Combined γ-Tocotrienol and Met Inhibitor Treatment Suppresses Mammary Cancer Cell Proliferation, Epithelial-to-Mesenchymal Transition, and Migration

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Abstract
Met is a receptor tyrosine kinase. Dysregulation of Met signaling is associated with aggressive cancer phenotypes characterized by highly invasive and metastatic growth. Low dose treatment of γ-tocotrienol, a rare form of vitamin E, has previously been shown to inhibit Met receptor activation and mammary tumor cell proliferation when combined with the Met inhibitor, SU11274. Experiments were conducted to determine the intracellular mechanisms involved in mediating the anticancer effects of combined γ-tocotrienol and SU11274 treatment in mammary cancer cell lines. Mouse (+SA) and human (MCF-7, and MDA-MB-231) mammary cancer cell lines, and CL-S1 (mouse) and MCF10A (human) immortalized normal 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 blotting. Immunofluorescent staining was used to characterize expression and localization of multiple epithelial and mesenchymal markers. Cell migration was determined using the wound healing assay. Combined treatment of subeffective concentrations of γ-tocotrienol and SU11274 resulted in a synergistic growth inhibition in +SA, MCF-7, and MDA-MB-231 cells, but had no effect on CL-S1 and MCF10A viability. Additional studies showed that combined treatment caused a large reduction in phosphorylated-Akt (active) levels and suppressed HGF-induced epithelial-to-mesenchymal transition (EMT) in +SA cells, as indicated by a characteristic increase in epithelial markers E-cadherin, membrane-bound β-catenin, and cytokeratins 8/18, and a corresponding decrease in mesenchymal marker vimentin expression, as compared to vehicle-treated controls.

Key words
Breast Cancer, Met, γ-Tocotrienol, Epithelial-to-Mesenchymal Transition

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Combined treatment with $\gamma$-tocotrienol and SU11274 blocked HGF-induced +SA and MDA-MB-231 cell motility. These results demonstrate that the anticancer effects of combined $\gamma$-tocotrienol and SU11274 treatment are mediated by a suppression in hepatocyte growth factor (HGF)-induced mitogenic signaling, EMT, and cell motility, and suggest that the use of these agents may provide significant benefits in the treatment of breast cancers characterized by aberrant Met activity. This work was supported, in part, by grants from First Tec International Ltd. (Hong Kong), the Malaysian Palm Oil Council (MPOC), and the Louisiana Cancer Foundation.

1. Introduction

Human breast cancer is characteristically composed of heterogeneous cell types that can display a wide range of histological features and malignancy that is often associated with aberrant activity of specific receptor tyrosine kinases\(^1\,^2\,^3\). Met is a receptor tyrosine kinase that is particularly relevant to oncogenic progression because enhanced Met activity is associated with a poor prognosis and an aggressive phenotype characterized by cancer cell invasion, metastasis, and robust angiogenesis\(^4\,^5\,^6\). Hepatocyte growth factor (HGF), also known as scatter factor, is the natural ligand for Met and stimulates cellular motility\(^5\). Excessive Met signaling is associated with an aggressive malignant phenotype due to the action of HGF, which is a potent inducer of epithelial-to-mesenchymal transition (EMT) in many different epithelial cell types\(^7\). Epithelial cells that undergo EMT lose their epithelial cell characteristics and acquire a mesenchymal phenotype that displays migratory and invasive characteristics\(^7\,^8\,^9\). $\gamma$-Tocotrienol is a member of the vitamin E family of compounds that displays potent anticancer activity at treatment doses that have little or no effect on normal cell function or viability\(^10\,^11\). Studies have shown that tocotrienol treatment attenuates EGF receptor tyrosine kinase downstream mitogenic signaling, including MAPK, PI3K/Akt, and JAKs/Stat and NFk$\beta$\(^12\,^13\). Recently, $\gamma$-tocotrienol treatment has been shown to reduce total Met levels and inhibit HGF-dependent Met activation in the highly malignant +SA mouse mammary epithelial cells\(^14\). Studies were conducted to further characterize the intracellular mechanisms involved in mediating the anticancer effects of combined $\gamma$-tocotrienol and SU11274, a specific Met inhibitor, treatment in a variety of normal and malignant mammary epithelial cell lines.

2. Materials and Methods

2.1 Cell lines: The highly malignant +SA cell line was derived from adenocarcinoma that developed spontaneously in BALB/c female mouse and CL-S1 cells are immortalized normal mouse mammary epithelial cells\(^15\,^16\). +SA and CL-S1 cells were maintained in serum-free defined media containing 10 ng/ml HGF. The estrogen-receptor, positive MCF-7 and estrogen-receptor, negative MDA-MB-231 human breast cancer cell lines were maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS). The MCF10A cell line is an immortalized normal non-tumorigenic human mammary epithelial cell line.

2.2 Cell growth and viability studies: +SA cells were initially seeded at a density of 5x10\(^4\) cells/well (6 replicates/group) and allowed to attach overnight. +SA cells were exposed to respective experimental treatments containing various concentrations of $\gamma$-tocotrienol or the Met inhibitor, SU11274, alone or in combination of subeffective concentrations for 3 days. MCF-7 and MDA-MB-231 cells were plated at a density of 1x10\(^3\) cells/well (6 replicates/group) in 96-well plates and treated in a similar manner with $\gamma$-tocotrienol or SU11274 as described above. Parallel studies were also conducted using immortalized normal mouse (CL-S1) and human (MCF10A) mammary epithelial cell lines. Viable cell count was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay.

2.3 Western blot analysis: At the end of treatment period, cells were isolated with trypsin, washed, and then whole cell lysates were prepared. Equal amount of protein (30-50 \(\mu\)g/lane) of each sample was subjected to electrophoresis through 7.5%-15% SDS-
polyacrylamide minigels and each gel was transblotted PVDF membrane, blocked with 2% BSA, and then incubated with specific primary antibodies. Blots were then visualized by chemiluminescence and images of protein bands were acquired using Kodak Gel Logic 1500 Imaging System.

2.4 Immunocytochemical fluorescent staining: +SA cells were 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 washed, fixed with 4% formaldehyde/PBS and permeabilized with 0.2% triton X-100 in PBS. Cells were then incubated with specific primary antibodies. Afterwards, cells were embedded in Vectashield mounting medium with DAPI. Fluorescent images were obtained by using confocal laser scanning microscope.

2.5 Migration assay: The 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 subconfluent cell monolayer per well overnight. Wounds were then scratched in each cell monolayer using a sterile 200 μL pipette tip. Media was removed and cells were washed twice with PBS and once with fresh serum-free media to remove floating cells. Cells were then treated with γ-tocotrienol and SU11274 alone or in combination. After a 24 h culture period, media was removed and cells were washed, fixed with methanol, and then stained with Giemsa. The distance travelled by the cells was determined by measuring the wound width at time 24 h and subtracting it from the wound width at the start of treatment.

2.6 Statistical analysis: Differences among various treatment groups were determined by the analysis of variance (ANOVA) followed by Dunnett’s test or Tukey HSD test using PASW statistics® version 18. A difference of P<0.05 was considered statistically significant as compared to the vehicle-treated control group. IC_{50} values (concentrations that induce 50% cell growth inhibition) were determined using non-linear regression curve fit analysis using GraphPad Prism software version 5. The level of interaction between γ-tocotrienol and SU11274 was determined by combination index (CI), dose reduction index (DRI), and isobologram analysis.17,18

3. Results

3.1 Effects of γ-tocotrienol and SU11274 on mammary tumor cell growth: Various doses of γ-tocotrienol and SU11274 on the growth of mouse (+SA) and human (MCF-7 and MDA-MB-231) mammary tumor cell lines after a 3-day culture period showed that treatment with 5-5.5 μM γ-tocotrienol or 4-5.5 μM SU11274 significantly inhibited HGF-dependent +SA cell growth in a dose-responsive manner as compared to cells in the vehicle-treated control group (Figure 1). The IC_{50} values for γ-tocotrienol and SU11274 were 4.85 and 4.81 μM, respectively, in +SA cells. Similarly, treatment with 0-30 μM γ-tocotrienol or 0-10 μM SU11274 resulted in a dose-dependent inhibition of MCF-7 cell growth as compared to the vehicle-treated control group, with IC_{50} values of 26.72 and 7.15 μM for γ-tocotrienol and SU11274, respectively (Figure 1). Treatment with 20-25 μM γ-tocotrienol or 8-12 μM SU11274 significantly inhibited MDA-MB-231 cells cell growth in a dose-responsive manner as 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 (Figure 1).

3.2 Effects of combined treatment of γ-tocotrienol with SU11274 on mammary tumor cell growth: Combined treatment of subeffective concentrations of γ-tocotrienol and SU11274 on the growth of mouse (+SA) and human (MCF-7 and MDA-MB-231) mammary cancer cell lines after a 3 day culture period showed that treatment with 1-3.5 μM γ-tocotrienol or 3 μM SU11274 had no effect on +SA cell viability (Figure 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. Similarly, combined treatment with subeffective doses γ-tocotrienol (5-20 μM) with a subeffective concentration of SU11274 (3 μM) resulted in a significant inhibition of MCF-7 cell growth as compared to cells in the vehicle-treated control group.
Figure 1: Effects of γ-tocotrienol and SU11274 on mammary tumor cell growth

Figure 1

![Bar charts](image)

1 Figure 1: SA mouse mammary tumor cells were initially plated at a density of 5x10^4 cells/well (6 replicates/group) in 24-well plates and maintained on defined serum-free media containing 10 ng/ml HGF. MCF-7 and MDA-MB-231 human mammary cancer cells were initially plated at a density of 1x10^4 cells/well in 96-well plates and maintained on DMEM/F-12 media containing 10% FBS. The 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 period, viable cell number was determined by the MTT colorimetric assay. Each bar indicates the mean number of cells/well ± SEM in each treatment group from a given experiment. Each experiment was repeated 3 times. *P < 0.05 compared to respective vehicle-treated control group.
Figure 2: Effects of combined treatment of γ-tocotrienol with SU11274 on mammary tumor cell growth

Figure 2 (A) +SA mouse mammary tumor cells were initially plated at a density of 5x10⁴ cells/well (6 replicates/group) in 24-well plates and maintained on defined serum-free media containing 10 ng/ml HGF. MCF-7 and MDA-MB-231 human mammary cancer cells were initially plated at a density of 1x10⁴ cells/well in 96-well plates and maintained on DMEM/F-12 media containing 10% FBS. The 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 treatment period, viable cell number was determined by the MTT colorimetric assay. Each experiment was repeated 3 times. *P < 0.05 compared to respective vehicle-treated control group. (B) Isobolograms of γ-tocotrienol and SU11274 antiproliferative effects on multiple mammary cancer cell lines. IC₅₀ 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 three 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 antiproliferative effect for the various combinations of γ-tocotrienol and SU11274 used.
Treatment of 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 and 4 µM SU11274 caused a significant dose-responsive inhibition of MDA-MB-231 cell growth as compared to vehicle-treated controls (Figure 2a). Isobologram analysis of combined treatment effects of γ-tocotrienol and SU11274 in the different cancer cell lines showed that the growth inhibitory effect of combined treatment of γ-tocotrienol and SU11274 in +SA, MCF-7, and MDA-MB-231 cancer cells was synergistic (Figure 2b). Similarly, CI analysis for the 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). The CI value for the combination of γ-tocotrienol and SU11274 in mouse +SA mammary tumor cells was 0.88, whereas the 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 a multifold reduction of the growth inhibitory dosage for combined γ-tocotrienol and SU11274 as compared to each compound alone (Table 1). The IC₅₀ dose of γ-tocotrienol was reduced by approximately 4-fold with combination treatment in +SA cell, reduced more than 3-fold in MCF-7 cells, and nearly 5-fold in MDA-MB-231 cells when used in combination with SU11274 as compared to γ-tocotrienol treatment alone.

**3.3 Effects of SU11274 and γ-tocotrienol on normal mouse and human mammary epithelial cell growth:** Treatment on the growth of immortalized normal mouse (CL-S1) and human (MCF10A) mammary epithelial cells over a 3-day culture period showed that 0-30 µM γ-tocotrienol had no effect on CL-S1 and MCF10A cell viability as compared to their respective vehicle treated control groups (Figure 3a). In combination studies, similar treatment doses as those used on cancer cell lines showed that combined treatment of 0-3.5 µM or 0-20 µM γ-tocotrienol with 3µM SU11274 had no effect on CL-S1 or MCF-10A cell growth, as compared to their respective vehicle-treated controls (Figure 3b).

**3.4 Effects of γ-tocotrienol and SU11274 on Ki-67 labelling in +SA mammary tumor cells.** Positive Ki-67 staining is a marker of proliferating cells and was observed in 88% of +SA cells grown in control medium (Figure 4a and 4b). Treatment with subeffective doses of γ-tocotrienol (2 µM) or SU11274 (3 µM) alone had no significant effect on Ki-67 staining as compared to cells in the vehicle-treated control group (Figure 4a and 4b). However, combined treatment with these agents resulted in less than 5% positive Ki-67 staining in +SA cells (Figure 4a and 4b).

**3.5 Effects of combined γ-tocotrienol and SU11274 treatment on HGF/Met downstream signaling.** Treatment with subeffective doses of γ-tocotrienol (2µM) or SU11274 (3µM) alone had little or no effect, whereas combined treatment with these agents resulted in a large relative reduction in total levels of PI3K, STAT1, and STAT5, but not Akt in +SA mammary tumor cells (Figure 5a and 5b). Combined treatment with these agents also caused a large reduction in phosphorylated (activated) STAT1, STAT5, Akt and NFκB levels as compared to individual compounds or vehicle-treated controls (Figure 5a and 5b). Western blot analysis showed that intracellular levels of MAPK, p-MAPK, MEK, and p-MEK were similar in all treatment groups (Figure 5a and 5b).

**3.6 Effects of γ-tocotrienol and SU11274 on HGF-induced mammary tumor cell migration:** Mouse (+SA) and human (MDA-MB-231) mammary cancer cell motility was determined using the wound healing assay. HGF-induced cellular migration was observed with more than 85% wound closure for both +SA and MDA-MB-231 mammary tumor cells after a 24 h treatment period in their respective vehicle-treated control groups (Figure 6a and 6b). Treatment with subeffective doses of γ-tocotrienol (2µM) or SU11274 (3µM) alone resulted in a slight reduction in HGF-induced migration of +SA and MDA-MB-231 mammary tumor cells, whereas combined treatment with these agents significantly inhibited +SA and MDA-MB-231 mammary tumor cells migration to an approximate 26% wound closure in both...
groups, as compared to their respective vehicle-treated control groups (Figure 6a and 6b).

3.7 Effects of γ-tocotrienol and SU11274 on epithelial and mesenchymal cell marker expression: +SA mammary tumor cells grown in media containing 10 ng/ml HGF as a mitogen, displayed a strong transition from epithelial to mesenchymal cell phenotype, as evidenced by a low expression in epithelial markers and a 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 a relatively high level of expression of the mesenchymal protein marker vimentin (Figure 7a).

Figure 3: Effects of SU11274 and γ-tocotrienol on normal mouse and human mammary epithelial cell growth

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Figure 3: (A) Antiproliferative 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 a density of 1x10⁴ cells/well (6 replicates/group) in 96-well culture plates and exposed to treatment media containing 10% BCS, while MCF10A cells were initially plated at a density of 1x10⁴ 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 a density of 1x10⁴ cells/well (6 replicates/group) and exposed to various treatments for a 3 day culture period. Afterwards, viable cell number was determined by the MTT colorimetric assay. Each bar indicates the mean number of cells ± SEM in each treatment group. Each experiment was repeated at least 3 times. *P < 0.05 as compared to their respective vehicle-treated control group.
Table 1: Combination index (CI) and drug reduction index (DRI) values for combined treatment of \( \gamma \)-tocotrienol and SU11274 resulting in 50% reduction in the growth of various mammary cancer cell lines

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Figure 4: Effects of \( \gamma \)-tocotrienol and SU11274 on Ki-67 labelling in +SA mammary tumor cells

*B* Figure 4: (A) +SA cells were plated on 4-chamber culture slides at a density of 1x105 cells/chamber (3 replicates/group) and allowed to attach in complete growth media supplemented with 10% BCS overnight. Cells were then washed with PBS and incubated with defined serum-free media containing 10 ng/ml HGF as a mitogen and 0-2 \( \mu \)M \( \gamma \)-tocotrienol (\( \gamma \)T3) and/or 0-3 \( \mu \)M SU11274 (SU) for a 3 day culture period. Afterwards, cells were fixed with 4% formaldehyde/PBS and permeabilized with 0.2% triton X-100. Fixed cells were then blocked and incubated with specific primary antibody for Ki-67 followed by incubation with Alexa Fluor 594-conjugated secondary antibody as described in Materials and Methods. The red color in the photomicrographs indicates positive fluorescence staining for Ki-67, while the blue color represents counter-stained of the cellular nucleus with DAPI. Magnification of each image is 200x. (B) The percentage of +SA cells displaying positive Ki-67 staining in proportion to the total number of cells in each treatment group. The vertical bars represent percent positive Ki-67 staining ± SEM in each treatment group. *P* < 0.05 as 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 3 times.
**Figure 5:** Effects of combined γ-tocotrienol and SU11274 treatment on HGF/Met downstream signaling

**Figure 5**

A

Control 2μM γ-T<sub>3</sub> 3μM SU 3μM SU

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B

Control 2μM γ-T<sub>3</sub> 3μM SU 3μM SU

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5 Figure 5: α-SA mammary tumor cells were plated at a density of 1x10^6 cells/100 mm culture dish. Cells were then incubated with serum-free defined media containing 10 ng/ml HGF as a mitogen and 0-2 μM γ-tocotrienol (γ-T<sub>3</sub>) and/or 0-3 μM SU11274 (SU) for a 3 day culture period. Afterwards, 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, Akt, p-Akt, p-NFκB105, PTEN, and p-PTEN 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.
Figure 6: Effects of γ-tocotrienol and SU11274 on HGF-induced mammary tumor cell migration

Figure 6

(A) Photomicrographs of γ-tocotrienol (γT3) and SU11274 (SU) treatment effects on +SA and MDA-MB-231 mammary tumor cell migration in response to HGF stimulation using the in vitro wound healing assay. Cells in each treatment group cells 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. Media was then removed, the cells washed, and then exposed to their respective treatments for a 24 hr culture period. Photomicrographs (100X 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 the wound distance at time 0. Vertical bar represent percent migration ± SEM. Each experiment was done in triplicate and the distance migrated was calculated in three or more randomly selected fields per treatment group. *P<0.05 as compared to their respective vehicle-treated control group. #P<0.05 as compared to γ-tocotrienol or SU11274 treatment alone.

Figure 7: Effects of γ-tocotrienol and SU11274 on epithelial and mesenchymal cell marker expression
Figure 7: (A) Western blot analysis of treatment effect of γ-tocotrienol (γ-T3) and/or SU11274 (SU) on the expression of major epithelial and mesenchymal cellular markers in +SA mammary tumor cells. +SA cells were plated at a density of 1x10^6 cells/100 mm culture dish. Cells were then incubated with control or treatment media containing subeffective doses of γ-tocotrienol (2 µM) and SU11274 (3 µM) either alone or in combination containing 10 ng/ml HGF as a mitogen for a three-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. (B) Immunocytochemical fluorescence staining of epithelial and mesenchymal markers in +SA mammary tumor cells treated with γ-tocotrienol and/or SU11274 after a 3 day culture period. +SA cells were seeded on 4-chamber culture slides at a density of 1x10^5 cells/chamber (3 replicates/group) and allowed to attach in complete growth media supplemented with 10% BCS overnight. Cells were then washed with PBS 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 Methods section. In the confocal images, the red or green color indicates the positive fluorescence staining for target proteins and the blue color represents counter staining of the +SA cell nuclei DAPI. Magnification of each image is 200X.
Treatment with subeffective doses of γ-tocotrienol (2 μM) or SU11274 (3 μM) alone resulted in a slight reduction in EMT, characterized by a slight increase in the level of some epithelial markers (cytokeratin-8 and cytokeratin-18), but little or no change in vimentin expression as compared to SA cells in the vehicle-treated control group (Figure 7a). In contrast, combined treatment with subeffective doses of γ-tocotrienol and SU11274 reversed EMT and cells in this group were characterized by displaying a relatively high level of expression of epithelial protein markers (E-cadherin, β-catenin, cytokeratin-8, and cytokeratin-18), and a corresponding large relative decrease in the expression of the mesenchymal protein marker, vimentin, as compared to vehicle-treated controls (Figure 7a). Similar to the results observed in the Western blot analysis, SA cell maintained in control media display a relatively low level of positive immunofluorescent staining for epithelial markers E-cadherin, β-catenin, cytokeratin-8, and cytokeratin-18, and a corresponding relatively high level of positive staining for the mesenchymal marker, vimentin (Figure 7b). Treatment with subeffective doses of γ-tocotrienol (2 μM) or SU11274 (3 μM) alone resulted in a slight increase in positive immunofluorescent staining for epithelial protein markers and slight decrease in mesenchymal marker expression as compared to cells in the vehicle-treated control group (Figure 7b). However, combined treatment with these same agents resulted in a reversal of HGF-induced EMT, characterized by a relatively large increase in epithelial markers (E-cadherin, β-catenin, cytokeratin-8, and cytokeratin-18) expression and a corresponding large relative decrease in mesenchymal marker (vimentin) expression as compared to cells in the vehicle-treated control group (Figure 7b).

4. Summary and conclusion
Results from these studies demonstrated that combined treatment with subeffective doses of γ-tocotrienol and the specific Met inhibitor, SU11274, results in a significant and synergistic inhibition of mouse and human mammary tumor cell growth, while having little or no effect on immortalized normal mouse and human mammary epithelial cells growth. The anti-proliferative effects of combination treatment with these agents was found to be associated with a suppression in HGF-dependent mitogenic signaling characterized by a suppression in Akt, STAT5 and NFκB activation. This same combination treatment was also found to reverse HGF-dependent epithelial-mesenchymal transition (EMT) as demonstrated by a decreased in mesenchymal (vimentin) and corresponding increase in epithelial (cytokeratin, E-cadherin and β-catenin) cellular marker expression. Furthermore, this reversal in EMT was characteristically associated with a large reduction in the cellular motility of highly malignant mouse and human mammary cancer cell lines. In summary, these findings demonstrate that the antiproliferative effects of combined low-dose treatment of γ-tocotrienol and SU11274 is mediated by suppression in HGF-dependent Met activation and mitogenic signalling and this growth inhibitory effect is associated with a blockade in HGF-dependent EMT and reduction in cellular motility, and strongly suggests that combined treatment of γ-tocotrienol with SU11274 may provide some benefit in the treatment of highly invasive and metastatic forms of breast cancer.

References


