First evidence that γ-tocotrienol inhibits the growth of human gastric cancer and chemosensitizes it to capecitabine in a xenograft mouse model through the modulation of NF-κB pathway

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Running title: γ-tocotrienol enhances the effect of capecitabine in gastric cancer.

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**Key words:** gastric cancer, \( \gamma \)-tocotrienol, apoptosis, proliferation, NF-\( \kappa \)B.

#Both KAM and MKS contributed equally to this work.
Abstract:

Purpose: Because of poor prognosis and development of resistance against chemotherapeutic
drugs, the existing treatment modalities for gastric cancer are ineffective. Hence, novel agents
that are safe and effective are urgently needed. Whether γ-tocotrienol can sensitize gastric
cancer to capecitabine in vitro and in a xenograft mouse model was investigated.

Experimental Design: The effect of γ-tocotrienol on proliferation of gastric cancer cell lines, was
examined by mitochondrial dye-uptake assay, apoptosis by esterase staining, NF-κB activation
by DNA binding assay and gene expression by western blot. The effect of γ-tocotrienol on the
growth and chemosensitization was also examined in subcutaneously implanted tumors in nude
mice.

Results: γ-tocotrienol inhibited the proliferation of various gastric cancer cell lines, potentiated
the apoptotic effects of capecitabine, inhibited the constitutive activation of NF-κB and
suppressed the NF-κB regulated expression of COX2, cyclin D1, Bcl-2, CXCR4, VEGF, and
MMP-9. In a xenograft model of human gastric cancer in nude mice, we found that
administration of γ-tocotrienol alone (1 mg/kg body weight, i.p. thrice/week) significantly
suppressed the growth of the tumor and this effect was further enhanced by capecitabine. Both
the markers of proliferation index Ki-67 and for microvessel density CD31 were downregulated
in tumor tissue by the combination of capecitabine and γ-tocotrienol. As compared to vehicle
control, γ-tocotrienol also suppressed the NF-κB activation and the expression of cyclin D1,
COX-2, ICAM-1, MMP-9, survivin, Bcl-xL, and XIAP.

Conclusions: Overall our results demonstrate that γ-tocotrienol can potentiate the effects of
capcitabine through suppression of NF-κB-regulated markers of proliferation, invasion,
angiogenesis and metastasis.
Introduction:

Gastric cancer remains one of the most common malignancies and the second leading cause of cancer mortality accounting for more than 600,000 deaths annually worldwide (1, 2). Chemotherapy constitutes an important treatment regimen for gastric cancers besides surgical resection (3). Unfortunately, only few patients experience complete pathologic response to chemotherapeutic drugs like capecitabine, mainly because of their resistance to chemotherapy (4). Hence, novel approaches to enhance the effects of chemotherapeutic drugs and reduce their resistance are imperative.

Various lines of evidence suggest that the activation of master transcription factor NF-κB and its-regulated gene products play a pivotal role in growth, metastasis and chemoresistance of gastric cancer. First, NF-κB is constitutively activated in human gastric cancer tissue and is associated with tumor progression (5). Second, it promotes gastric cancer growth by inhibiting apoptosis (6). Third, NF-κB mediates the induction of mitogenic gene products such as cyclin D1, which is overexpressed in human gastric cancer tissue and is inversely correlated with poor prognosis and poor survival (7). Fourth, NF-κB plays an important role in regulating the CXC motif receptor 4 (CXCR4) (8) and cyclooxygenase-2 (COX-2), (9) that are associated with gastric cancer metastasis. Finally, it is well established that *H. pylori*, a well-known risk factor for gastric carcinoma, is a potent activator of NF-κB in gastric epithelial cells (10). Moreover, NF-κB pathway is also responsible for the increased generation of several cell adhesion molecules including ICAM-1, whose expression is significantly correlated with an increase in *H. pylori* induced gastritis (11). Together, these findings implicate the involvement of NF-κB pathway in gastric cancer and thus the agents that can modulate NF-κB and NF-κB regulated gene products have enormous potential for the treatment of gastric cancer.
One such agent derived from natural sources that can have a great potential for gastric cancer prevention and treatment is Vitamin E constituent, \( \gamma \)-tocotrienol derived from palm oil and rice bran. Vitamin E family of compounds primarily consists of four tocotrienols and four tocopherols (12). However, while tocopherols had been intensively studied for their health benefits, many novel benefits of tocotrienols are only beginning to be brought to light by research in the last decade (13, 14). For instance, \( \gamma \)-tocotrienol has been reported to suppress the proliferation of a wide variety of tumor cells (15), including gastric (16-19), hepatocellular carcinoma (20), melanoma (21), breast (22), colorectal (23), and prostate (24). In vivo mice studies have demonstrated that \( \gamma \)-tocotrienol can suppress the growth of breast tumor (25), prostate (26) lung cancer and melanoma (27) and also inhibit the growth of liver, and pancreatic cancer either alone or in combination with chemotherapeutic drugs and radiation (28, 29). How \( \gamma \)-tocotrienol mediates its anticancer effects is not completely understood, but the roles of various signaling cascades/kinases/transcription factors such as mitogen activated protein kinases (17), PI3-K/Akt (30), NF-\( \kappa \)B (13), STAT3(20) telomerase (31), peroxisome proliferators-activated receptor gamma (32), hypoxia inducible factor-1alpha (33), \( \beta \)-catenin (23), epidermal growth factor (24) and inhibitor of differentiation family proteins (34) have been implicated.

Although \( \gamma \)-tocotrienol has been found to suppress proliferation, to inhibit invasion/migration and induce apoptosis in human gastric cancer SGC-7901 cells (28-31), but its potential to act as a chemosensitizing agent in gastric cancer cell lines and xenograft models has never been explored before (16-19). Thus, in the present study, we investigated whether \( \gamma \)-tocotrienol could sensitize human gastric cancer to capecitabine in vitro and in a xenograft mouse model. Our observations indicate for the first time that \( \gamma \)-tocotrienol can inhibit the proliferation of various gastric cancer cells, enhanced capecitabine -induced apoptosis, and
potentiated the antitumor activity of capecitabine in human xenograft gastric cancer model through the modulation of NF-κB and NF-κB-regulated gene products.
Translational Relevance

Despite advances in earlier detection and therapy for gastric cancer, it still remains the second leading cause of cancer death worldwide, killing over 600,000 people annually worldwide. Existing drugs lack efficacy and yet are highly toxic. For example, although capecitabine is used routinely in the treatment of gastric cancer, development of resistance to this treatment in the patients is one of the major problems. Thus agents which can overcome the resistance and can enhance the effect of capecitabine are urgently needed. Through in vitro and in vivo experiments, we demonstrate for the first time that γ-tocotrienol, a Vitamin E analogue, is one such agent that can reduce the resistance and can potentiate the effect of capecitabine against gastric cancer. Because γ-tocotrienol is already in clinical trials, the present study may form the basis of novel therapeutic options for the treatment of gastric cancer patients.
Materials and Methods

Reagents

γ-tocotrienol with purity > 97% was obtained from Davos Life Science, Singapore. MTT, Tris base, glycine, NaCl, SDS, BSA, β-actin antibody, and corn oil were purchased from Sigma-Aldrich (St. Louis, MO). γ-tocotrienol was dissolved in dimethylsulfoxide as a 10 mM stock solution and stored at 4°C for in vitro and in corn oil for in vivo experiments respectively. Further dilution was done in cell culture medium. RPMI-1640 media, fetal bovine serum (FBS), 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Invitrogen (Carlsbad, CA). Antibodies against p65, MMP-9, Bcl-2, Bcl-xL, COX-2, ICAM-1, cyclin D1, survivin, Mcl-1, VEGF, XIAP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). CXCR4 antibody obtained from Abcam (Cambridge, MA, USA). CD31 antibody was purchased from and Cell Signaling Technology (Danvers, MA). Ki-67 antibody was purchased from BD PharMingen, Inc. (San Diego, CA). Goat anti-rabbit-horse radish peroxidase (HRP) conjugate and goat anti-mouse HRP were purchased from Invitrogen (Carlsbad, CA). Capecitabine was obtained from Duheng International Trading Company Ltd. Shanghai China and dissolved in sterile phosphate buffered saline (PBS) on the day of use.

Cell lines

The gastric cancer cell lines SNU-5 and SNU-16 were obtained from the American Type Culture Collection (Manassas, VA). MKN45 cells were obtained from JCRB (Japanese Collection of Research Bioresources), Japan. All the gastric cancer cell lines were cultured in RPMI-1640 media supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin.
**Western blotting**

For detection of various proteins gastric cancer cells (2x10⁶/ml) were treated with γ-tocotrienol for different time intervals. The cells were then washed and extracted by incubation for 30 min on ice in 0.05 ml buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM NaCl, 0.1% NP-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF, 0.5 μg/ml benzamidine, 1 mM DTT, and 1 mM sodium vanadate. The lysate was centrifuged and the supernatant was collected. Whole-cell extract protein (30 μg) was resolved on 12% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, blotted with antibodies against survivin, Bcl-2, Bcl-xL, cyclin D1, VEGF, procaspase-3, and PARP and then detected by chemiluminescence (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

**Cell proliferation MTT assay**

The effect of γ-tocotrienol on cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method as described previously (20). The cells (5,000/well) were incubated with γ-tocotrienol in triplicate in a 96-well plate and then incubated for indicated time points at 37°C. A MTT solution was added to each well and incubated for 2 h at 37°C. A lysis buffer (20% SDS and 50% dimethylformamide) was added, and the cells were incubated overnight at 37°C. The absorbance of the cell suspension was measured at 570 nm by Tecan (Durham, NC) plate reader.
Live/Dead Assay

To investigate whether γ-tocotrienol could potentiate the apoptotic effects of capecitabine in gastric cancer cells, we used a LIVE/DEAD cell viability assay kit (Invitrogen), which is used to determine intracellular esterase activity and plasma membrane integrity. This assay uses calcein, a polyanionic, green fluorescent dye that is retained within live cells, and a red fluorescent ethidium homodimer dye that can enter cells through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membranes of live cells (20). Briefly, gastric cancer cells (5,000/well) were incubated in chamber slides, pretreated with γ-tocotrienol for 4 h, and treated with capecitabine for 24 h. Cells were then stained with the assay reagents for 30 min at room temperature. Cell viability was determined under a fluorescence microscope by counting live (green) and dead (red) cells.

Flow cytometric analysis

To determine the effect on the cell cycle, cells were exposed to combination of γ-tocotrienol for 4 h, and treated with capecitabine for 24 h. Thereafter cells were washed, fixed with 70% ethanol, and incubated for 30 min at 37°C with 0.1%RNase A in PBS. Cells were then washed again, resuspended, and stained in PBS containing 25 μg/ml propidium iodide (PI) for 30 min at room temperature. Cell distribution across the cell cycle was analyzed with a CyAn ADP flow cytometer (Dako Cytomation).

Gastric Tumor model

All procedures involving animals were reviewed and approved by NUS Institutional Animal Care and Use Committee. Six week-old athymic nu/nu female mice (Animal Resource Centre,
Australia) were implanted subcutaneously in the right flank with SNU-5 cells (3 X 10^6 cells/100 μl saline). When tumors have reached 0.25 cm in diameter, the mice were randomized into the following treatment groups (n = 5/group) (a) untreated control (corn oil, 100 μL daily); (b) toco-trienol (1 mg/kg bodyweight, suspended in corn oil, intraperitoneal [i.p.] injection) thrice/week; (c) capecitabine alone (60 mg/kg bodyweight, suspended in corn oil, twice weekly by gavage; and (d) combination (toco-trienol, 1 mg/kg bodyweight, suspended in corn oil, intraperitoneal [i.p.] injection) thrice/week and capecitabine, 60 mg/kg bodyweight, suspended in corn oil, twice weekly by gavage). Therapy was continued for 4 weeks, and the animals were euthanized 1 week later. Primary tumors were excised and the final tumor volume was measured as V = 4/3πr^3, where r is the mean radius of the three dimensions (length, width, and depth). Half of the tumor tissue was fixed in formalin and embedded in paraffin for immunohistochemistry and routine hematoxylin and eosin (H&E) staining. The other half was snap frozen in liquid nitrogen and stored at -80°C.

**Immunohistochemical analysis of gastric tumor samples**

Solid tumors from control and various treatment groups were fixed with 10% phosphate buffered formalin, processed and embedded in paraffin. Sections were cut and deparafinized in xylene, and dehydrated in graded alcohol and finally hydrated in water. Antigen retrieval was performed by boiling the slide in 10 mM sodium citrate (pH 6.0) for 30 min. Immunohistochemistry was performed following manufacturer instructions (DAKO LSAB kit). Briefly, endogenous peroxidases were quenched with 3% hydrogen peroxide. Non-specific binding was blocked by incubation in the blocking reagent in the LSAB kit (Dako, Carpinteria, CA) according to the manufacturer’s instructions. Sections were incubated overnight with primary antibodies as
follows: anti-p65, anti-COX-2, ant-VEGF, anti-MMP-9, anti-Ki-67 and anti-CD31 (each at 1:100 dilutions). Slides were subsequently washed several times in Tris buffered saline with 0.1% Tween 20 and were incubated with biotinylated linker for 30 min, followed by incubation with streptavidin conjugate provided in LSAB kit according to the manufacturer’s instructions. Immunoreactive species were detected using 3, 3-diaminobenzidine tetrahydrochloride (DAB) as a substrate. Sections were counterstained with Gill’s hematoxylin and mounted under glass cover slips. Images were taken using an Olympus BX51 microscope (magnification, 20x). Positive cells (brown) were quantitated using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.).

**Preparation of nuclear extract from gastric tumor samples**

Gastric tumor tissues (100 mg/mouse) from mice in the control and treatment groups were minced and incubated on ice for 30 min in 0.5 mL of ice-cold buffer A [10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.9), 1.5 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L dithiothreitol (DTT), 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF)]. The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 16,000 × g at 4°C for 10 min. The resulting nuclear pellet was suspended in 0.2 mL of buffer B [20 mmol/L HEPES (pH 7.9), 25% glycerol, 1.5 mmol/L MgCl₂, 420 mmol/L NaCl, 0.5 mmol/L DTT, 0.2 mmol/L EDTA, 0.5 mmol/L PMSF, 2 µg/mL leupeptin] and incubated on ice for 2 h with intermittent mixing. The suspension was then centrifuged at 16,000 × g at 4°C for 30 min. The supernatant (nuclear extract) was collected and stored at -70°C until used as previously described (35). Protein concentration was measured by the Bradford assay with BSA as the standard.
Measurement of NF-κB activation in gastric cancer cells and tumor samples

To determine NF-κB activation, we performed DNA binding assay using TransAM NF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer’s instructions and as described previously (20). Briefly, nuclear extracts from γ-tocotrienol treated gastric cancer cell lines and tumor tissues were incubated in a 96-well plate coated with oligonucleotide containing the NF-κB consensus-binding sequence 5'-GGGACTTTCC-3'. Bound NF-κB was then detected by a specific primary antibody. An HRP-conjugated secondary antibody was then applied to detect the bound primary antibody and provided the basis for colorimetric quantification. The enzymatic product was measured at 450 nm with a microplate reader (Tecan Systems, San Jose, CA, USA). Specificity of this assay was tested by the addition of wild-type or mutated NF-κB consensus oligonucleotide in the competitive or mutated competitive control wells before the addition of the nuclear extracts.

Statistical analysis

Statistical analysis was performed by Student’s t-test and one way analysis of variance, (ANOVA). A p value of less than 0.05 was considered statistically significant.
Results:

The purpose of this study was to determine whether γ-tocotrienol, a component of Vitamin E (with chemical structure shown in Fig. 1A) might have a role in the treatment of gastric cancer either alone or in combination with capecitabine and if so, through what mechanism(s). For this, we used four different well-characterized human gastric cancer cell lines. To facilitate the monitoring of tumor growth in mice, one of these cell lines, SNU-5 was subcutaneously injected and used in the xenograft transplant model in mice.

γ-tocotrienol inhibits the proliferation and potentiates the effect of capecitabine in gastric cancer cells in vitro

We first investigated the effect of γ-tocotrienol on the proliferation of three different gastric cancer cell lines. γ-tocotrienol inhibited the growth of all three human gastric cancer cells (SNU-5, MKN-45, SNU-16) in a dose- and time-dependent manner (Fig. 1B). Whether γ-tocotrienol can potentiate the effect of capecitabine against these three cell lines was also examined. We employed flow cytometric analysis and an esterase staining assay (live/dead assay) to establish whether γ-tocotrienol can potentiate the apoptosis induced by capecitabine. As shown in Figs. 1C and 1D, the dose of γ-tocotrienol (10 μM) or capecitabine (10 μM) that had minimum effect on apoptosis alone produced enhancement of apoptosis when used in combination.

γ-tocotrienol inhibits constitutive and capecitabine-induced NF-κB activation in gastric cancer cells

We next examined that how γ-tocotrienol potentiates the effects of capecitabine in gastric cancer cells. NF-κB has been shown to be constitutively expressed in gastric cancer and mediates
resistance to apoptosis (5, 36, 37). Whether γ-tocotrienol induces downregulation of constitutive NF-κB activation in SNU-16 and SNU-5 cells was examined by using an ELISA-based DNA binding assay. Results show that the treatment with γ-tocotrienol inhibited NF-κB expression in a dose-dependent manner (Figs. 2A and B). We further observed that chemotherapeutic drug capecitabine was also able to further induce NF-κB activation in a dose-dependent manner in MKN45 cells, with maximum activation observed at 25 μM (Fig. 2C). Interestingly, γ-tocotrienol was also found to suppress capecitabine-induced NF-κB activation in a dose-dependent manner in MKN45 cells (Fig. 2D), thereby indicating that it is a potent modulator of both constitutive and inducible NF-κB activation in gastric cancer cells. Whether γ-tocotrienol can also modulate the expression of various NF-κB-regulated gene products was also examined. We found that the γ-tocotrienol suppressed the constitutive expression of anti-proliferative (cyclin D1), anti-apoptotic (Bcl-2), invasive/metastatic (ICAM-1, MMP-9, CXCR4) and angiogenic (VEGF) protein expression in a time-dependent manner in SNU16 cells (Fig. 2E). γ-tocotrienol also induced the cleavage of PARP in SNU-16 cells (Fig. 2E). Based on these observations in vitro, we decided to study the effect of γ-tocotrienol and capecitabine either alone or in combination in an in vivo gastric cancer xenograft model.

γ-tocotrienol potentiates the antitumor effects of capecitabine in a xenograft gastric cancer model in nude mice

We examined the therapeutic potential of γ-tocotrienol and capecitabine either alone or in combination on the growth of subcutaneously implanted human gastric cancer cells in nude mice. The experimental protocol is depicted in Fig. 3A. SNU-5 cells were implanted
subcutaneously in the right flank of nude mice. When tumors have reached 0.25 cm in diameter after a week, the mice were randomized into 4 groups and started the treatment as per the experimental protocol. The treatment was continued for 4 weeks and animals were sacrificed after 5 weeks. We found that \(\gamma\)-tocotrienol alone when given at 1 mg/kg body weight significantly inhibited the growth of the tumor (p<0.001 when compared to control) (Figs. 3B and 3C). Capecitabine alone was also very effective (p<0.001 when compared to control; p>0.05 when compared to \(\gamma\)-tocotrienol alone group); and the combination of the two agents were more effective in reducing the tumor burden. The tumor volume in the combination of \(\gamma\)-tocotrienol and capecitabine group was significantly lower than \(\gamma\)-tocotrienol alone group (p<0.001) or capecitabine alone group (p<0.001) on day 35 (Figs. 3C and 3D).

\(\gamma\)-tocotrienol inhibits CD31 and Ki-67 expression in gastric tumor tissues

While Ki-67-positive index is used as a marker for cell proliferation, the CD31 index is a marker for microvessel density. Whether \(\gamma\)-tocotrienol and capecitabine modulate these markers, was examined. Fig. 4A shows that both \(\gamma\)-tocotrienol (p<0.05) and capecitabine (p<0.05) alone significantly downregulated the expression of Ki-67 in gastric cancer SNU-5 tissue and the combination of the two was most effective (p<0.001). Similarly when examined for CD31, we found that both agents significantly reduced the CD31 expression as compared to control group and two together were most effective (p<0.001 when compared to capecitabine alone) (Fig. 4B).
**γ-tocotrienol inhibited the constitutive NF-κB expression and NF-κB-regulated gene products in gastric tumor tissues**

We also evaluated the effect of γ-tocotrienol and capecitabine on NF-κB levels in gastric tumor tissue. Fig. 5A shows that γ-tocotrienol either alone or in combination with capecitabine was quite effective in suppressing the constitutive expression of NF-κB in gastric cancer tissue. Capecitabine alone had no significant effect on constitutive NF-κB activation in gastric tissue. A Western blot analysis for p65 in extracts from tumor samples showed that γ-tocotrienol alone inhibited NF-κB (p65) activation (Fig. 5B).

NF-κB is known to regulate the expression of number of proteins, including those involved in proliferation (cyclin D1, COX-2), invasion/metastasis (ICAM-1, MMP-9) and survival (Bcl-xL, survivin, XIAP) (38). Whether γ-tocotrienol and capecitabine can modulate the expression of these NF-κB-regulated gene products in tumor tissues, was examined by western blot analysis. We found that treatment with combination of γ-tocotrienol and capecitabine was effective in down regulating the over expression of various gene products regulated by NF-κB (Fig. 5C) and also involved in various aspects of gastric cancer growth, survival, invasion and metastasis.

Whether modulation of nuclear NF-κB, COX2, VEGF and MMP-9 can also be detected by immunohistochemical methods was also examined. As shown in Fig. 6, these gene products were significantly downregulated in gastric tumor samples treated with γ-tocotrienol in combination with capecitabine. The downregulation was more impressive with either γ-tocotrienol or capecitabine alone. The immunohistochemical analysis results further supports the data obtained from western blot. These results collectively indicate that γ-tocotrienol suppresses
the activation of NF-κB thereby inhibiting the expression of genes involved in proliferation, survival, invasion and angiogenesis.
Discussion:

Despite the major improvements in diagnosis and treatment regimens, gastric cancer remains one of the most lethal cancers, with less than 20% of patients surviving up to 5 years. Thus, novel agents that are nontoxic, efficacious and can significantly enhance the effects of existing chemotherapeutic drugs are urgently needed. The aim of the present study was to investigate whether \( \gamma \)-tocotrienol, a component of vitamin E, could enhance the antitumor activity of capecitabine against human gastric cancer. We found that \( \gamma \)-tocotrienol suppressed the proliferation of various gastric cancer cell lines, potentiated capecitabine -induced apoptosis, and inhibited constitutively active and inducible NF-\( \kappa \)B activation as well as NF-\( \kappa \)B-regulated gene products. We also found that in a xenograft mouse model \( \gamma \)-tocotrienol effectively suppressed the growth of gastric cancer alone and also when used in combination with capecitabine.

We first observed that \( \gamma \)-tocotrienol can suppress the proliferation of various gastric cancer cell lines in a dose- and time dependent manner. Our results are in part agreement with those of Sun et al. (17) who reported that \( \gamma \)-tocotrienol could suppress the proliferation of gastric adenocarcinoma SGC-7901 cells. The inhibitory effects of \( \gamma \)-tocotrienol were correlated with the DNA damage and cell cycle arrest at G(0)/G(1) phase, although the detailed molecular mechanism(s) were not elucidated. We found that \( \gamma \)-tocotrienol caused downregulation of cell proliferative gene products such as cyclin D1, which may explain its potent anti-proliferative effects in gastric cancer.

Specifically, we also found for the first time that \( \gamma \)-tocotrienol when used in combination with capecitabine, is highly effective in inducing apoptosis in gastric cancer cell lines. This is very intriguing because although \( \gamma \)-tocotrienol has been previously reported to induce apoptosis in SGC-7901 cells (16, 17), its effect in combination with chemotherapeutic agents like
capecitabine has never been investigated before in gastric cancer. We observed that this effect may be mediated due to the downregulation of cell survival proteins such as Bcl-2 in gastric cancer. Interestingly, we also observed for the first time that both constitutive and capecitabine-induced NF-κB activation was suppressed by γ-tocotrienol in gastric cancer cells. These results are consistent with those previously reported with other dietary agents like curcumin (39) and phenethyl isothiocyanate (40). Also, γ-tocotrienol was found to downregulate the expression of various invasive, metastatic and angiogenic gene products (ICAM-1, MMP-9, CXCR4, and VEGF) which may account for its recently reported inhibitory effects on invasion, metastasis and angiogenesis (18, 41).

We found for the first time that the intraperitoneal administration of γ-tocotrienol alone inhibited the growth of human gastric tumors when examined in vivo in a xenograft nude mice model. Tumor growth was inhibited by more than 50% on treatment with γ-tocotrienol and capecitabine alone respectively. Also, when the two agents were used in combination, they were found to be much more effective and potent. When examined for the mechanism by which γ-tocotrienol manifests its effects in the mice against gastric cancer, we found that the proliferation marker Ki67 as well as microvessel density indicator CD31 was downregulated by γ-tocotrienol. Further investigation, also revealed the downregulation of NF-κB and NF-κB regulated Cyclin D1, COX-2, survivin, Bcl-xL, XIAP, ICAM-1, MMP-9 and VEGF. All of these effects were further enhanced by capecitabine. γ-tocotrienol has been used in combination therapy with several chemotherapeutic agents/targeted therapies such as statins in breast and colorectal cancers (42, 43), celecoxib in breast cancer (44), gemcitabine in pancreatic cancer (29), EGFR inhibitors erlotinib and gefitinib in breast cancer (45, 46), but so far its effects on gastric cancer mice models either alone or in combination has never been investigated before. Our results
overall suggest for the first time that γ-tocotrienol has significant potential for the treatment of gastric cancer and its effects can be further enhanced by capecitabine. A number of clinical trials with tocotrienols in pancreatic, prostate, and breast cancer patients are already in progress, and based on our results, well designed clinical studies are required for potential translation of our preclinical findings also in gastric cancer patients.
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Figure legends:

**Figure 1.** γ-tocotrienol inhibits the growth and proliferation, potentiates the apoptotic effects of capecitabine in gastric cancer cells *in vitro*. A, structure of γ-tocotrienol. B, MTT assay results showed dose-dependent suppression of cell proliferation in all three gastric cancer cell lines treated with γ-tocotrienol. Points, mean of triplicate; bars, SE. Data are a representative of two independent experiments. C and D, Flow cytometric analysis and Live/Dead assay results indicate that γ-tocotrienol (γ-toco, 10μM) potentiates capecitabine (Cape, 10μM)-induced apoptosis in gastric cancer cells. Data indicated as percentage proportions of apoptotic gastric cancer cells for Live/Dead assay. Values are mean ± SE of triplicate. Data are a representative of two independent experiments.

**Figure 2.** A and B, DNA binding assay results showing that γ-tocotrienol suppresses the constitutive activation of NF-κB in SNU-16 and SNU-5 cells in a dose-dependent manner. SNU-16 and SNU-5 (1 x 10⁶) cells were treated with γ-tocotrienol (10, 25 and 50 µM) for 4 h, and nuclear extracts were prepared and assayed for NF-κB activation by ELISA linked DNA binding assay. C, MKN45 (1 x 10⁶) cells were treated with capecitabine (10, 20 and 25 µM) for 4 h, and nuclear extracts were prepared and assayed for NF-κB activation by ELISA linked DNA binding assay. D, MKN45 (1 x 10⁶) cells were pre-treated with γ-tocotrienol (10, 25 and 50 µM) for 4 h, stimulated with capecitabine 25 µM) for 4 h, and then the nuclear extracts were prepared and assayed for NF-κB activation by ELISA linked DNA binding assay. E, γ-tocotrienol suppressed the constitutive expression of gene products involved in proliferation, metastasis and antiapoptosis in gastric cancer cells. SNU-16 (1 x 10⁶) cells were treated with 50 µM γ-tocotrienol for the indicated time points and western blot was performed as described under Materials and Methods section. * indicates p<0.05.
Figure 3. γ-tocotrienol potentiates the effect of capecitabine to inhibit the growth of gastric cancer in nude mice. A, schematic representation of experimental protocol described in Materials and Methods. Group I was given corn oil (100 µL, p.o., daily), group II was given γ-tocotrienol (1 mg/kg body weight, i.p. thrice/week), group III was given capecitabine (60 mg/kg body weight, twice weekly by gavage), and group IV was given γ-tocotrienol (1 mg/kg body weight, i.p. thrice/week), and capecitabine (60 mg/kg body weight, twice weekly by gavage). B, necropsy photographs of mice bearing subcutaneously implanted gastric tumors; C, tumor volumes in mice measured during the course of experiment and calculated using the formula $V = \frac{4}{3} \pi r^3$, *** indicates p<0.001. D, tumor volumes in mice measured on the last day of the experiment at autopsy using Vernier calipers and calculated using the formula $V = \frac{4}{3} \pi r^3$ ($n = 5$). Columns, mean; bars, SE. *** indicates p<0.001.

Figure 4. γ-tocotrienol enhances the effect of capecitabine against tumor cell proliferation and angiogenesis in gastric cancer. A left panel, immunohistochemical analysis of proliferation marker Ki-67 indicates the inhibition of gastric cancer cell proliferation in γ-tocotrienol either alone or in combination with capecitabine-treated groups of animals. A right panel, quantification of Ki-67+ cells as described in Materials and Methods. Columns, mean of triplicate; bars, SE. *** indicates p<0.001. B left panel, immunohistochemical analysis of CD31 for microvessel density in gastric cancer tumors indicates the inhibition of angiogenesis by either γ-tocotrienol alone and in combination with capecitabine; B right panel, quantification of CD31+ microvessel density as described in Materials and Methods. Columns, mean of triplicate; bars, SE. *** indicates p<0.001.

Figure 5. γ-tocotrienol enhances the effect of capecitabine against the expression of NF-κB and NF-κB–regulated gene products in gastric cancer tissue samples. A, detection of NF-κB by DNA
binding assay in tumor tissue samples showed the significant inhibition of NF-κB by combination, *** indicates p<0.001. B, Western blot analysis showed the inhibition of NF-κB (p65) by γ-tocotrienol in whole cell extracts from animal tissue. C, Western blot showing that combination of γ-tocotrienol and capecitabine inhibit the expression of NF-κB–dependent gene products cyclin D1, COX-2, ICAM-1, MMP-9, Bcl-xL, survivin, XIAP and pro-caspase-3 in gastric tumor tissues. Samples from three mice in each group were analyzed and representative data are shown.

**Figure 6.** Immunohistochemical analysis of nuclear p65, COX-2, MMP-9 and VEGF showed the inhibition of NF-κB, COX-2, MMP-9 and VEGF by either γ-tocotrienol alone or in combination with capecitabine. *Percentage*, positive staining for the given biomarker.
A. γ-Tocotrienol

B. 

![Graph showing the effect of γ-Toco at different concentrations (0, 10, 25, 50 µM) on SNU-5, SNU-16, and MKN-45 cell lines over time (0, 24, 48, 72 h).]

C. 

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Control</th>
<th>γ-Toco</th>
<th>Cape</th>
<th>γ-Toco + Cape</th>
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<td><img src="image" alt="Histogram" /></td>
<td><img src="image" alt="Histogram" /></td>
<td><img src="image" alt="Histogram" /></td>
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<tr>
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<td>S phase</td>
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<td>14.53%</td>
<td>18.1%</td>
<td>5.4%</td>
</tr>
<tr>
<td>G2/M</td>
<td>22.32%</td>
<td>19.29%</td>
<td>28.87%</td>
<td>17.13%</td>
</tr>
</tbody>
</table>

| SNU-16     | ![Histogram](image) | ![Histogram](image) | ![Histogram](image) | ![Histogram](image) |
| Sub G0/G1  | 4.14%   | 7.86%  | 11.16% | 43.25% |
| Go/G1      | 45.02%  | 42.04% | 38.21%| 39.02% |
| S phase    | 15.04%  | 15.75% | 15.81%| 8.79% |
| G2/M       | 35.92%  | 30.97% | 29.4% | 9.38%  |

| MKN-45     | ![Histogram](image) | ![Histogram](image) | ![Histogram](image) | ![Histogram](image) |
| Sub G0/G1  | 5.78%   | 8.28%  | 13.04% | 48.81% |
| Go/G1      | 55.81%  | 46.48% | 52.79%| 46.84% |
| S phase    | 15.45%  | 13.46% | 13.3% | 3.7% |
| G2/M       | 22.96%  | 29.46% | 20.3% | 0.82% |

D. 

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Control</th>
<th>γ-Toco</th>
<th>Cape</th>
<th>γ-Toco + Cape</th>
</tr>
</thead>
<tbody>
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<td><img src="image" alt="Apoptosis image" /></td>
<td><img src="image" alt="Apoptosis image" /></td>
<td><img src="image" alt="Apoptosis image" /></td>
</tr>
<tr>
<td>Apoptosis %</td>
<td>3.8 ± 2.5</td>
<td>9.7 ± 3.1</td>
<td>11.8 ± 2.6</td>
<td>41.8 ± 4.3</td>
</tr>
</tbody>
</table>

| SNU-16     | ![Apoptosis image](image) | ![Apoptosis image](image) | ![Apoptosis image](image) | ![Apoptosis image](image) |
| Apoptosis %| 3 ± 1.2  | 7.1 ± 2.5 | 13.1 ± 2.2 | 42.3 ± 5.9 |

| MKN-45     | ![Apoptosis image](image) | ![Apoptosis image](image) | ![Apoptosis image](image) | ![Apoptosis image](image) |
| Apoptosis %| 5.7 ± 1.7 | 8.3 ± 2.3 | 15.8 ± 3.1 | 47.7 ± 6.1 |
A. Ki67 immunohistochemistry

- Vehicle
- \( \gamma \)-Toco
- Cape
- \( \gamma \)-Toco + Cape

B. CD31 immunohistochemistry

- Vehicle
- \( \gamma \)-Toco
- Cape
- \( \gamma \)-Toco + Cape

*** p<0.001
A.

B.

- [Graph showing Optical Density (450nm) for different treatments with significance levels marked with stars.]

- [Western blot images for p65 and β-actin, showing treatment conditions: - - + + γ-Toco Cape - - - + Cape - + - + γ-Toco Cape - + + - Cape - + - + γ-Toco Cape - + + + Cape]

- [Proteins in the blot: Cyclin D1, COX-2, MMP-9, ICAM-1, Bcl-xL, Survivin, XIAP, β-actin]
First evidence that g-tocotrienol inhibits the growth of human gastric cancer and chemosensitizes it to capecitabine in a xenograft mouse model through the modulation of NF-κB pathway


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