Combined γ -tocotrienol and Met inhibitor treatment suppresses mammary cancer cell proliferation, epithelial-to-mesenchymal transition and migration

N. M. Ayoub*, M. R. Akl* and P. W. Sylvester*

*College of Pharmacy, University of Louisiana at Monroe, Monroe, 71291, LA, USA

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Abstract

Objectives: Dysregulation of Met signalling is associated with malignant transformation. Combined treatment has been shown to reduce Met activation and mammary tumour cell proliferation. Experiments here, were conducted to determine mechanisms involved in mediating anti-cancer effects of combined γ -tocotrienol and SU11274 (Met inhibitor) treatment in various mammary cancer cell lines.

Materials and methods: Treatment effects on mouse (+SA) and human (MCF-7, and MDA-MB-231) mammary cancer cell lines, and normal mouse (CL-S1) and human (MCF10A) mammary epithelial cell lines were compared. Cell proliferation and survival were determined by MTT assay and Ki-67 staining; protein expression was determined by western blot analysis. Immunofluorescence staining was also used to characterize expression and localization of multiple epithelial and mesenchymal markers. Cell migration was determined using a wound-healing assay.

Results: Combined treatment with γ -tocotrienol and SU11274 resulted in synergistic inhibition of +SA, MCF-7, and MDA-MB-231, but not CL-S1 or MCF10A cell growth that was associated with reduction in Akt STAT1/5 and NF κ B activation and corresponding blockade in epithelial-to-mesenchymal transition, as indicated by increased expression of E-cadherin, β -catenin, and cytokeratins 8/18 (epithelial markers) and corresponding reduction in vimentin (mesenchymal marker) and reduction in cancer cell motility.

Conclusions: Suggest that combined γ -tocotrienol and Met inhibitor treatment may provide benefit in treatment of breast cancers characterized by aberrant Met activity.

Introduction

Human breast cancer tumours are characteristically composed of heterogeneous cell types that can display a wide range of histological features and malignancy, often associated with aberrant activity of specific receptor tyrosine kinases (1–3). Met is a receptor tyrosine kinase particularly relevant to oncogenic progression as enhanced Met activity is associated with poor prognosis and an aggressive phenotype characterized by cancer cell invasion, metastasis and robust angiogenesis (1,4–6). Hepatocyte growth factor (HGF), also known as scatter factor, is the natural ligand for Met and stimulates cell motility (5). HGF is a disulphide-linked heterodimeric molecule produced predominantly by mesenchymal cells and acts in a paracrine manner to stimulate surrounding Met-expressing epithelial cells (7).

Hepatocyte growth factor activation of the Met receptor results in its dimerization, activation of tyrosine kinase activity and initiation of down-stream signalling that promotes cell proliferation and survival (5,8,9). Adaptor proteins such as Grb2, Shc, Src and the regulatory subunit phosphatidylinositol-3-kinase (PI3K) can interact directly with phosphorylated Met receptor or indirectly through scaffolding protein, Gab1, to activate downstream signalling molecules such as MAPK and transcription factors (STATs) (10). Met dysregulation can result from nearly all known oncogenic transformation mechanisms, including point mutations, and can eventually lead to constitutive activation of the tyrosine kinase domain (11). Met has been shown to be among the most mutated receptor tyrosine kinases in human cancer, with more than 20 different somatic or germ-line point mutations described so far (11). Aberrant signalling

Correspondence: P. W. Sylvester, College of Pharmacy, University of Louisiana at Monroe, 700 University Avenue, Monroe, LA 71209, USA. Tel.: +318 342 1958; Fax: +318 342 1737; E-mail: sylvester@ulm.edu

can also result from Met overexpression and gene amplification (12). Excessive Met signalling is associated with aggressive malignant phenotype (1,4) due to action of HGF, a potent inducer of epithelial-to-mesenchymal transition (EMT) in many different epithelial cell types (6,13). Epithelial cells that undergo EMT lose their epithelial cell characteristics and acquire a mesenchymal phenotype that displays migratory and invasive characteristics (7,8,14). Because of its clinical significance, Met has become a target for anti-cancer drug development. SU11274 and PHA-665752 were the first small Met inhibitor molecules developed, and provided *in vivo* evidence that inhibition of Met is an effective anti-cancer therapy (15). However, SU11274 lacks drug-like properties that apparently prevented its further development for clinical use and has only been used experimentally for *in vitro* studies and a limited number of *in vivo* studies (15).

γ -Tocotrienol is a member of the vitamin E family of compounds that displays potent anti-cancer activity at treatment doses that have little or no effect on normal cell function or viability (16,17). It is now well established that γ -tocotrienol acts to interfere with hormone and growth factor-dependent mitogenic signalling (18,19). Specially, γ -tocotrienol has been found to significantly inhibit epidermal growth factor (EGF)-dependent activation and phosphorylation of ErbB3, ErbB4 and, to a lesser extent, ErbB2, but not ErbB1 (20–22). Studies have shown that tocotrienol treatment attenuates receptor tyrosine kinase downstream mitogenic signalling, including MAPK, PI3K/Akt and JAKs/Stat and NF κ B (21,23). Recently, γ -tocotrienol treatment has been shown to reduce total Met levels and inhibit HGF-dependent Met activation in highly malignant +SA mouse mammary epithelial cells (24). Studies here, were conducted to further characterize intracellular mechanisms involved in mediating anti-cancer effects of combined γ -tocotrienol and SU11274, a specific Met inhibitor, treatment in a variety of normal and malignant mammary epithelial cell lines.

Materials and methods

Reagents and antibodies

All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Purified γ -tocotrienol was generously provided by First Tech International Ltd. (Hong Kong, Special Administrative Region of the People's Republic of China). All antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), unless otherwise stated. Antibodies for Ki-67 and cytokeratin 8/18 were purchased

from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for α -tubulin was purchased from EMD Biosciences (LA Jolla, CA, USA). Goat anti-rabbit and anti-mouse secondary antibodies were purchased from PerkinElmer Biosciences (Boston, MA, USA). Alexa Fluor 594-conjugated anti-goat antibody, Alexa Fluor 594-conjugated anti-rabbit antibody, Alexa Fluor 488-conjugated anti-mouse antibody, Alexa Fluor 488-conjugated anti-rabbit antibody and EGF were purchased from Invitrogen (Carlsbad, CA, USA). HGF was purchased from PeproTech Inc. (Rocky Hill, NJ, USA).

Cell line and culture conditions

The highly malignant +SA cell line was derived from an adenocarcinoma developed spontaneously in a BALB/c female mouse (25,26). This mammary tumour cell line is characterized as being highly malignant, oestrogen-independent and displays anchorage-independent growth when cultured in soft agarose gels (26). +SA cells were maintained in serum-free defined media containing 10 ng/ml HGF as the mitogen, Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 5 mg/ml bovine serum albumin (BSA), 10 μ g/ml transferrin, 100 U/ml soybean trypsin inhibitor, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 μ g/ml insulin at 37 °C in an environment of 95% air and 5% CO₂ in a humidified incubator (24). CL-S1 cells are immortalized normal mouse mammary epithelial cells derived from hyperplastic D1 cell line that spontaneously arose in BALB/c mice (25,27). The CL-S1 cell line is immortal in culture and forms only hyperplastic nodules, not solid tumours, upon transplantation into the mouse mammary gland fat pad (25,27). CL-S1 mammary epithelial cells were maintained in DMEM/F12 supplemented with 10% bovine calf serum (BCS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 μ g/ml insulin. Oestrogen-receptor positive MCF-7 and oestrogen-receptor negative MDA-MB-231 human breast cancer cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in DMEM/F12 supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 μ g/ml insulin. The MCF10A cell line is an immortalized normal non-tumorigenic human mammary epithelial cell line, which was purchased from ATCC. MCF10A cells were maintained in DMEM/F12 supplemented with 5% horse serum, 0.5 μ g/ml hydrocortisone, 20 ng/ml EGF, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 μ g/ml insulin. All cells were maintained at 37 °C in an environment of 95% air and 5% CO₂ in humidified incubator. For subculturing, cells were rinsed in sterile Ca²⁺ and Mg²⁺-free phosphate-buffered saline

(PBS) and incubated in 0.05% trypsin containing 0.025% EDTA in PBS for 3–15 min at 37 °C. Released cells were centrifuged, resuspended in medium and counted using a haemocytometer.

Experimental treatments

To dissolve the highly lipophilic γ -tocotrienol in aqueous culture media, stock solution of γ -tocotrienol was prepared by suspending it in a solution of sterile 10% BSA as described previously (27,28). Briefly, an appropriate amount of γ -tocotrienol was dissolved in 100 μ l absolute ethyl alcohol then added to a small volume of sterile 10% BSA in water, and incubated overnight at 37 °C. This stock solution was used to prepare various concentrations of treatment media for all experiments. Ethanol was added to all treatment groups in a given experiment in such a way that final concentration of ethanol never exceeded 0.1%. Stock solution of Met inhibitor, SU11274 was prepared in DMSO, and DMSO was added to all treatment media so that final concentration was the same in all treatment groups, never exceeding concentration of 0.1%.

Measurement of viable cell number

Viable cell count was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously (27,28). Briefly, control and treatment media in various treatment groups were replaced with fresh media containing 0.41 mg/ml MTT. After 3- to 4-h incubation, media were removed and MTT crystals were dissolved in isopropanol (1 ml/well for 24-well plates) or DMSO (100 μ l/well for 96-well plates). Optical density of each sample was measured at 570 nm on a microplate reader (SpectraCount; Packard BioScience Company, Meriden, CT, USA). Number of cells/well was calculated against a standard curve prepared by plating various concentrations of cells, as determined using a haemocytometer at the start of each experiment.

Cell growth and viability studies

+SA cells were initially seeded at 5×10^4 cells/well (6 replicates/group) in 24-well plates in serum-free defined media containing 10 ng/ml HGF as mitogen and allowed to attach overnight. Cells were then exposed to respective experimental treatments containing various concentrations of γ -tocotrienol or SU11274, alone or in combination, of subeffective concentrations for 3 days as indicated in the figure legends. MCF-7 and MDA-MB-231 cells were plated at 1×10^4 cells/well (6 replicates/

group) in 96-well plates in complete growth medium supplemented with 10% foetal bovine serum. The next day, cells were divided into different treatment groups and fed fresh treatment media containing various concentrations of individual compounds alone or in combination, for 3 days as indicated in the figure legends. To evaluate selectivity of anti-cancer effects of γ -tocotrienol and SU11274, similar studies were conducted in parallel using immortalized normal mouse (CL-S1) and human (MCF10A) mammary epithelial cell lines. CL-S1 cells were seeded at 1×10^4 cells/well (6 replicates/group) in 96-well plates in complete growth medium supplemented with 10% BCS. After attachment, CL-S1 cells were exposed to various concentrations of γ -tocotrienol and SU11274 alone or in combination for 3-days culture. MCF10A cells were seeded at 1×10^4 cells/well (6 replicates/group) in 96-well plates in complete growth medium supplemented with 5% horse serum, allowed to attach overnight, then exposed to similar experimental treatments for 3 days, as described in the Figure legends. At the end of experimentation, viable cell number was determined by MTT assay.

Western blot analysis

+SA cells were initially plated at 1×10^6 cells/100 mm culture plate, allowed to attach overnight then incubated in respective control or treatment media containing 10 ng/ml HGF for 3 days. At the end of the treatment period, cells were isolated with trypsin, washed in PBS, then whole cell lysates were prepared as described previously, in Laemmle buffer (28). Protein concentration in each sample was determined using Bio-Rad protein assay kit (Bio Rad, Hercules, CA, USA). Equal amount of protein (30–50 μ g/lane) of each sample was subjected to electrophoresis through 7.5–15% SDS-polyacrylamide minigels. Minigels within a given treatment were run simultaneously using AccuPower model 500 power supply unit (VWR, Suwanee, GA, USA). Each gel was then equilibrated in transfer buffer and transblotted at 30 V for 12–16 h at 4 °C on to a polyvinylidene fluoride membrane (PerkinElmer Lifesciences, Wellesley, MA, USA) in a Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA) according to the method of Towbin *et al.* (29). These polyvinylidene fluoride membranes were then blocked with 2% BSA in 10 mM Tris-HCl containing 50 mM NaCl and 0.1% Tween 20, pH 7.4 (TBST) then, incubated with specific primary antibodies against MEK, phospho-MEK (p-MEK), MAPK, phospho-MAPK (p-MAPK), PI3K, PDK1, phospho-PDK1 (p-PDK1), Akt, phospho-Akt (p-Akt), phospho-NF κ B (p-NF κ B), PTEN, phospho-PTEN (p-PTEN), STAT1, phospho-STAT1 (p-STAT1), STAT5, phospho-STAT5

(p-STAT5), E-cadherin, β -catenin, cytokeratin-8, cytokeratin-18, vimentin or α -tubulin at a 1:1000 to 1:5000 dilution in 2% BSA in TBST, and incubated overnight at 4 °C. At the end of the incubation period, membranes were washed 5 times in TBST then incubated in respective horseradish peroxidase-conjugated secondary antibody at 1:3000 to 1:5000 dilution in 2% BSA in TBST, for 1 h at room temperature, followed by rinsing 5 times in TBST. Blots were then visualized by chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Images of protein bands from all treatment groups within a given experiment were acquired using Kodak Gel Logic 1500 Imaging System (Carestream Health Inc, New Haven, CT, USA). Visualization of α -tubulin was used to ensure equal sample loading in each lane. All experiments were repeated at least 3 times and a representative western blot image from each experiment is shown in the figures. Densitometric analysis was performed using Kodak Molecular Imaging Software 4.5 (Carestream Health Inc). For quantification, values obtained from densitometry of western blot images for the various treatment groups were normalized to their respective α -tubulin and control densitometric values to clearly visualize differences between treatment groups.

Immunocytochemical fluorescent staining

+SA cells were seeded on 4-chamber culture slides (Becton Dickinson and Company, NJ, USA) at 1×10^5 cells/chamber (3 replicates/group) and allowed to attach in complete growth medium supplemented with 10% BCS, overnight. Cells were then washed in PBS and incubated with vehicle control or treatment-defined medium containing 10 ng/ml HGF, for 3 days. At the end of treatments, cells were washed in pre-cooled PBS, fixed in 4% formaldehyde/PBS for 6 min and permeabilized with 0.2% triton X-100 in PBS for 2 min. Fixed cells were washed in PBS and blocked with 5% donkey or goat serum in PBS for 1 h at room temperature. Cells were then incubated in specific primary antibodies to Ki-67 (1:500), E-cadherin (1:400), β -catenin (1:400), cytokeratin-8 (1:200), cytokeratin-18 (1:200) and vimentin (1:250) overnight at 4 °C in 5% donkey or goat serum in PBS. Thereafter, cells were washed five times in pre-cooled PBS followed by incubation with Alexa Fluor 594 or Alexa Fluor-488-conjugated secondary antibody (1:3000 to 1:20 000) in 5% donkey or goat serum in PBS for 1 h at room temperature. After final washing, cells were embedded in Vectashield mounting medium with DAPI (Vector Laboratories IN, Burlingame, CA, USA). Fluorescent images were obtained using confocal laser scanning microscopy (Carl Zeiss Microimaging

Inc., Thornwood, NY, USA). Percentage +SA cells displaying Ki-67 labelling was determined by counting numbers of positive Ki-67 staining cells as a proportion of total number of cells counted (stained with DAPI). Cells were counted manually in 5 photomicrographs taken randomly in each chamber, for every treatment group.

Migration assay

In vitro wound-healing assay was used to assess directional cell motility in two dimensions. +SA and MDA-MB-231 cells were plated in sterile flat-bottom 24-well plates (6 replicates/group) and allowed to form a sub-confluent cell monolayer per well, overnight. Wounds were then scratched in each cell monolayer using a sterile 200 μ l pipette tip. Medium was removed and cells were washed twice in PBS and once in fresh serum-free medium to remove floating cells. Cells were then incubated in culture media containing γ -tocotrienol and SU11274 alone or in combination, at desired concentrations in serum-free defined medium containing 10 ng/ml HGF as the mitogen. Cells were incubated for 24-h and thereafter, medium was removed and cells were washed in pre-cooled PBS, fixed in methanol previously cooled to -20 °C, and stained with Giemsa reagent. Wound healing was visualized at 0 and 2 h by Nikon ECLIPSE TE200-U microscopy (Nikon Instruments Inc., Melville, NY, USA) coupled with CoolSNAP cf CCD camera (Roper Scientific Inc., Photometrics, Tucson, AZ, USA). Digital images were captured using Nikon NIS Elements software (Nikon Instruments Inc.). Distance travelled by cells was determined by measuring wound width at 24 h and subtracting it from the wound width at the start of treatment (time zero). Values obtained were then expressed as % migration, setting the gap width at t_0 as 100%. Each experiment was performed in triplicate and distance migrated was calculated in three or more randomly selected fields per treatment group.

Statistical analysis

Differences between various treatment groups were determined by analysis of variance (ANOVA) followed by Dunnett's test or Tukey HSD test, using PASW statistics version 18. Difference of $P < 0.05$ was considered statistically significant compared to vehicle-treated control group or as defined in the figure legends. IC₅₀ values (concentrations that induce 50% cell growth inhibition) were determined using non-linear regression curve fit analysis using GraphPad Prism software version 5. Level of interaction between γ -tocotrienol and SU11274 was determined by combination index (CI),

dose reduction index (DRI) and isobologram analysis. CI is a quantitative representation of pharmacological interaction between two compounds (30,31). CI values of less than 1, 1 and greater than 1 indicate synergistic, additive, and antagonistic effects, respectively. CI values were calculated as follows (30): $CI = [S_c/S + T_c/T]$

T and S stand for the IC_{50} concentrations of individual γ -tocotrienol and SU11274, respectively, which induce 50% cell growth inhibition; T_c and S_c are concentrations of γ -tocotrienol and SU11274 that also inhibit cell growth by 50% when used in combination. DRI value represents fold decrease in dose of individual compounds when used in combination, compared to concentration of a single agent needed to achieve the same effect level (30). Favourable DRI (>1) allows dose reduction that leads to reduced toxicity, while therapeutic efficacy is retained (30,31). DRI values for γ -tocotrienol (DRIT) and SU11274 (DRIS) were calculated as the following:

$$DRIT = T/T_c \text{ and } DRIS = S/S_c$$

Isobologram analysis is a simple graphical method that can be used to evaluate the effect of equally effective dose pairs for a single effect level (32,33). An isobologram is constructed on a coordinate system composed of individual drug doses and commonly contains a straight 'line of additivity' that is employed to distinguish additive from synergistic and antagonistic interactions (33). The straight line in each isobologram was formed by plotting IC_{50} doses of γ -tocotrienol and SU11274 on x - and y -axes, respectively. The solid line connecting these points represents drug doses for each compound that induced the same relative growth inhibition when used in combination, if the interaction between these compounds is additive. The data point in each isobologram corresponds to IC_{50} dose of γ -tocotrienol and SU11274 given in combination. If a data point is on the line, this represents additive treatment effect, whereas a data point that lies below or above the line indicates synergism or antagonism, respectively.

Results

Effects of γ -tocotrienol and SU11274 on mammary tumour cell growth

Effects of various doses of γ -tocotrienol and SU11274 on the growth of mouse (+SA) and human (MCF-7 and MDA-MB-231) mammary tumour cell lines after 3-days culture are shown in Fig. 1. Treatment with 5–5.5 μ M γ -tocotrienol or 4–5.5 μ M SU11274 significantly inhibited HGF-dependent +SA cell growth in a dose-responsive manner compared to cells in the vehicle-treated control

Figure 1. +SA mouse mammary tumour cells were initially plated at 5×10^4 cells/well (6 replicates/group) in 24-well plates and maintained on defined serum-free media containing 10 ng/ml hepatocyte growth factor. MCF-7 and MDA-MB-231 human mammary cancer cells were initially plated at 1×10^4 cells/well in 96-well plates and maintained on DMEM/F-12 media containing 10% fetal bovine serum. Next day, all cells were divided into different treatment groups and exposed to various doses of γ -tocotrienol or SU11274 throughout a 3-day culture period. At the end of treatment, viable cell number was determined by the MTT colorimetric assay. Each bar indicates mean number of cells/well $\pm$ SEM in each treatment group from a given experiment. Each experiment was repeated three times. * $P < 0.05$ compared with respective vehicle-treated control group.

group. IC_{50} values for γ -tocotrienol and SU11274 were 4.85 and 4.81 μ M, respectively, in +SA cells. Similarly, treatment with 0–30 μ M γ -tocotrienol or 0–10 μ M SU11274 resulted in dose-dependent inhibition of MCF-7

cell growth compared to the vehicle-treated control group, with IC_{50} values of 26.72 and 7.15 μM for γ -tocotrienol and SU11274, respectively (Fig. 1). Treatment with 20–25 μM γ -tocotrienol or 8–12 μM SU11274 significantly inhibited MDA-MB-231 cell growth in a dose-responsive manner compared to cells in the vehicle-treated control group, with IC_{50} values of 21.12 and 9.96 μM for γ -tocotrienol and SU11274, respectively (Fig. 1).

Effects of combined treatment of γ -tocotrienol with SU11274 on mammary tumour cell growth

Effects of combined treatment of subeffective concentrations of γ -tocotrienol and SU11274 on growth of mouse

(+SA) and human (MCF-7 and MDA-MB-231) mammary cancer cell lines after 3-days culture are shown in Fig. 2. Treatment with 1–3.5 μM γ -tocotrienol or 3 μM SU11274 had no effect on +SA cell viability (Fig. 2a). However, combined treatment of 3 μM SU11274 with 1–3.5 μM γ -tocotrienol significantly inhibited HGF-dependent +SA cell growth in a dose-responsive manner (Fig. 2a). Similarly, combined treatment with subeffective doses γ -tocotrienol (5–20 μM) with subeffective concentration of SU11274 (3 μM) resulted in significant inhibition of MCF-7 cell growth compared to cells in the vehicle-treated control group (Fig. 2a). Treatment with 5–15 μM γ -tocotrienol or 4 μM SU11274 alone had no effect on MDA-MB-231 cell viability, whereas combined treatment with subeffective doses of γ -tocotrienol

Figure 2. (a) +SA mouse mammary tumour cells were initially plated at 5×10^4 cells/well (6 replicates/group) in 24-well plates and maintained on defined serum-free media containing 10 ng/ml hepatocyte growth factor. MCF-7 and MDA-MB-231 human mammary cancer cells were initially plated at 1×10^4 cells/well in 96-well plates and maintained on DMEM/F-12 media containing 10% fetal bovine serum. Next day, all cells were divided into different treatment groups and exposed to various doses of γ -tocotrienol and/or SU11274 throughout a 3-day culture period. At the end of the treatment, viable cell number was determined by the MTT colorimetric assay. Each experiment was repeated three times. * $P < 0.05$ compared with respective vehicle-treated control group. (b) Isobolograms of γ -tocotrienol and SU11274 anti-proliferative effects on multiple mammary cancer cell lines. IC_{50} concentrations (dose that induced a 50% inhibition of cell growth following a 3-day culture period) for γ -tocotrienol and SU11274 were plotted on the y and x axis, respectively. The solid line connecting these points represents the concentration of each compound required to induce the same relative growth inhibition when used in combination if the interaction between the compounds is additive. The data point on each isobologram represents the actual doses of γ -tocotrienol and SU11274, which, when used in combination, result in 50% inhibition of mammary cancer cell growth over a period of 3 days in culture. The isobolograms of the three different mammary cancer cell lines show data points to be positioned below the line, indicating a strong synergistic anti-proliferative effect for the various combinations of γ -tocotrienol and SU11274 used.

and 4 μM SU11274 caused significant dose-responsive inhibition of MDA-MB-231 cell growth compared to vehicle-treated controls (Fig. 2a).

Isobologram analysis of combined treatment effects of γ -tocotrienol and SU11274 in the different cancer cell lines is shown in Fig. 2b. Results showed that growth inhibitory effects of combined treatment of γ -tocotrienol and SU11274 in +SA, MCF-7, and MDA-MB-231 cancer cells was synergistic, as the data point in each isobologram was below the line of additivity (Fig. 2b). Similarly, CI analysis for growth suppressive effects of combined γ -tocotrienol and SU11274 indicates high level of synergism with CI values less than 1 in all mammary cancer cell lines examined (Table 1). CI value for combination of γ -tocotrienol and SU11274 in mouse +SA mammary tumour cells was 0.88, whereas CI value for their respective combined treatments in MCF-7 and MDA-MB-231 human breast cancer cell lines were 0.71 and 0.61, respectively (Table 1). In addition, DRI analysis showed multifold reduction of growth inhibitory dosage for combined γ -tocotrienol and SU11274 compared to each compound alone. IC_{50} dose of γ -tocotrienol was reduced approximately 4-fold with combination treatment in +SA cells, reduced more than 3-fold in MCF-7 cells, and nearly 5-fold in MDA-MB-231 cells, when used in combination with SU11274 compared to γ -tocotrienol treatment alone (Table 1).

Effects of SU11274 and γ -tocotrienol on normal mouse and human mammary epithelial cell growth

Treatment effects on growth of immortalized normal mouse (CL-S1) and human (MCF10A) mammary epithelial cells over 3-days culture are shown in Fig. 3. Results show that treatment with 0–30 μM γ -tocotrienol had no effect on CL-S1 and MCF10A cell viability compared to their respective vehicle treated control groups (Fig. 3). In contrast, treatment with 15–20 μM and 10–15 μM SU11274 on CL-S1 and MCF10A cells, respectively, caused significant inhibition of growth in these cell lines, with IC_{50} values of 17.27 and 13.83 μM in CL-S1 and MCF10A cells, respectively (Fig. 3). In combination

studies, similar treatment doses to those used on cancer cell lines showed that combined treatment of 0–3.5 μM or 0–20 μM γ -tocotrienol with 3 μM SU11274 had no effect on CL-S1 or MCF-10A cell growth, compared to their respective vehicle-treated controls (Fig. 3).

Effects of γ -tocotrienol and SU11274 on Ki-67 labelling in highly malignant mouse +SA mammary tumour cells

Positive Ki-67 staining is a marker of proliferating cells (35). This was observed in 88% of +SA mammary tumour cells grown in control medium containing 10 ng/ml HGF after a 3-day culture period (Fig. 4). Treatment with subeffective doses of γ -tocotrienol (2 μM) or SU11274 (3 μM) alone resulted in positive Ki-67 nuclear staining in approximately 83% and 82%, respectively, in +SA cells (Fig. 4). In contrast, combined treatment with these agents resulted in less than 5% positive Ki-67 staining in +SA cells (Fig. 4).

Effects of combined γ -tocotrienol and SU11274 treatment on HGF/Met downstream signalling

Treatment with subeffective doses of γ -tocotrienol (2 μM) or SU11274 (3 μM) alone had little or no effect, whereas combined treatment with these agents resulted in large relative reduction in total levels of PI3K, STAT1, and STAT5, but not Akt, in +SA mammary tumour cells (Fig. 5). Combined treatment with these agents also caused large reduction in phosphorylated (activated) STAT1, STAT5, PI3K, Akt and NF κ B levels compared to individual compounds or vehicle-treated controls (Fig. 5). Western blot analysis showed that intracellular levels of MAPK, p-MAPK, MEK and p-MEK displayed some reductions, but these differences were not found to be significantly different from those levels of their respective vehicle-treated control group (Fig. 5).

Effects of γ -tocotrienol and SU11274 on HGF-induced mammary tumour cell migration

Treatment effects on mouse (+SA) and human (MDA-MB-231) mammary cancer cell motility were determined using the wound-healing assay as shown in Fig. 6. HGF-induced cell migration was observed with more than 85% wound closure for both +SA and MDA-MB-231 mammary tumour cells after 24-h treatment period, over their respective vehicle-treated control groups (Fig. 6). Treatment with subeffective doses of γ -tocotrienol (2 μM) or SU11274 (3 μM) alone resulted in slight reduction in HGF-induced migration of +SA and MDA-MB-231 mammary tumour cells, whereas combined treatment with these agents significantly inhibited +SA

Table 1. CI and DRI values for combined treatment of γ -tocotrienol and SU11274 resulting in 50% reduction in the growth of various mammary cancer cell lines.

Mammary cancer cell line	CI	DRI SU11274	DRI γ -tocotrienol
+SA	0.88	1.6	3.8
MCF-7	0.71	2.4	3.5
MDA-MB-231	0.61	2.5	4.9

CI, Combination index; DRI, drug reduction index.

and MDA-MB-231 mammary tumour cell migration to an approximate 26% wound closure in both groups, compared to their respective vehicle-treated control groups (Fig. 6).

Effects of γ -tocotrienol and SU11274 on epithelial and mesenchymal cell marker expression

+SA mammary tumour cells grown in medium containing 10 ng/ml HGF as mitogen displayed strong transition from epithelial to mesenchymal cell phenotype, as

evidenced by low expression of epithelial markers and prominent expression of mesenchymal markers. Specifically, +SA cells in the control group expressed relatively low levels of the epithelial protein markers E-cadherin, β -catenin, cytokeratin-8 and cytokeratin-18, and relatively high level of expression of mesenchymal protein marker vimentin (Fig. 7a). Treatment with subeffective doses of γ -tocotrienol (2 μ M) or SU11274 (3 μ M) alone resulted in slight reduction in EMT, characterized by slight increase in the level of some epithelial markers (cytokeratin-8 and cytokeratin-18), but little or no

Figure 3. (a) Anti-proliferative effects of γ -tocotrienol and/or SU11274 on the growth of immortalized normal CL-S1 (mouse) and MCF10A (human) mammary epithelial cells. CL-S1 cells were initially plated at 1×10^4 cells/well (6 replicates/group) in 96-well culture plates and exposed to treatment media containing 10% bovine calf serum, while MCF10A cells were initially plated at 1×10^4 cells/well (6 replicates/group) in 96-well culture plates and exposed to treatment in media containing 5% horse serum for a period of 3 days. (b) Effects of combined treatment of a subeffective dose of SU11274 with a range of subeffective doses of γ -tocotrienol on the growth of CL-S1 and MCF10A cells. All cells were initially plated at 1×10^4 cells/well (6 replicates/group) and exposed to various treatments for a 3-day culture period. Thereafter, viable cell number was determined by the MTT colorimetric assay. Each bar indicates the mean number of cells $\pm$ SEM in each treatment group. Each experiment was repeated at least three times. * $P < 0.05$ as compared with their respective vehicle-treated control group.

Figure 4. (a) +SA cells were plated on 4-chamber culture slides at 1×10^5 cells/chamber (3 replicates/group) and allowed to attach in complete growth media supplemented with 10% bovine calf serum overnight. Cells were then washed in phosphate-buffered saline and incubated in defined serum-free media containing 10 ng/ml HGF as a mitogen and 0–2 μM γ -tocotrienol (γT^3) and/or 0–3 μM SU11274 (SU) for a 3-day culture period. Thereafter, cells were fixed in 4% formaldehyde/PBS and permeabilized with 0.2% triton X-100. Fixed cells were then blocked and incubated in specific primary antibody for Ki-67 followed by incubation with Alexa Fluor 594-conjugated secondary antibody as described in the Materials and methods section. Red colour in the photomicrographs indicates positive fluorescence staining for Ki-67, while the blue represents counterstaining of cell nuclei, with DAPI. Magnification of each image is 200 $\times$. (b) Percentage of +SA cells displaying positive Ki-67 staining in proportion to total number of cells in each treatment group. Vertical bars represent per cent positive Ki-67 staining $\pm$ SEM in each treatment group. * $P < 0.05$ compared to vehicle-treated control group. Cells were counted manually in five photomicrographs selected randomly in each chamber for each treatment group. This experiment was repeated at least three times.

change in vimentin expression compared to +SA cells in the vehicle-treated control group (Fig. 7a). In contrast, combined treatment with subeffective doses of γ -tocotrienol and SU11274 reversed EMT and cells in this group were characterized by displaying relatively high level of expression of epithelial protein markers (E-cadherin, β -catenin, cytokeratin-8 and cytokeratin-18)

and corresponding large relative reduction in expression of mesenchymal protein marker, vimentin, compared to vehicle-treated controls (Fig. 7a).

Treatment effects on immunofluorescence staining of epithelial and mesenchymal protein markers in +SA mammary tumour cells are shown in Fig. 7b. Similar to results observed in the western blot analysis, +SA cell

Figure 5. (a) +SA mammary tumour cells were plated at 1×10^6 cells/100-mm culture dish. Cells were then incubated in serum-free defined media containing 10 ng/ml HGF as a mitogen and 0–2 μ M γ -tocotrienol (γ T³) and/or 0–3 μ M SU11274 (SU) for a 3-day culture period. Thereafter, cells were isolated with trypsin and whole cell lysates were prepared and then subjected to polyacrylamide gel electrophoresis and western blot analysis for total MEK, p-MEK, MAPK, p-MAPK, STAT1, p-STAT1, STAT5, p-STAT5, PI3K, PDK1, p-PDK1, Akt, p-Akt, p-NF κ B105, PTEN and p-PTEN (Ser380/Thr382/383) levels. α -tubulin was visualized to ensure equal sample loading in each lane. Each western blot is a representative image of the data obtained for experiments that were repeated at least three times. **(b)** Scanning densitometric analysis was performed for each blot to visualize the relative levels of proteins. Integrated optical density of each band was normalized with their corresponding α -tubulin and control treatment bands and then shown in bar graphs. Vertical bars indicate the fold-change in protein levels in various treatment groups $\pm$ SEM as compared with their respective vehicle-treated control group. * $P < 0.05$ compared to their respective vehicle-treated control group.

maintained in control medium displayed relatively low levels of positive immunofluorescence staining for epithelial markers E-cadherin, β -catenin, cytokeratin-8 and cytokeratin-18, and corresponding relatively high level of positive staining for mesenchymal marker, vimentin (Fig. 7b). Treatment with subeffective doses of γ -tocotrienol (2 μ M) or SU11274 (3 μ M) alone resulted in a slight increase in positive immunofluorescence staining for epithelial protein markers and slight reduction in mesenchymal marker expression, compared to cells in the vehicle-treated control group (Fig. 7b). However, combined treatment with these same agents resulted in reversal of HGF-induced EMT, characterized by relatively large increase in epithelial marker (E-cadherin, β -catenin, cytokeratin-8 and cytokeratin-18) expression and corresponding large relative reduction in mesenchymal marker (vimentin) expression compared to cells in the vehicle-treated control group (Fig. 7b).

Discussion

Results from these studies demonstrated that combined treatment with subeffective doses of γ -tocotrienol and specific Met inhibitor, SU11274, resulted in significant

and synergistic inhibition of mouse and human mammary tumour cell growth, while having little or no effect on immortalized normal mouse and human mammary epithelial cell growth. Anti-proliferative effects of combination treatment with these agents were found to be associated with suppression in HGF-dependent mitogenic signalling characterized by suppression in Akt, STAT5 and NF κ B activation. This same combination treatment was also found to reverse HGF-dependent EMT as demonstrated by reduction in mesenchymal (vimentin) and corresponding increased in epithelial (cytokeratin, E-cadherin and β -catenin) cell marker expression. Furthermore, this reversal in EMT was characteristically associated with a large reduction in cell motility of highly malignant mouse and human mammary cancer cell lines.

Numerous studies during the past decade have established the potent anti-cancer action of tocotrienols (16–18). Anti-proliferative and apoptotic effects of γ -tocotrienol against breast cancer cells appear to be mediated, at least in part, through inhibition of receptor tyrosine kinase activation and mitogenic signalling. Similarly, SU11274 has been shown to be 50 times more selective in inhibiting Met activity than any other receptor tyrosine kinase (35). SU11274 acts by competing with

Figure 6. (a) Photomicrographs of γ -tocotrienol (γT^3) and SU11274 (SU) treatment effects on +SA and MDA-MB-231 mammary tumour cell migration in response to hepatocyte growth factor stimulation using the *in vitro* wound-healing assay. Cells in each treatment group were plated in sterile flat-bottom 24-well plates (6 replicates/group) and allowed to form a subconfluent cell monolayer overnight. Wounds were then scratched in each cell monolayer using a sterile 200 μl pipette tip. Medium was then removed, cells were washed and then exposed to their respective treatments for a 24-h culture period. Photomicrographs (100 $\times$ magnification) were taken at the beginning and end of the treatment period. (b) Quantitative analysis of wound closure in each treatment group was calculated relative to wound distance at time 0. Vertical bar represents per cent migration $\pm$ SEM. Each experiment was performed in triplicate and the distance migrated was calculated in three or more randomly selected fields per treatment group. * $P < 0.05$ compared to their respective vehicle-treated control group. # $P < 0.05$ compared to γ -tocotrienol or SU11274 treatment alone.

ATP at the catalytic site on Met and thereby suppressing Met tyrosine kinase activity and ultimately attenuating downstream mitogenic signalling, particularly the PI3K/PDK/Akt pathway (35). Previous findings have shown that combined treatment with subeffective doses of γ -tocotrienol and SU11247 significantly inhibited HGF-dependent mouse +SA mammary tumour cell growth, and this effect was associated with large reduction in total and phosphorylated (activated) levels of Met (24). Recommended safe dose limit is 200–1000 mg/day in humans for various tocotrienol isoforms (36). Treatment doses of

γ -tocotrienol in the present study are physiologically relevant based on serum concentrations that were found to range between 2 and 4 μM in human subjects given a single 300 mg oral dose of mixed tocotrienols, under fed or fasting conditions (37). Similarly, SU11274 and other Met inhibitors have been found to display potent anti-cancer effects using daily treatment doses from 10 to 100 mg/kg days, in mouse xenograph tumour studies (38). Results in the present study demonstrate that low-dose combined treatment with γ -tocotrienol and SU11275 displays synergistic inhibition of Met activation

and signalling and strongly suggests that this type of therapy using tocotrienols together with Met inhibitors may greatly improve therapeutic responsiveness in patients with metastatic breast cancer.

Alterations in receptor tyrosine kinase expression, activation, and signalling play an important role in aetiology of many types of cancer (3). In normal mammary tissue, HGF is prominently expressed in the surrounding

Figure 7. (a) Western blot analysis of treatment effect of γ -tocotrienol (γT^3) and/or SU11274 (SU) on expression of major epithelial and mesenchymal cell markers in +SA mammary tumour cells. +SA cells were plated at 1×10^6 cells/100 mm culture dish. Cells were then incubated with control or treatment media containing subeffective doses of γ -tocotrienol (2 μ M) and SU11274 (3 μ M) either alone or in combination containing 10 ng/ml HGF as a mitogen for a 3-day culture period. Following treatment exposure, whole cell lysates were prepared and then subjected to polyacrylamide gel electrophoresis and western blot analysis for E-cadherin, β -catenin, cytokeratin-8, cytokeratin-18 and vimentin. α -tubulin was visualized to ensure equal sample loading in each lane. Each western blot is a representative image of the data obtained for experiments that were repeated at least three times. Scanning densitometric analysis was performed for each blot to visualize the relative levels of proteins. Integrated optical density of each band was normalized with their corresponding α -tubulin and control treatment bands and then shown in bar graphs. Vertical bars indicate the fold-change in protein levels in various treatment groups $\pm$ SEM as compared with their respective vehicle-treated control group. * $P < 0.05$ as compared with their respective vehicle-treated control group. (b) Immunocytochemical fluorescence staining of epithelial and mesenchymal markers in +SA mammary tumour cells treated with γ -tocotrienol and/or SU11274 after a 3-day culture period. +SA cells were seeded on 4-chamber culture slides 1×10^5 cells/chamber (3 replicates/group) and allowed to attach in complete growth media supplemented with 10% BCS overnight. Cells were then washed with phosphate-buffered saline and incubated with vehicle control or treatment defined media containing 10 ng/ml HGF for 3 days in culture. At the end of treatments, cells were fixed with 4% formaldehyde/PBS and permeabilized with 0.2% triton X-100. Fixed cells were blocked and incubated with specific primary antibodies for E-cadherin, β -catenin, cytokeratin-8, cytokeratin-18 and vimentin followed by incubation with Alexa Fluor 594- or 488-conjugated secondary antibodies as described in the Materials and methods section. In the confocal images, the red or green colour indicates the positive fluorescence staining for target proteins and the blue colour represents counter staining of the +SA cell nuclei DAPI. Magnification of each image is 200 $\times$.

stroma of epithelial cells (1,6). Together with other growth factors, HGF stimulates tubulogenesis of the mammary gland in a tightly controlled paracrine manner (6). However, deregulation of the HGF/Met axis causes a shift from transient activation of Met to sustained and/or elevated Met activation, which, in cooperation with other receptor tyrosine kinases such as HER2, can promote expression of a malignant mammary epithelial phenotype (6). Development of the malignant phenotype is a multi-step process, characterized by loss of epithelial polarity, dispersion of cell–cell junctions, degradation of basement membrane, and increased cell migration and invasion (1). EMT is one of the first steps in metastatic progression and is essential for migration of tumour cells away from primary tumours, and invasion into surrounding tissues (4,6,13,14,39,40). Previous studies have shown that Met is a key promoter of EMT and sustained activation of HGF–Met signalling is associated with dissociation of cadherin-based adherens junctions, followed by loss of E-cadherin and cytokeratins 8/18 expression, and upregulation of mesenchymal protein vimentin (4,6,13,14,38–43). Eventually, epithelial cells that undergo EMT lose their epithelial cell characteristics to acquire a mesenchymal phenotype and become migratory and invasive (14).

Results of the present study show that treatment with moderate doses of γ -tocotrienol or SU11274 significantly inhibited growth of mouse (+SA) and human (MCF-7 and MDA-MB-231) mammary cancer cell lines. The +SA murine mammary tumour cell line is characterized as being highly malignant and oestrogen-independent (25,26,44,45). MCF-7 cells are oestrogen receptor-positive (46), whereas MDA-MB-231 are oestrogen receptor-negative breast cancer cells (47). Combined treatment with subeffective doses of γ -tocotrienol with SU11274 was found to induce significant and synergistic inhibition of growth in all mammary cancer cell lines as indicated

by evaluation of their respective CI, DRI and isobologram analyses. These same treatments were also shown to have no significant effect on corresponding mouse (CL-S1) and human (MCF10A) normal mammary epithelial cell growth or viability. The findings are of particular interest as they demonstrate that combination treatment with γ -tocotrienol and SU11274 may be effective against a variety of heterogeneous mammary cancer cell types, particularly very aggressive forms that are oestrogen receptor-negative and highly malignant.

The inhibitory effect of combined γ -tocotrienol and SU11274 treatment appears to be mediated through suppression in HGF-dependent mitogenesis, as indicated by relative large reduction in positive Ki-67 staining in +SA mammary tumour cells. Ki-67 is a nuclear antigen localized at the periphery of the chromosome scaffold and nuclear cortex (48). It is expressed in all phases of the cell cycle of proliferating cells (G1 phase, S phase, G2 phase, and M phase), but not in cells in the resting phase (G0 phase) (48–50). Combined treatment with subeffective doses of γ -tocotrienol and SU11274 resulted in nearly 80% reduction in positive Ki-67 staining in +SA mammary tumour cells. Subsequent studies showed that combined treatment with these same agents also caused corresponding significant reduction in HGF-dependent mitogenic signalling characterized by relatively large reduction in phosphorylated (activated) levels of Akt, STAT1/5 and NF κ B. Specifically, combination treatment was found to reduce total levels of PI3K and cause corresponding decrease in phosphorylated Akt levels, but not in total Akt levels. These findings suggest that treatment-induced suppression of Akt activity results from both reduction in Met activation and reduction in total STAT1/5 levels. This suggestion is further strengthened by the finding that combination treatment with subeffective doses of γ -tocotrienol and SU11274 had little or no effect

on total or phosphorylated (activated) PTEN levels. PTEN is a specific phosphatase associated with regulating Akt and NF κ B activity. Similarly, treatment with these same agents was also found to cause reduction in total STAT1/5 levels and corresponding decrease in phosphorylated (activated) STAT1/5, again suggesting that treatment-induced suppression of activated STAT1/5 results from both reduction in Met activation and reduction in total STAT1/5 levels. A summary of treatment effects on Met signalling is shown in Fig. 8.

Results of the present study demonstrate that HGF-dependent growth of +SA mammary cancer cells is associated with low expression levels of E-cadherin, β -catenin and cytokeratins 8/18 and increased expression of vimentin. However, combination treatment with low doses of γ -tocotrienol and SU11274 resulted in blockade of HGF-dependent EMT and reversal in expression of mesenchymal *versus* epithelial cell protein markers in +SA tumour cells. Specifically, combined treatment with these agents was found to enhance expression, and localization of E-cadherin in adherent cell–cell junctions in +SA cells; this is of particular interest because functional loss of E-cadherin is considered the most critical event linked with EMT (41). Immunoprecipitation and cross-linking studies have shown that Met and E-cadherin are physically located at cell–cell junctions in several cancer cell types, suggesting a direct role for Met in regulation of intercellular adhesion (42,51,52). Molecular mechanisms mediating HGF-dependent reduction in E-cadherin expression involve activation of transcrip-

tional repressors and downregulation and internalization of E-cadherin (53–56). Studies have also shown that HGF-dependent Met activation leads to phosphorylation of β -catenin, which reduces its affinity for the E-cadherin complex and leads to its rapid degradation (6).

Cytokeratins are epithelium-specific intermediate filament proteins that are expressed in a tissue-specific manner (57). In normal mammary epithelium, luminal cells usually express cytokeratins 8, 18 and 19, which is typical for simple epithelia (57). Cytokeratins 8 and 18 are markers of luminal differentiation (57–60). Generally, human breast cancer arises from luminally differentiated epithelial cells as witnessed by strong expression of cytokeratins 8, 18 and 19 (59). Results showed that expression of cytokeratins 8 and 18 have very low expression in +SA mammary tumour cells maintained in medium containing HGF as mitogen. However, combined treatment with subeffective doses of γ -tocotrienol and SU11274 was shown to increase expression of cytokeratins 8/18, a characteristic associated with differentiated luminal epithelial cells. Furthermore, other studies have provided evidence, indicating that functionally differentiated epithelial cells display loss of proliferative potential (61), whereas breast cancer cell lines became more aggressive as keratin filaments are replaced by vimentin, the intermediate filament-protein of mesenchymal cells (57). Taken together, these findings demonstrate that elevated keratin expression is associated with differentiated epithelial phenotypes that display less proliferative and invasive potential.

Figure 8. Schematic representation of inhibitory effects of γ -tocotrienol and SU11274 on Met signalling. The Met receptor has an extracellular α -chain that binds HGF and a transmembrane β -chain that contains the tyrosine kinase domain and autophosphorylation sites that are essential for interacting with substrates. Activation of Met by hepatocyte growth factor leads to receptor dimerization and recruitment of adaptor (GAB1, Grb2, Shc) and signalling (Ras/MAPK, PI3K/Akt, Src, STAT, Shp2) proteins. Downstream signalling promotes cell proliferation, altered cytoskeletal function, decreased cellular adhesion, increased cellular invasion, decreased apoptosis and enhanced DNA transcription. Combined treatment with γ -tocotrienol and SU11274 significantly reduces Met levels and activation (autophosphorylation) that ultimately result in significant inhibition of various downstream signalling pathways involved with tumour cell proliferation, motility, viability, morphology and epithelial-to-mesenchymal transition.

HGF-dependent Met activation and enhanced cell motility are characterized by formation and retraction of filopodia/lamellipodia, as well as uropod alteration in actin formation (7). Although this mechanism is not completely understood, HGF activation of Met and increased cell motility appear to involve the PI3K/Akt signalling pathway and p21 GTPases including Ras, Rac and Rho (7). Results in the present study confirm and extend these previous studies and demonstrate that combined treatment with low doses of γ -tocotrienol and SU11274 inhibited HGF-dependent Akt activation and cell motility in mouse (+SA) and human (MDA-MB-231) mammary tumour cells.

In conclusion, the present findings strongly suggest that anti-proliferative effects of combined low-dose treatment of γ -tocotrienol and SU11274 are mediated by suppression in HGF-dependent Met activation and mitogenic signalling, and this growth inhibitory effect is associated with blockade in HGF-dependent EMT and reduction in cell motility. These findings suggest that combined treatment of γ -tocotrienol with Met inhibitors may provide benefit in treatment of highly invasive and metastatic forms of breast cancer.

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