory effects on +SA mammary tumor cell growth, confirming previous findings in other studies (4), and therefore the IC50 value for α-tocopherol was not determined and was not included in Table I.

Nanoemulsion characterization. Nanoemulsions preparations used for all treatment groups were characterized for size and PI to assess emulsion stability (Table II). The droplet diameter of all nanoemulsion treatment preparations was found to be 240−250 nm. This droplet size has been shown to be optimal for resistance to physical destabilization that can result from gravitational separation, flocculation or coalescence, and greatly reduce loss to the reticuloendothelial system when used in in vivo studies (24, 25). All treatment formulations were also found to have low PI values, ranging from 0.19 to 0.25, indicating a desirable narrow droplet size that is comparable to those of the majority of commercially available parenteral lipid emulsions (26).

Anticancer effects of natural tocotrienols and their oxazine derivatives in vivo. Comparative anti-proliferative effects of γ- and δ-tocotrienols and their respective oxazine derivatives, as shown in in vitro studies described above were then tested in an in vivo animal tumor model. Female BALB/c mice bearing syngeneic +SA mammary tumors were divided into different treatment groups and treated with nanoemulsion preparations that contained equal concentrations of α-tocopherol (negative control), γ-tocotrienol, γ-tocotrienol oxazine derivatives 26, 31 or 39, δ-tocotrienol, or δ-tocotrienol oxazine derivatives 40 or 44. An untreated control group was also added to ensure that intraleisional injection of the α-tocopherol nanoemulsion did not influence +SA mammary tumor growth through a nonspecific mechanism. +SA mammary tumor growth in mice treated with the nanoemulsion preparation containing α-tocopherol displayed continuous and rapid growth throughout the 11-day treatment period, and this growth was not found to differ significantly from tumors grown in the untreated control group (Figure 3). +SA mammary tumor growth in mice treated with γ-tocotrienol or δ-tocotrienol nanoemulsion preparations was reduced compared to the α-tocopherol-treated negative control group, but these differences were not found to be significant (p<0.06). In contrast, +SA mammary tumor growth in mice treated with the γ-tocotrienol oxazine derivatives 26, 31 or 39, or the δ-tocotrienol oxazine derivatives 40 or 44, was significantly inhibited compared to tumor growth in the α-tocopherol-treated negative control group and compared to their respective natural tocotrienol parent compounds (Figure 3). Animal body weight was not found to be significantly different between any of the different treatment groups at any time during the 11-day treatment period (data not shown).

Effects of γ- and δ-tocotrienol and their oxazine derivatives on +SA mammary tumor cell-cycle regulatory protein expression. At the conclusion of the 11-day experimental...
period, animals in the various treatment groups were sacrificed and tumors were collected for subsequent western blot analysis. Treatment with nanoemulsions containing the natural parent γ- and δ-tocotrienol compounds caused slight, but insignificant effects on +SA tumor levels of cyclin D1, CDK2, CDK4, and CDK6, cell-cycle regulatory proteins that are associated with the promotion of cell-cycle progression, compared to tumors collected from mice in the α-tocopherol-treated negative control group (Figure 4). Similarly, tumors from these same animals did not display any relative changes in levels of p21 and p27, proteins associated with inducing cell-cycle arrest, compared to α-tocopherol-treated negative control group (Figure 4). In contrast, +SA mammary tumors from mice treated with γ-tocotrienol oxazine derivatives 26, 31 or 39, or the δ-tocotrienol oxazine derivatives 40 or 44, displayed large relative decreases in cyclin D1, CDK2, CDK4, and CDK6 levels, and a corresponding large increases in p21 and p27 levels compared to +SA mammary tumors from mice in the α-tocopherol-treated negative control group (Figure 4).

Effects of γ- and δ-tocotrienol and their oxazine derivatives on +SA mammary tumor AKT and NFκB levels and activation. Western blot analysis showed that treatment with nanoemulsions containing the parent natural γ- and δ-tocotrienol compounds had little effect on levels of PI3K, AKT, phosphorylated-AKT, phosphorylated-NFκB, phosphorylated-IκBα, or COX2 in +SA mammary tumors compared to tumors collected from mice in the α-tocopherol-treated negative control group (Figure 5A and B). However, +SA mammary tumors from mice treated with the γ-tocotrienol oxazine derivatives 26, 31 or 39, or the δ-tocotrienol oxazine derivatives 40 or 44, displayed a relatively large decrease in levels of phosphorylated (active) AKT, a mitogenic signaling protein associated with promoting cell proliferation and survival, and a relatively large decrease in the intracellular ratio of phosphorylated-AKT/total AKT levels, compared to tumors collected from mice in the α-tocopherol-treated negative control group (Figure 5A). Similarly, tumors from mice treated with γ- and δ-tocotrienol oxazine derivatives displayed a relatively large
decrease in levels of phosphorylated-NFκB (active), phosphorylated-IκB (inactive), and COX2 as compared to tumors collected from mice in the α-tocopherol-treated negative control group (Figure 5B).

Discussion

Results in this study demonstrate that synthetic γ- and δ-tocotrienol oxazine derivatives display a significantly enhanced anticancer activity when compared to their respective natural parent compounds. These anti-proliferative effects of tocotrienol oxazine derivatives were associated with a reduction in protein markers associated with mitogenic signaling (activated AKT and NFκB) and cell-cycle progression (cyclin D1, CDK2, CDK4, and CDK6). Specifically, treatment with the γ-tocotrienol oxazine derivatives 26 or 31, but not 39, and δ-tocotrienol oxazine derivative 44, but not 40, were found to be more potent in suppressing in vitro +SA mammary tumor cell growth as compared to γ- and δ-tocotrienol, respectively. However, in vivo studies demonstrated that all γ- and δ-tocotrienol oxazine derivatives were significantly more potent than their respective natural tocotrienol parent compounds in suppressing +SA mammary tumor growth in syngeneic mice. Although it is not presently understood why tocotrienol oxazine derivatives are more potent anticancer agents in vivo...
than in vitro, these findings suggest that tocotrienol oxazine derivatives may provide significant benefit in the treatment of breast cancer.

A possible explanation for the greater anticancer potency of tocotrienol oxazine derivative in vivo versus in vitro may be related to their formulation as lipid nanoemulsion as used in these studies. Studies have shown that tocotrienols display low bioavailability following oral administration because these compounds are highly lipophilic and are poorly-soluble in aqueous solution (9-11). Recently, studies have shown that emulsification of lipophilic compounds not only enhances their solubility and stabilization in aqueous solutions, but also improves their biological activity and the efficacy of chemotherapeutic agents (12, 13, 25). Additional studies have shown that intraliesional injection of lipophilic nanoemulsions were extremely effective in concentrating drugs in breast carcinoma tissue and reducing systemic toxicity, providing a promising approach for drug targeting of chemotherapy in breast cancer treatment (14). The present study confirms and extends these findings by demonstrating that intraliesional injection of tocotrienols and tocotrienol oxazine derivatives is an effective strategy for optimizing the therapeutic action of these agents, and appears to bypass the natural limitations that are responsible for causing low bioavailability/rapid metabolic clearance when administered orally or systemically. It is also possible that nanoemulsion formulations of tocotrienol and tocotrienol oxazine derivatives allow a less restricted passage of these compounds through the mammary tumor cell membrane. Enhanced tumor cell uptake of these

Figure 5. Western blot analysis of PI3K, protein kinase B (AKT), phosphorylated-AKT (p-AKT), phosphorylated-nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (p-NFκB), phosphorylated-nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (p-IkBa) and cyclooxygenase 2 (COX2) levels in +5A mammary tumors grown in syngeneic BALB/c mice treated with tocotrienol and tocotrienol oxazine derivatives. Lysates prepared from each tumor were separated by polyacrylamide gel electrophoresis (40 μg/lane) followed by western blot analysis, α-Tubulin was visualized to ensure equal sample loading in each lane. Each western blot is a representative image of data obtained for experiments that were repeated at least three times. Scanning densitometry analysis was performed on all blots in triplicate and the optical density of each band was normalized with that of the α-tubulin, as shown in bar graphs. Vertical bars indicate the p-AKT/AKT ratio of optical density in each treatment group±SEM. Vertical bars in other graphs indicate the normalized integrated optical density of bands visualized in each lane +SEM. *p<0.05, as compared with the α-tocopherol-treated negative control group. C: Untreated control; α-T: α-tocopherol-treated negative control; γT3: γ-tocotrienol; δT3: δ-tocotrienol; 26, 31, and 39 γ-tocotrienol oxazine derivatives; and 40 and 44 δ-tocotrienol oxazine derivatives.
compounds would allow higher levels of these compounds to be present at their intracellular sites of action, and thereby enable a greater biological response. Additional studies are required to determine if this suggestion is correct.

Previous studies synthesized a total of 42 tocotrienol oxazine derivatives by subjecting the chroman ring to electrophilic substitution reactions using the Mannich and Lederer-Manasse procedures (8). Preliminary screening of these compounds indicated that the γ-tocotrienol oxazine derivatives 26, 31 and 39, and δ-tocotrienol oxazine derivatives 40 and 44 displayed the highest potential for further development as possible anticancer therapeutic agents (8). Therefore, these agents were selected in the present study for further characterization of their anticancer activity in vivo. Experimental findings in the present study provide additional insight on the possible intracellular mechanism of action mediating the anticancer effects of these agents.

Cell proliferation requires progression through the cell cycle. The cell cycle consists of a series of events, highly regulated by various stimulatory and inhibitory proteins that control progression through the different phases of mitosis in an orderly manner. Increased expression of cyclins along with their partner CDKs at specific stages is a hallmark of cell-cycle progression. Cyclins are bound with CDKs in response to mitogenic signaling and further phosphorylate various target proteins responsible for cell-cycle promotion (27, 28). In addition, CDK inhibitors, such as p21 and p27, bind to different cyclin–CDK complexes and function as inhibitors of cell-cycle progression at different phases of mitosis (29). However, cancer cells are often characterized as having defective cell-cycle regulation, which can be expressed by an overproduction of cyclins and CDKs, coupled to a low expression of CDK inhibitors (30, 31). Intralesional injection γ- and δ-tocotrienol oxazine derivatives significantly inhibited +SA mammary tumor growth and western blot analysis of tumor samples taken from these treatment groups showed a relatively large reduction in cyclin D1, CDK2, CDK4 and CDK6, and a corresponding increase in the expression of CDK-inhibitory proteins, p21 and p27. It is well-established that the formation of cyclin D1–CDK complexes is essential for transition from the G1 to S phase during cell-cycle progression (27, 28). Taken together, these data indicate that the anticancer effects of tocotrienol oxazine derivatives on +SA mammary tumors in mice are mediated by the inhibition of cell-cycle progression.

NF-κB is a transcription factor that is overexpressed in many forms of cancer and plays an important role in tumor progression and in enhancing expression of COX2, an enzyme that stimulates prostaglandin synthesis and which plays a role in mediating the inflammatory response (32). In normal cells, NF-κB activity is restricted by the inhibitor protein, IκBα. IκBα binds to NF-κB in the cytoplasm and prevents its translocation to the nucleus, thereby preventing NFκB transcription activity (33). Phosphorylation of IκBα leads to its ubiquitination and degradation by proteasomes, and elevation in phosphorylated IκBα levels is associated with a corresponding increase in NF-κB activity (33). Treatment with tocotrienol oxazine derivatives was found to induce a relatively large decrease in phosphorylated-IκBα and phosphorylated-NFκB, as well as a corresponding decrease in COX2 level, and these effects appear to be associated with the suppression of +SA tumor growth displayed in these treatment groups.

It is also well-established that the PI3K/AKT pathway plays an important role in cell survival and growth, and enhanced PI3K/AKT signaling is associated with a poor prognosis in patients with breast cancer (34-36). Phosphorylated-AKT, the active form, has also been shown to phosphorylate and activate downstream signaling molecules that include NF-κB in the cytoplasm and its transcription activity (33). Phosphorylation of IκBα, as well as a corresponding increase in phosphorylated-AKT levels have been shown to be associated with the inhibition of receptor tyrosine kinase activation and signaling, including suppression in the activation of a number of epidermal growth factor (EGF) receptor family members and hepatocyte growth factor receptor (c-Met) receptors (19, 40-43).

In summary, oxazine derivatives of γ- and δ-tocotrienols display enhanced anticancer activity in vivo as compared to their parent natural compounds. The growth inhibitory effects of these agents appear to be mediated by a reduction in mitogenic signaling and inhibition of cell-cycle progression. These results suggest that intralesional injection of oxazine derivatives of γ- and δ-tocotrienols formulated as lipid nanoemulsion may be an effective and promising approach for the treatment of breast cancer.

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References


