Mol. Nutr. Food Res. 2008, *52*, 419 – 426 DOI 10.1002/mnfr.200700222

Research Article

β-Sitosterol enhances tamoxifen effectiveness on breast cancer cells by affecting ceramide metabolism

Atif B. Awad[1], Stephanie L. Barta[1], Carol S. Fink[1] and Peter G. Bradford[2]

[1] Department of Exercise and Nutrition Sciences, School of Public Health and Health Professions at the University at Buffalo, Buffalo, NY, USA
[2] Department of Pharmacology and Toxicology, School of Medicine at the University at Buffalo, Buffalo, NY, USA

The objective of this study was to investigate the effects of the dietary phytosterol β-sitosterol (SIT) and the antiestrogen drug tamoxifen (TAM) on cell growth and ceramide (CER) metabolism in MCF-7 and MDA-MB-231 human breast cancer cells. The MCF-7 and MDA-MB-231 cell lines were studied as models of estrogen receptor positive and estrogen receptor negative breast cancer cells. Growth of both cell lines as determined using the sulforhodamine B assay was inhibited by treatment with 16 μM SIT but only MCF-7 cell growth was inhibited by treatment with 1 μM TAM. The combination of SIT and TAM further inhibited growth in both cell lines, most significantly in MDA-MB-231 cells. CER is a proapoptotic signal and CER levels were increased in both MCF-7 and MDA-MB-231 cells by individual treatment with SIT and TAM and the combined treatment raised cellular CER content even further. SIT and TAM raised CER levels by different means. SIT potently activated *de novo* CER synthesis in both MCF-7 and MDA-MB-231 cells by stimulating serine palmitoyltransferase activity; whereas TAM promoted CER accumulation in both cell types by inhibiting CER glycosylation. These results suggest that the combination regimen of dietary SIT and TAM chemotherapy may be beneficial in the management of breast cancer patients.

Keywords: Breast cancer therapy / Ceramide / Phytosterols / Serine palmitoyltransferase / Tamoxifen

Received: June 19, 2007; revised: August 22, 2007; accepted: September 8, 2007

1 Introduction

Breast cancer is the most common cancer in women and the second leading cause of cancer death in women of developed countries. Data show that there are more than 200 000 new breast cancer diagnoses and over 40 000 deaths from breast cancer annually in the United States [1]. It has been established that estrogen plays a role in the development of breast cancer especially in its early stages [2]. Recent studies have suggested that dietary factors also play a role in the development of breast cancer [3, 4]. Accordingly, dietary recommendations have been offered as potential protection from the disease. For example, it has been recommended that lower dietary fat and a reduction in total caloric intake, in addition to maintaining a physically active lifestyle, may reduce the likelihood of developing breast cancer [5, 6].

Both controlled clinical studies and epidemiologic data suggest that certain dietary phytochemicals may offer protection from specific cancers. Included among these phytochemicals are polyphenols, phytoestrogens, and phytosterols [7 – 12]. On the other hand, there has been little research on the mechanisms of cancer development in which the effects of specific dietary components in conjunction with standard chemotherapies on cancer were examined. The most commonly used drug in the treatment of breast cancer is the selective estrogen receptor modulator, tamoxifen (TAM). TAM has been shown to be most effective when combined with surgery and/or radiation in the early stages of breast tumor development, particularly in estrogen receptor positive tumors. Moreover, TAM is also potentially beneficial in the prevention of breast cancer in women at high

Correspondence: Professor Atif B. Awad, 15 Farber Hall, State University of New York at Buffalo, Buffalo, New York 14214, USA
E-mail: awad@buffalo.edu
Fax: +1-716-829-3700

Abbreviations: CER, ceramide; **GalCer,** galactosylceramide; **GluCer,** glucosylceramide; **GSL,** glycosphingolipid; **LacCer,** lactosylceramide; **SIT,** β-sitosterol; **SM,** sphingomyelin; **SPH,** sphingosine; **SRB,** sulforhodamine B; **TAM,** tamoxifen

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

risk for the disease [13]. It would be significant to know whether cancer protective dietary components, such as phytosterols, might influence the efficacy of TAM in breast cancer treatment.

Phytosterols are plant sterols chemically similar to cholesterol but found exclusively in plants, particularly legumes. The most common phytosterol in the diet is β-sitosterol (SIT) [6]. Controlled laboratory experiments have shown that SIT at dietary relevant levels inhibits the growth *in vitro* and *in vivo* of several types of tumor cells including breast cancer cells [14 – 16]. SCID mice fed diets rich in phytosterols had reduced rates of growth and metastasis of human breast cancer cells [17]. Proposed mechanisms to account for these actions of phytosterols include their promotion of cell cycle arrest, induction of apoptosis, and stimulation of sphingomyelin (SM) turnover [7].

The objective of the present study was to investigate the effects of combining the dietary phytosterol SIT with the breast cancer drug TAM on cell growth as well as on ceramide (CER) metabolism in MCF-7 and MDA-MB-231 human breast cancer cells. Since both SIT and TAM affect CER metabolism in breast cancer cells [7, 18], we hypothesized that the combination of the two compounds might potentiate their effects on tumor cell apoptosis by stimulating CER production and/or reducing its conversion to metabolites that are less toxic to the tumor cells such as glycosylated CERs.

2 Materials and methods

2.1 Cell culture

MCF-7 and MDA-MB-231 breast cancer cells were maintained in complete RPMI 1640 media (5% FBS, 1% antibiotic/antimycotic, 2.0 g/L sodium bicarbonate, pH 7.4) in a humidified environment (37°C, 5% CO_2, 95% air). Media were changed every 48 h and cultures were split and passed every 5 – 7 days or when they reached 80 – 90% confluence.

2.2 Cell growth and cytotoxicity analysis

Cell growth of MCF-7 and MDA-MB-231 cells along with the cytotoxicity of SIT, TAM, and cholesterol treatments were measured by the sulforhodamine B (SRB) assay [19]. Cholesterol is the main sterol in the blood and thus it was as control for SIT in these studies. Cells were seeded at 1000 cells/well in 96-well plates, allowed to adhere for 24 h, and then treated with cholesterol (0.5 – 16 μM), SIT (0.5 – 16 μM), TAM (1 – 10 μM), or a combination of treatments for 3 days. Ethanol vehicle served as the control. After treatment, cells were fixed in 10% trichloroacetic acid for 45 min at 4°C, washed five times with water, and allowed to air dry. Cells and appropriate blank wells were stained with 0.4% w/v SRB dye in 1% acetic acid for 25 min. After washing five times with 1% acetic acid, the plates were air dried, and the SRB dye was solubilized with 150 μL of 10 mM Tris base (pH 10.5). The absorbance at 570 nm was compared against vehicle-treated control cells.

2.3 Sphingolipid analysis

The effect of SIT and TAM treatments on CER synthesis was assessed by monitoring [^{3}H]-serine incorporation into cellular sphingolipids, including SM, sphingosine (SPH), and CER. Sphingolipid isolation methods were adapted from Jones and Murray [20] and Hubscher *et al.* [21]. Cells were seeded (3000 cells/cm^2) in 6-well plates. After 48 h, media were replaced with complete RPMI 1640 containing vehicle or TAM (1 or 5 μM). SIT (4 or 16 μM) was added as indicated for specific treatment combinations. On day 4, media were replaced with MEM (1% FBS, 1% antibiotic/antimycotic, 2.0 g/L sodium bicarbonate, 2.0 g/L glucose) also containing treatments. [^{3}H]-Serine (2 μCi/ml) was added for 24 h. On day 5 (3 days of treatment and after 24 h of [^{3}H]-serine exposure), cells were incubated for 5 h with fresh MEM containing 5 mM nonradioactive serine. Nonradiolabeled cells, grown in parallel wells and treated with SIT and/or TAM, were trypsinized and counted. Radiolabeled cells were washed in PBS, precipitated in ice-cold methanol containing 20 μg each of nonradiolabeled SM, SPH, and CER as carriers and scraped into glass test tubes. Chloroform lipid extracts were prepared and saponified with 0.2 M KOH at 37°C. Sphingolipids were separated by TLC in a solvent system of chloroform/methanol/ammonium hydroxide/water (80:20:1:1) to 9.5 cm followed by further separation in a solvent system of anhydrous diethyl ether/methanol (99:1) to 19.5 cm. Sphingolipids were visually identified by iodine staining using authentic sphingolipid standards. Silica gel areas corresponding to SM, CER, and SPH were scraped into scintillation vials and 0.5 mL water was added. After 10 min of sonication, scintillation fluid was added and the [^{3}H]-radioactivity was measured by liquid scintillation spectrometry. Sample DPMs were normalized to the number of cells *per* well.

2.4 Serine palmitoyltransferase (SPT) assay

MCF-7 and MDA-MB-231 cells were seeded (5000 cells/cm^2) into T-150 flasks and after 2 days, supplemented with 16 μM SIT, 1 μM TAM, SIT plus TAM, or vehicle. After 3 days of treatment, microsomes were prepared. SPT activity was quantified by measuring [^{3}H]-serine substrate incorporation into 3-ketosphinganine and other sphingolipids [22]. Microsomes (50 μg protein) were preincubated in a reaction buffer of 0.1 M HEPES (pH 8.3), 2.5 mM EDTA, 50 μM pyridoxyl phosphate, 5 mM DTT, and 1.0 mM L-serine for 10 min at 37°C. Reactions were initiated by the addition of 10 μL of palmitoyl CoA (0.2 mM) and [^{3}H]-serine (1.0 μCi) substrate. After 20 min, the reaction was terminated by the addition of 0.5 N NH_4OH. After the addition of

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

SPH carrier (25 μg), sphingolipid products were extracted with chloroform/methanol (2:1) and 0.5 N NH_4OH (2 mL). The chloroform phase containing the lipids was dried, and the radioactivity was measured by liquid scintillation spectrometry. SPT activity is expressed as pmol sphingolipid formed/min/mg protein.

2.5 Glycosphingolipid (GSL) analysis

MCF-7 and MDA-MB-231 GSLs were radiolabeled *in situ* with [^{3}H]-galactose and measured in lipid extracts after separation on TLC [23]. Cells were seeded at the same density and treated as described for sphingolipid analysis. After 48 h of treatment, media were replaced with 2 mL of RPMI 1640 containing SIT and/or TAM, or vehicle, and [^{3}H]-galactose (2 μCi/mL). After an additional 24 h, cells were washed in PBS, scraped in methanol, and the GSLs extracted with 4 mL chloroform and 2 mL water. Carrier GSLs (20μg each of glucosylceramide (GluCer), galactosylceramide (GalCer), lactosylceramide (LacCer), and gangliosides) were added to improve recovery. The lower chloroform phase was dried and the extracts spotted for TLC. GSLs were separated in a solvent system of chloroform/methanol/water (40:10:1) and identified by iodine staining and autoradiography after treatment with EN^{3}HANCE (PerkinElmer). Corresponding areas of silica gel were scraped, placed in scintillation vials and radioactivity was measured by liquid scintillation spectrometry. Sample DPMs were normalized to the number of cells *per* well.

2.6 Statistical analyses

Values are expressed as means ± the standard errors of the means. Comparison data of growth curves were analyzed using a one-way analysis of variance (ANOVA). All other data were analyzed using a two-way ANOVA. Significance between means was tested using either a T-test (one-way) or the Bonferroni *post hoc* test (two-way). Differences between means were deemed statistically significant at $p < 0.05$. ProStat (Poly Software International, Pearl River, NY) was used to calculate the one-way ANOVA and SigmaStat (SPSS) was used to calculate the two-way ANOVA.

3 Results

3.1 SIT inhibits growth of MCF-7 and MDA-MB-231 cells

Breast cancer cells were treated with varying concentrations of SIT for 3 days to determine effects on cell growth. MCF-7 cell growth was decreased to 87% of control by 8 μM SIT and to 71% of control by 16 μM SIT. MDA-MB-231 cell growth was decreased to 50% of control by 8 μM SIT and to 38% of control by 16 μM SIT (Fig. 1).

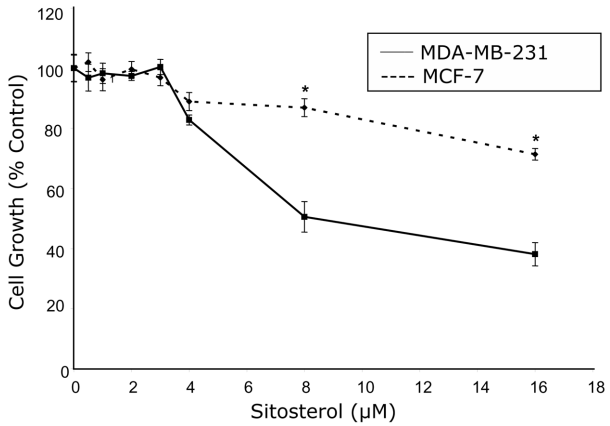

Figure 1. Effect of SIT on the growth of MCF-7 and MDA-MB-231 breast cancer cells. Cells (1000 cells/well) were seeded in 96-well plates and after 24 h, treated with SIT for 3 days. Cell growth was determined by the SRB assay. Values (mean ± SE, $n = 6$) for MCF-7 and MDA-MB-231 growth are significantly different (*$p < 0.001$) from each other (*t*-test).

3.2 TAM inhibits growth of MCF-7 and MDA-MB-231 cells

Growth of both MCF-7 and MDA-MB-231 cells was inhibited by 3 days TAM treatment (Fig. 2). Growth was inhibited to approximately the same extent and in a dose-dependent manner in both cell types. However, TAM was more potent in inhibiting the growth of MCF-7 cells compared to MDA-MB-231 cells, although maximal growth inhibition occurred at 10 μM in both cell lines. At 10 μM TAM, MDA-MB-231 cell growth was decreased to 17% of controls and MCF-7 growth was decreased to 7% of controls. Although MDA-MB-231 cells are reported to be estrogen receptor alpha-negative, they do express small but detectable levels of estrogen receptor beta [24]. Thus, it is possible the inhibition of MDA-MB-231 cell growth by TAM may be being mediated by estrogen receptor beta or TAM may be acting to inhibit growth by means in addition to its role as an antiestrogen.

3.3 The combination of SIT and TAM potentiates inhibition of MCF-7 and MDA-MB-231 cell growth

The effect of combined treatment with SIT and TAM on breast cancer cell growth was determined at two concentrations of each substance. TAM concentrations (1 and 5 μM) were chosen to reflect the low and mid-portion of the TAM dose-response curve. SIT concentrations (4 and 16 μM) were also chosen based on growth effects, but these concentrations were also selected because they reflect the physiologic levels found in the blood of individuals who consume characteristically western (4 μM) and primarily plant based (16 μM) diets.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

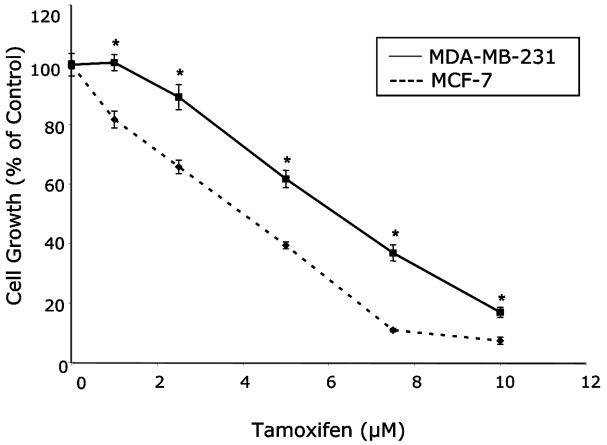

Figure 2. Effect of TAM on the growth of MCF-7 and MDA-MB-231 breast cancer cells. Cells (1000 cells/well) were seeded in 96-well plates and after 24 h, treated with TAM for 3 days. Cell growth was determined by the SRB assay. Values (mean ± SE, $n = 7$) for MCF-7 and MDA-MB-231 growth are significantly different (*$p < 0.05$) from each other (*t*-test).

Table 1. Effect of TAM and SIT on the growth of MCF-7 and MDA-MB-231 cells

SIT	TAM		
	–	1 μM	5 μM
MCF-7			
–	100 ± 2.4[a)]	71.1 ± 0.8[c)]	13.3 ± 0.6[e)]
4 μM	103.4 ± 1.2[a)]	73.3 ± 2.7[c)]	10.8 ± 0.8[e)]
16 μM	63.6 ± 1.8[b)]	58.9 ± 1.2[d)]	11.0 ± 0.8[e)]
MDA-MB-231			
–	100 ± 1.8[a)]	104.2 ± 4.3[a)]	19.4 ± 1.0[d)]
4 μM	96.2 ± 2.5[a)]	80.3 ± 1.8[c)]	16.4 ± 1.6[d)]
16 μM	40.8 ± 2.1[b)]	35.2 ± 1.9[b)]	10.6 ± 0.9[e)]

Values are numbers of viable cells as percents of vehicle-treated controls. Values (means ± SE, $n = 6$) within the same cell type with different superscripts are significantly different ($p < 0.05$) by two-way ANOVA followed by the Bonferroni *post hoc* test.

The effects of TAM and SIT on MCF-7 cell growth are summarized in Table 1. MCF-7 cell growth was unaffected by 4 μM SIT alone but was significantly reduced ($p < 0.05$) to 64% of control by 16 μM SIT. Low dose 1 μM TAM alone reduced MCF-7 growth to 71% of control (Table 1). When 1 μM TAM was combined with 4 μM SIT, there was no further reduction of growth by the inclusion of SIT (73 and 71% of control). However, when 1 μM TAM was combined with 16 μM SIT there was a further significant reduction in growth, although much less than an additive effect (TAM 73%, SIT 64%, TAM + SIT 59% of control, $p < 0.05$). The combination of 5 μM TAM with either 4 or 16 mM SIT significantly inhibited cell growth ($p < 0.05$) to 13% (5 μM TAM), 11% (5 μM TAM plus 4 μM SIT), and 11% (5 μM TAM plus 16 μM SIT) of control. There was no significant additive effect of SIT with 5 μM TAM, perhaps because of the dramatic growth inhibition induced by each agent alone.

With MDA-MB-231 cells, there was a significant inhibitory effect on growth by combining SIT with TAM (Table 1). When examined alone, only the highest concentration of SIT (16 μM) and TAM (5 μM) inhibited MDA-MB-231 cell growth (to 41 and 20% of control, respectively). However, combined treatments of low dose 1 μM TAM plus 4 μM SIT or 1 μM TAM plus 16 mM SIT inhibited cell growth ($p < 0.05$) to 80 and 35% of control, respectively. The combination of 5 μM TAM with 4 or 16 μM SIT was significantly more effective than either agent alone: 5 μM TAM decreased cell growth to 20% of control, and the inclusion of 4 μM or 16 μM SIT with 5 μM TAM decreased cell growth to 17 and 11% of control, respectively.

Considering the effects on cell growth, MCF-7 and MDA-MB-231 cells reacted somewhat differently to the treatments of 4 μM SIT and to the combination of 5 μM TAM plus 16 μM SIT relative to their respective controls (Table 1). MCF-7 cells were affected by both concentrations of TAM to a greater extent than MDA-MB-231 cells. Conversely, MDA-MB-231 cell growth was inhibited to a greater extent by SIT, as seen with 16 μM SIT treatment alone, as well as in its combination with 1 μM TAM.

3.4 SIT and TAM increase CER production in MCF-7 and MDA-MB-231 cells

CER is an intracellular modulator of cell growth. The effects of SIT and TAM alone and in combination on intracellular CER production were examined in breast cancer cells. Treatment of MCF-7 cells for 3 days with 4 μM SIT had no effect on CER formation as assessed by [^{3}H]-serine incorporation into CER (Table 2). However, treatment with

Table 2. Effect of TAM and SIT on the total [^{3}H]-CER content of MCF-7 and MDA-MB-231 cells

SIT	TAM	
	–	1 μM
MCF-7		
–	100 ± 25.2[a)]	234.2 ± 59.3[c)]
4 μM	127.6 ± 23.7[a)]	339.2 ± 95.8[c)]
16 μM	185.1 ± 38.6[b)]	516.7 ± 91.6[c)]
MDA-MB-231		
–	100 ± 6.8[a)]	178.9 ± 27.0[a)]
4 μM	103.5 ± 11.8[a)]	265.2 ± 16.4[a)]
16 μM	936.7 ± 150.0[b)]	2010.2 ± 360.4[c)]

Values are total [^{3}H]-CER *per* 10^6 cells expressed as percents of vehicle-treated controls. Values (means ± SE, $n = 3$) within the same cell type with different superscripts are significantly different ($p < 0.05$) by two-way ANOVA followed by the Bonferroni *post hoc* test.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

16 μM SIT increased CER formation by 85% compared to controls. The effect of TAM on CER formation in MCF-7 cells was not affected by the presence or absence of SIT. The combined effect of TAM (pooled data from 1 μM TAM, 1 μM TAM plus 4 μM SIT, and 1 μM TAM plus 16 μM SIT) showed a 3.6-fold increase ($p < 0.05$) in CER formation compared to the vehicle treatment control.

In MDA-MB-231 cells, CER formation was affected by 16 μM SIT treatment alone and by combination treatment of 16 μM SIT plus 1 μM TAM (Table 2). A nine-fold increase in CER levels was observed in MDA-MB-231 cells after 3 days treatment with 16 μM SIT and a 20-fold increase was observed after 3 days combined treatment with 16 μM SIT plus 1 μM TAM. CER levels were relatively higher and affected to greater extents in MDA-MB-231 cells compared to MCF-7 cells.

3.5 SIT and TAM increase serine palmitoyltransferase activity in MCF-7 and MDA-MB-231 cells

Serine palmitoyltransferase (SPT) is the rate-limiting enzyme in CER synthesis. The effects of SIT and TAM on SPT activity were determined by measuring [^{3}H]-serine incorporation into sphingolipids using microsomes of cells treated for 3 days with SIT and TAM. Treatment of MCF-7 cells with 1 μM TAM had no significant effect on microsomal SPT activity (140 ± 8 *vs.* 161 ± 15 pmol/min/mg; Table 3). However, treatment with 16 μM SIT significantly increased SPT activity (180 ± 9 pmol/min/mg) and this effect was potentiated by the inclusion of 1 μM TAM (221 ± 5 pmol/min/mg).

SPT activity of MDA-MB-231 cell microsomes was considerably lower than that of MCF-7 cell microsomes (Table 3): basal activities of 49 ± 4 *versus* 140 ± 8 pmol/min/mg. With MDA-MB-231 cells, significant differences in SPT activity were observed after treatment with either 1 μM TAM or 16 μM SIT; however, the effect of the combination of TAM plus SIT was no different than that of SIT alone (Table 3).

3.6 SIT and TAM affect GSL levels in MCF-7 and MDA-MB-231 cells

CER can be glycosylated with simple or complex sugars within the sphingolipid biosynthetic pathway to produce GSLs including GluCer, GalCer, LacCer, and gangliosides. This biosynthesis is catalyzed by the GluCer synthase enzyme and TAM has been reported to be an inhibitor of GluCer synthase [25]. An inhibitory effect of TAM on GluCer synthase would prolong the lifetime of CER within cells. It is not known how SIT affects CER glycosylation in breast cancer cells.

In MCF-7 cells, SIT alone (4 or 16 μM) had no significant effect on total GSL levels in MCF-7 cells (Table 4).

Table 3. Effect of TAM and SIT on serine palmitoyltransferase activity in MCF-7 and MDA-MB-231 cells

Treatment	SPT activity (pmol/min/mg protein)	
	MCF-7	MDA-MB-231
Vehicle	140 ± 8[a)]	49 ± 4[a)]
1 μM TAM	161 ± 15[a)]	90 ± 5[b)]
16 μM SIT	180 ± 9[b)]	178 ± 11[c)]
1 μM TAM + 16 μM SIT	221 ± 5[c)]	187 ± 9[c)]

Values are total pmol [^{3}H]-sphingolipid formed/min/mg protein by microsomes prepared from treated cells. Values (means ± SE, $n = 4$) within the same cell type with different superscripts are significantly different ($p < 0.05$) by two-way ANOVA followed by the Bonferroni *post hoc* test.

Table 4. Effect of TAM and SIT on the total [^{3}H]-GSL content of MCF-7 and MDA-MB-231 cells

SIT	TAM	
	–	1 μM
MCF-7		
–	100 ± 10.1[a)]	25.8 ± 2.3[b)]
4 μM	100.8 ± 28.4[a)]	38.4 ± 4.5[b,c)]
16 μM	153.6 ± 43.8[a)]	55.7 ± 13.5[c)]
MDA-MB-231		
–	100 ± 8.2[a)]	64.8 ± 5.9[b)]
4 μM	113.0 ± 14.9[a)]	37.3 ± 5.3[b)]
16 μM	116.6 ± 9.1[a)]	53.3 ± 4.7[b)]

Values are total [^{3}H]-glycosylated sphingolipid *per* 10^6 cells expressed as percents of vehicle-treated controls. Values (means ± SE, $n = 3$ [MCF-7], $n = 4$ [MDA-MB-231]) within the same cell type with different superscripts are significantly different ($p < 0.05$) by two-way ANOVA followed by the Bonferroni *post hoc* test.

However, 1 μM TAM reduced GSL levels to 26% of controls and this effect of TAM was countered partially by 16 μM SIT (56% of control). Similarly in MDA-MB-231 cells, SIT alone (4 and 16 μM) had no effect on total GSL levels and TAM (1 μM) inhibited GSL levels (to 65% of the control) (Table 4). However, unlike MCF-7 cells, the combination of TAM plus SIT was not significantly different than TAM alone.

4 Discussion

It is important to investigate the potential effects of combining SIT, a dietary phytosterol, with TAM, a widely prescribed breast cancer drug, on breast cancer cell growth. Combining dietary and pharmacologic therapies in the treatment of breast cancer may show potentiating activity. MCF-7 and MDA-MB-231 breast cancer cell lines were chosen for this study. MCF-7 cells are estrogen receptor-

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

positive and are sensitive to hormonal regulation of cell growth and metabolism typical of early breast cancers. MDA-MB-231 cells are estrogen receptor-negative and are used to model late-stage breast cancers.

Previous research has indicated that SIT has growth inhibitory effects on MCF-7 and MDA-MB-231 breast cancer cells [14, 15, 24] and on tumors of other organs (*e.g.*, prostate and colon) [26–28] *in vitro* and *in vivo*. This effect may be mediated by the proapoptotic effect of SIT [14, 16]. The current study supports these findings in that SIT was found to inhibit the growth of MCF-7 and MDA-MB-231 breast cancer cells. SIT was found to be more effective in inhibiting MDA-MB-231 cell growth. At the highest concentration used, SIT (16 μM) inhibited MDA-MB-231 cell growth by 62% and MCF-7 cell growth by 29%.

The growth inhibitory effect of TAM on estrogen receptor positive tumors as well as on MCF-7 breast cancer cells has been documented [29, 30]. Since TAM is a selective estrogen receptor modulator, its effects would be expected to be more pronounced in the estrogen receptor-positive MCF-7 breast cancer cells. The present study indicates that growth of both MCF-7 and MDA-MB-231 cells is inhibited by TAM in a dose-dependent fashion. However, at the lowest TAM concentration used (1 μM), cell growth inhibition occurred only in MCF-7 cells and not in MDA-MB-231 cells, agreeing with other reports [31, 32].

The combination of TAM and SIT on breast cancer cell growth has not previously been investigated. However, other TAM-nutrient and TAM-drug combinations have been studied and have demonstrated growth inhibition in various cancer cell lines, including MCF-7 and MDA-MB-231 [33–36]. For example, Aoyama [33] investigated the effect of combining TAM with the synthetic retinoid, *N*-(4-hydroxyphenyl)retinamide (4-HPR), on MDA-MB-231 breast cancer cells. This TAM-drug combination potently inhibited cell growth with arrest at the G_2/M phase transition and was associated with increased cellular glycosylated lipid (GM3) levels. Similarly, Cover *et al.* [34] demonstrated that the combination of TAM with the naturally occurring phytochemical, indole-3-carbinol, inhibited DNA synthesis and reduced anchorage-dependent cell growth in MCF-7 and BM breast cancer cells to a greater extent than either compound alone.

The present study demonstrates that the combination of TAM and SIT inhibited breast cancer cell growth more than either agent alone. In the case of MCF-7 cells, TAM had a significant growth inhibitory effect on its own. Potentiation of the effect of 1 μM TAM was seen only with the high concentration (16 μM) of SIT. The effect on MDA-MB-231 cells was more pronounced: 4 μM SIT enhanced the growth inhibitory effect of 1 μM TAM and 16 μM SIT enhanced the inhibitory effect of 5 μM TAM.

CER has been implicated as a second messenger molecule and may be a mediator of numerous cancer chemotherapeutic agents [37]. CER inhibits cell growth, induces cell cycle arrest, and promotes apoptosis. SIT treatment increases CER production in LNCaP prostate cancer cells and in HT-29 colon cancer cells [7, 27]. This action was accompanied by SM breakdown and increased apoptosis. The present study demonstrates increased CER production in breast cancer cells treated with the combination of SIT and TAM: 1 μM TAM plus 16 μM SIT in MDA-MB-231 cells produced a 20-fold increase in CER levels compared to the control. This increase in CER in MDA-MB-231 cells was synergistic and not merely additive. In MCF-7 cells, 1 μM TAM had no effect on CER production on its own; however, 1 μM TAM significantly potentiated CER production by 4 μM and 16 μM SIT (combined data).

CER can be metabolized into GSLs by glycosylation with the addition of one or more monosaccharides. CER glycosylation attenuates the growth inhibition and apoptotic characteristics of CER, potentially allowing cancer cells to escape CER-induced apoptosis, to proliferate, and to become potentially drug resistant [28–30]. Research shows that TAM inhibits GluCer synthase [31], which catalyzes CER glycosylation. The inhibition of GluCer synthase by TAM enables the levels of CER to accumulate, perhaps resulting in cell growth inhibition [32]. The effect of SIT in combination with TAM on the levels of glycosylated CER-containing lipids (GluCer, GalCer, LacCer, and gangliosides) in breast cancer cells have not been reported until this point. The present study demonstrates that TAM reduced the accumulation of GSLs in MCF-7 and MDA-MB-231 cells by 74 and 35%, respectively. Similar findings were reported with studies of LNCaP and PC-3 prostate cancer cells [38]. Compared to TAM alone, SIT and TAM in combination had no effect on GSL levels in MDA-MB-231 cells, but a small increase was observed at the higher concentrations of SIT treatment in MCF-7 cells. In the latter situation, the increase was mostly attributed to high ganglioside levels (data are not shown).

SPT is the rate-limiting enzyme for *de novo* CER synthesis. The combined effect of SIT and TAM on SPT activity has not been previously reported. Treatment of MDA-MB-231 cells with 1 μM TAM produced a 1.9-fold increase in SPT activity. Treatment with 16 μM SIT produced a 3.7-fold increase. The combination treatment of TAM and SIT did not generate a significantly greater increase in SPT activity than was produced by SIT treatment alone. This suggests that although TAM increased SPT activity slightly in MDA-MB-231 cells, the major effector of this enzyme was SIT. In MCF-7 cells, there was no significant effect of 1 μM TAM alone and SPT activity in these cells was less influenced by treatment with SIT (1.3-fold increase above its control) than in MDA-MB-231 cells. However, SIT in combination with TAM potentiated the stimulatory effects of either treatment alone, suggesting an interaction of these two treatments on SPT activity in MCF-7 cells. The mechanism underlying the synergistic actions of SIT and TAM on SPT is unknown.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

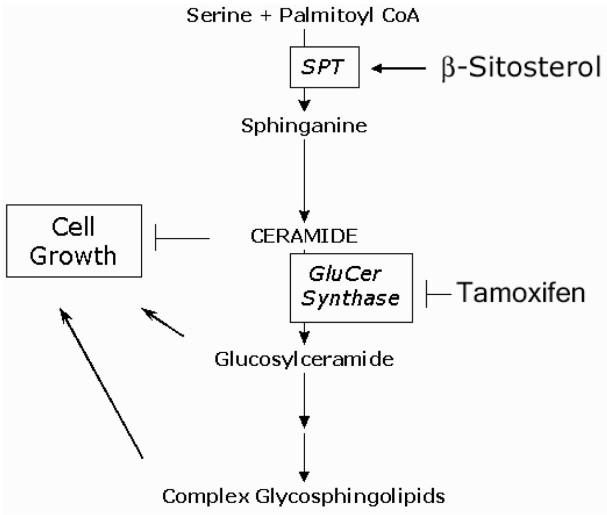

Figure 3. Proposed mechanism of SIT and TAM action within the sphingolipid metabolism pathway. SIT stimulates CER formation *via* activation of serine palmitoyl transferase, while TAM inhibits glucosyl CER synthase and the metabolism of CER to GSLs. The combination of SIT and TAM potentiates an increase in CER levels in breast cancer cells which may inhibit growth.

In conclusion, the results of the present study suggest possible mechanisms by which SIT and TAM might interact to affect sphingolipid metabolism, particularly *de novo* CER synthesis and CER glycosylation, and to inhibit cell growth in two breast cancer cell lines (Fig. 3). One mode of action for SIT is to increase SPT activity. Increased SPT activity would increase CER concentrations. The primary effect of TAM (1 μM), however, is to increase cellular CER levels by decreasing GSL levels. Although GluCer synthase was not directly measured in the current study, it is inferred from a previous study on breast cancer [23] that TAM is specifically inhibiting this enzyme to produce the observed decrease in GSLs and subsequent increase in CER.

Therapeutic implications of this study suggest that plant-based diets rich in phytosterols may offer protection against the development of breast cancer and may potentiate the antitumor activity of TAM during breast cancer therapy. Breast tumor tissue contains both hormone-sensitive and insensitive cells. Thus, the results here showing potentiation of breast cancer cell growth inhibition in MCF-7 and MD-MBA-231 cells by the combination of the dietary supplement SIT along with the anticancer drug TAM may be useful in the management of all breast cancer patients.

The authors have declared no conflict of interest.

5 References

[1] Jemal, A., Clegg, L. X., Ward, E., Ries, L. A. G., *et al*., Annual report to the nation on the status of cancer, 1975 – 2001, with a special feature regarding survival, *Cancer* 2004, *101*, 3 – 27.

[2] Ames, B. N., Gold, L. S., Willet, W. C., The causes and prevention of cancer, *Proc. Natl. Acad. Sci. USA* 1995, *92*, 5258 – 5265.

[3] Sieri, S., Krogh, V., Pala, V., Muti, P., *et al*., Dietary patterns and risk of breast cancer in the ORDET cohort, *Cancer Epidemiol. Biomarkers Prev.* 2004, *13*, 567 – 572.

[4] Greenwald, P., Clifford, C. K., Milner, J. A., Diet and cancer prevention, *Eur. J. Cancer* 2001, *37*, 948 – 965.

[5] World Cancer Research Fund and American Institute for Cancer Research, *Food, Nutrition and the Prevention of Cancer: A Global Perspective*, American Institute for Cancer Research, Washington DC 1997.

[6] Dietary Guidelines for Americans, US Department of Health and Human Services, Washington DC 2005.

[7] Bradford, P. G., Awad, A. B., Phytosterols: Bioactivity on Cancer, In: Awad, A. B., Bradford, P. G. (Eds.), *Nutrition and Cancer Prevention*, Taylor and Francis, Boca Raton 2006, pp. 251 – 270.

[8] Deapen, D., Liu, L., Perkins, C., Bernstein, L., Ross, R. K., Rapidly rising breast cancer incidence rates among Asian-American women, *Int. J. Cancer* 2002, *99*, 747 – 750.

[9] Moyers, S. B., Kumar, N., Green tea polyphenols and cancer chemoprevention: Multiple mechanisms and endpoints for phase II trials, *Nutr. Rev.* 2004, *62*, 204 – 211.

[10] Fulda, S., Debatin, K. M., Sensitization for anticancer drug-induced apoptosis by chemopreventive agent resveratrol, *Oncogene* 2004, *23*, 6702 – 6711.

[11] Aldercreutz, H., Phytoestogens and cancer, *Lancet Oncol.* 2002, *3*, 364 – 373.

[12] Talalay, P., Fahey, J. W., Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism, *J. Nutr.* 2001, *131*, 3027S – 3033S.

[13] Jordan, V. C., Chemoprevention of breast cancer with selective oestrogen-receptor modulators, *Nat. Rev. Cancer* 2007, *7*, 46 – 53.

[14] Awad, A. B., Downie, A. C., Fink, C. S., Inhibition of growth and stimulation of apoptosis by β-sitosterol treatment of MDA-MB-231 human breast cancer cells in culture, *Int. J. Mol. Med.* 2000, *5*, 541 – 545.

[15] Awad, A. B., Williams, H., Fink, C. S., Effect of phytosterols on cholesterol metabolism and MAP kinase in MDA-MB-231 human breast cancer cells, *J. Nutr. Biochem.* 2003, *14*, 111 – 119.

[16] Awad, A. B., Roy, R., Fink, C. S., β-Sitosterol, a plant sterol, induces apoptosis and activates key caspases in MDA-MB-231 human breast cancer cells, *Oncol. Reports* 2002, *10*, 497 – 500.

[17] Awad, A. B., Downie, A., Fink, C. S., Kim, U., Dietary phytosterol inhibits the growth and metastasis of MDA-MB-231 human breast cancer cells grown in SCID mice, *Anticancer Res.* 2000, *20*, 821 – 824.

[18] Cabot, M. C., Giuliano, A. E., Volner, A., Han, T.-Y., Tamoxifen retards glycosphingolipid metabolism in human cancer cells, *FEBS Lett.* 1996, *394*, 129 – 131.

[19] Skehan, P., Storeng, R., Scudiero, D., Monks, A., *et al*., New colorimetric cytotoxicity assay for anticancer-drug screening, *J. Natl. Cancer Inst.* 1990, *82*, 1107 – 1112.

[20] Jones, M. J., Murray, A. W., Evidence that ceramide selectively inhibits protein kinase C-α translocation and modulates bradykinin activation of phospholipase D, *J. Biol. Chem.* 1995, *270*, 5007 – 5013.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[21] Hubscher, G., Hawthorne, J. N., Kemp, P., The analysis of tissue phospholipids: Hydrolysis procedure and results with pig liver, *J. Lipid Res.* 1960, *1*, 433 – 438.

[22] Wang, H., Giuliano, A. E., Cabot, M. C., Enhanced de novo ceramide generation through activation of serine palmitoyltransferase by the *p*-glycoprotein antagonist SDZ PSC 833 in breast cancer cells, *Mol. Cancer Ther.* 2002, *1*, 719 – 726.

[23] Lavie, Y., Cao, H.-T., Volner, A., Lucci, A., *et al*., Agents that reverse multidrug resistance, tamoxifen, verapamil, and cyclosporine A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells, *J. Biol. Chem.* 1997, *272*, 1682 – 1687.

[24] Chen, B., Gajdos, C., Dardes, R., Kidwai, N., *et al*., Potential of endogenous estrogen receptor beta to influence the selective ER modulator ERbeta complex, *Int. J. Oncol.* 2005, *27*, 327 – 335.

[25] Awad, A. B., Williams, H., Fink, C. S., Phytosterols reduce in vitro metastatic ability of MDA-MB-231 human breast cancer cells, *Nutr. Cancer* 2001, *40*, 157 – 164.

[26] Choi, Y. H., Kong, K. R., Kim, Y.-A., Jung, K. O., *et al*., Induction of Bax and activation of caspases during β-sitosterol-mediated apoptosis in human colon cancer cells, *Int. J. Oncol.* 2003, *23*, 1657 – 1662.

[27] Awad, A. B., von Holtz, R. L., Cone, J. P., Fink, C. S., Chen, Y.-C., β-Sitosterol inhibits the growth of HT-29 human colon cancer cells by activating the sphingomyelin cycle, *Anticancer Res.* 1998, *18*, 471 – 479.

[28] Awad, A. B., Fink, C. S., Williams, H., Kim, U., *In vitro* and *in vivo* (SCID mice) effects of phytosterols on the growth and dissemination of human prostate cancer PC-3 cells, *Eur. J. Cancer Prev.* 2001, *10*, 507 – 513.

[29] MacGregor, J. I., Jordan, V. C., Basic guide to the mechanisms of antiestrogen action, *Pharm. Rev.* 1998, *50*, 151 – 196.

[30] Mandlekar, S. R., Yu, T.-H., Tan, A. N., Kong, T., Activation of caspase-3 and c-jun NH_2-terminal kinase-1 signaling pathways in tamoxifen-induced apoptosis of human breast cancer cells, *Cancer Res.* 2000, *60*, 5995 – 6000.

[31] Aldous, W. K., Marean, A. J., DeHart, M. J., Matej, L. A., Moore, K. H., Effects of tamoxifen on telomerase activity in breast carcinoma cell lines, *Cancer* 1999, *85*, 1523 – 1529.

[32] Seeger, H., Huober, J., Wallweiner, D., Mueck, A. O., Inhibition of human breast cancer cell proliferation with estradiol metabolites is as effective as with tamoxifen, *Horm. Metab. Res.* 2004, *36*, 277 – 280.

[33] Aoyama, Y., Experimental studies on the effects of the combined use of N-(4-hydroxyphenyl)retinamide (4-HPR) and tamoxifen (TAM) for estrogen receptor (ER)-negative breast cancer, *Kurume Med. J.* 2002, *49*, 27 – 33.

[34] Cover, C. M., Hsieh, S. J., Cram, E. J., Hong, C., *et al*., Indole-3-carbinol and tamoxifen cooperate to arrest the cell cycle of MCF-7 human breast cancer cells, *Cancer Res.* 1999, *59*, 1244 – 1251.

[35] Seeger, H., Diesing, D., Gückel, B., Wallwiener, D., *et al*., Effect of tamoxifen and 2-methoxyestradiol alone and in combination on human breast cancer cell prolife ration, *J. Steroid Biochem. Mol. Biol.* 2003, *84*, 255 – 257.

[36] Doisneau-Sixou, S. F., Cestac, P., Faye, J.-C., Favre, G., Sutherland, R. L., Additive effects of tamoxifen and the farnesyl transferase inhibitor FTI-277 on inhibition of MCF-7 breast cancer cell-cycle progression, *Int. J. Cancer* 2003, *106*, 789 – 798.

[37] Reynolds, C. P., Maurer, B. J., Kolesnick, R. N., Ceramide synthesis and metabolism as a target for cancer therapy, *Cancer Lett.* 2004, *206*, 169 – 180.

[38] Wang, H., Charles, A. G., Frankel, A. J., Cabot, M. C., Increasing intracellular ceramide: An approach that enhances the cytotoxic response in prostate cancercells, *Urology* 2003, *61*, 1047 – 1052.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim