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**Antiproliferative effect of sphingosylphosphorylcholine in thyroid FRO cancer cells mediated by cell cycle arrest in the G2/M phase**

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Running title: Antiproliferative effects of SPC in FRO cells.

**Abstract**

Among the group of bioactive sphingolipids, sphingosylphosphorylcholine (SPC) has been known to induce both antiproliferative and proliferative effects depending on cell type. In the present investigation we show that SPC (1-10  $\mu$ M) reduced the proliferation of FRO cells (an anaplastic thyroid carcinoma cell line) in a concentration dependent manner. The effect was pertussis toxin insensitive, and independent of phospholipase C, protein kinase C, p38 kinase, or jun kinase. In addition to inhibiting the migration of FRO cells, application of SPC induced a rapid ( $< 10$  min) rounding of the cells, which was dependent on extracellular sodium. However, DAPI staining and caspase-3 analysis could not reveal any apoptotic effects of SPC. Furthermore, when cells treated with SPC for 24 h were washed and replated, they continued to grow, albeit somewhat slower than control cells. Flow cytometry analysis revealed a significant increase in the population of cells in the G2-M phase, and a reduction in S phase. SPC reduced the phosphorylation of Akt with about 50% and evoked a substantial decrease in the amount of phosphorylated mitogen-activated protein (MAP) kinase. In cells treated with the PI3kinase inhibitor wortmannin, both migration and proliferation were inhibited, as well as the amount of phosphorylated MAP kinase. Treatment of the cells with either SPC or wortmannin increased the levels of p21, but decreased that of cyclin B1 and Cdc2. Taken together, SPC is an effective suppressor of thyroid cancer cell proliferation and migration, and this effect is, in part, mediated by inhibition of both the PI3K-Akt and the MAP kinase signalling pathways.

**Key words:** thyroid, cancer, receptors, lipids, cell cycle.

## Introduction

The sphingomyelin derivative sphingosylphosphorylcholine (SPC) has been shown to be involved in many cellular processes including proliferation, migration and cytoskeleton rearrangements and is produced in cells both in physiological and pathological conditions (Zu Heringdorf et al., 2002). It has been shown also that SPC is a naturally occurring lipid mediator in blood with plasma and serum concentrations being around 50 and 130 nM, respectively (Liliom et al., 2001). SPC may either stimulate or inhibit cell proliferation, depending on cell type (Yamada et al., 1997; Wakita et al., 1998). The inhibitory effect of SPC on the proliferation of cells is usually observed in different types of cancer cells, whereas the proliferation of normal cells (e.g. fibroblasts) is greatly enhanced (Desai et al., 1993). The stimulatory effect of SPC on proliferation has been attributed to acute stimulation of the mitogen-activated protein (MAP) kinase pathway (Seufferlein and Rozengurt, 1995), whereas the inhibitory effect is mediated by a prolonged stimulation of MAP kinase (Clark et al., 2004). Furthermore, SPC may modulate the cytoskeleton and stimulate or inhibit the migration of cells (Boguslawski et al., 2000; Beil et al., 2003). Compared to other sphingolipids, e.g. sphingosine 1-phosphate (S1P), little information is available regarding the mechanism of action of SPC on these cellular events (Zu Heringdorf et al., 2002; Spiegel and Milstein 2002). In particular, the lack of unambiguously specific receptors for SPC has hampered detailed investigations on the physiological significance of the lipid.

Information regarding the importance of sphingolipids in thyroid cells is scarce. Previous studies have shown that S1P evokes calcium transients in rat thyroid FRTL-5 cells (Okajima et al., 1997; Törnquist et al., 1997) and rat thyroid PC Cl3 cells

(Björklund et al., 2005), and that SPC evokes calcium responses and has a modest stimulatory effect on FRTL-5 cell proliferation (Törnquist and Ekokoski, 1994). In human thyroid cancer cells, SIP has either a stimulatory or inhibitory effect on cell migration, depending on cell type (Balthasar et al., 2006). We recently showed that SPC evoked entry of calcium in thyroid FRO cancer cells, and also potently attenuated proliferation of the cells (Afrasiabi et al., 2006).

The regulation of proliferation is an extremely complicated process. The progression through the cell cycle, when the cell mass is increased and DNA is doubled, is regulated by several different cyclin-dependent kinases (cdks). These are activated through the binding of different types of cyclins, which function as regulatory units (Damia and Borggini, 2004). Moreover, specific inhibitory proteins may inhibit the cdks. To ensure a smooth transition through the cell cycle, several checkpoints exist that monitor the progression of the cell. In the G2-M checkpoint, p53 and p21<sup>waf1/cip1</sup> are two important inhibitory proteins, which can inactivate the Cdc2-cyclin B1 complex, thus evoking cell cycle arrest. In addition to p21<sup>waf1/cip1</sup>, also Gadd45 and the 14-3-3 $\sigma$  protein are important inhibitors of Cdc2 (Damia and Borggini, 2004; Taylor and Stark, 2001). Upstream of the cdks and the inhibitory proteins, the phosphoinositide 3-kinase/Akt, also known as protein kinase B (PKB) pathway is an important regulator of proliferation, and PI3K/Akt has been shown to inactivate e.g. p21<sup>waf1/cip1</sup> (Liang and Slingerland, 2003). Thus, it has been concluded that PI3K/Akt activity is needed for the progression of the cells through G2-M.

In the present report we show that SPC potently attenuated both the proliferation and migration of thyroid FRO cancer cells, and evoked a decrease in the phosphorylation

of both Akt and MAP kinase. Additionally, SPC caused cell arrest in the G2/M phase of the cell cycle. We also detected an increase in the amount of the p21 oncoprotein upon stimulation of the cells with SPC, whereas both cyclin B1 and Cdc2 were decreased. No measurable changes were observed in the amount of p53. Our results suggest a role for both the PI3K-Akt/PKB and the MAP kinase signaling pathways in the inhibitory effect of SPC on cell proliferation and migration.

## Materials and methods

Sphingosylphosphorylcholine, and *D-erythro*-sphingosine-1-phosphate were purchased from Biomol (Plymouth, PA, USA). RPMI 1640 medium (without L-glutamine) and non-essential aminoacids were from Cambrex (Verviers, Belgium). L-glutamine, fetal bovine serum (FBS), penicillin and streptomycin were from GIBCO (Grand Island, NY, USA). DMEM (Dulbecco's modified Eagle's medium), pertussis toxin, U 73122, U 73343, GF 109203X, calphostin C, rottlerin, wortmannin, SB 203580, SP 600125, PD 98059, staurosporin, propidium iodide and MTT were from Sigma (St. Louis, MO, USA). BAPTA AM was from Molecular Probes Inc. (Eugene, OR, USA) DAPI and phalloidin stains were purchased from Invitrogen (Basel, Switzerland). Human Collagen type I was from Becton Dickinson Biosciences (Bedford, MA, USA). The FACE™ AKT ELISA kit was from Active Motif (Carlsbad, CA, USA). Primary antibodies against p53, p-p53 (Ser-392), p21, Cdc2, p-Cdc2 (Tyr-15), p-Cdc2 (Thr-161), cyclin B1, p44/42 MAP kinase, phospho-p44/42 MAP kinase (Thr-202/Tyr-204) and  $\beta$ -actin were obtained from Cell Signaling Technology (Beverly, MA, USA). The secondary antibodies used were

horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies (Sigma). Culture dishes were purchased from Falcon Plastics (Becton, NJ, USA).

*Cell culture.* Human anaplastic thyroid cancer cells (FRO) were a generous gift from Dr. James Fagin (University of Cincinnati, OH, USA), the NPA papillary thyroid cancer cell line was kindly provided by Dr. Sylvia Asa (University Health Network and Toronto Medical Laboratories, Canada) and the ML-1 follicular thyroid cancer cells were provided generously by Dr. Johann Schönberger (University of Rosenberg, Germany). FRO and NPA cells were cultured in RPMI 1640 medium and ML-1 cells were cultured in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. For NPA and FRO cells 1% non-essential amino acids was added to the medium. Cells were grown in a water-saturated atmosphere containing 5% CO<sub>2</sub> and 95% air at 37°C. Fresh medium was always added to the cells the day before an experiment.

*<sup>3</sup>H-thymidine incorporation assay.* Cell proliferation was determined using <sup>3</sup>H-thymidine incorporation (Amersham, Buckinghamshire, UK). For the assay 50 000 cells were cultured for 48 h on 35 mm plates in a water-saturated atmosphere containing 5% CO<sub>2</sub> and 95% air at 37°C followed by 24 h of treatment with indicated concentrations of SPC in low (5%) dextran-treated-charcoal-stripped FBS containing medium. At the day of the experiment the cultures were pulsed with <sup>3</sup>H-thymidine (0.4 µCi/ml) for 4 h of incubation. Cells were washed three times with ice cold PBS followed by a 10 min incubation with 5% perchloric acid and another 10 min with 0.1 N NaOH. Cells were

harvested and radioactivity was counted using a Wallac 1410 liquid scintillation counter (Wallac OY, Turku, Finland).  $^3\text{H}$ -thymidine uptake was expressed as mean counts per minute (cpm) of triplicate samples. The results were confirmed by cell counting using trypan blue.

*3-(4,5 dimethylthiazol-2-yl) 2-5 diphenyltetrazolium bromide (MTT) proliferation assay.*

The cells (10 000 cells/well), seeded into 96-well plates for 24 h, were incubated with different concentrations of SPC for 1-20 h at 37°C in 5% CO<sub>2</sub> and 95% air. After adding 100 µl/well of MTT solution (5mg/ml), the plates were incubated for another 4 h. Supernatants were then removed and the formazan crystals were solubilized in 100 µl DMSO. Optical density was determined at 540 nm using the Viktor fluorescence analyser (PerkinElmer Life Sciences, Turku, Finland).

*Cell migration assay.* Migration experiments were performed on 6.5 mm-diameter Transwell (Corning Costar, Bodenheim, Germany) chambers with 8 µM pore size. The filters were coated with collagen I (5µg/cm<sup>2</sup>) and then placed into the lower chamber. Lipid-stripped FBS was placed in the lower chamber as a chemoattractant. Cells were harvested and diluted with RPMI medium containing 0.1% BSA, 100 000 cells were added to the upper chamber and treated as indicated. The chambers were incubated in a humidified incubator at 37°C in 5% CO<sub>2</sub> / 95% air for 6 h. The cells that traversed the filter and spread on the lower surface of the filter were fixed with 2% paraformaldehyde in PBS for 10 min, and stained with 0.1% crystal violet in 20% methanol for 5 min. The membranes were rinsed and allowed to dry. Nonmigratory cells on the upper membrane

surface were removed with a cotton swab. The number of migratory cells/membrane was counted with 40x magnification in eight microscopic fields in a straight line bisecting the membrane.

*Western blot analysis.* After stimulation for the indicated times, FRO cells were washed 3 times with ice-cold PBS and lysed in cell lysis buffer [10mM Tris (pH 7.7), 150 mM NaCl, 7 mM EDTA, 0.5% NP-40, 0.2 mM PMSF (phenylmethylsulfonyl fluoride) and 0.5 µg/ml leupeptin], the lysate was centrifuged at 13 000 rpm for 15 minutes at 4°C and the supernatant was collected. The protein concentration was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). The samples were then stored at –20°C. Samples (15 µg of protein/sample) were subjected to SDS/PAGE (10% polyacrylamide). The proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by wet-blotting. Western blot analysis was performed using specific antibodies to the indicated proteins. The secondary antibodies used were horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies (Sigma). The proteins were detected by enhanced chemiluminescence. For loading controls, membranes were washed with 1M Tris (pH 7.6), stripped for 20 min in 0.1 M glycine (pH 2.5) and subjected to anti-β actin antibody. Densitometric analysis was performed using MCID+ software for data acquisition and image analysis (Imaging Research, St. Catherine's, Ontario, Canada) and results were corrected for protein loading by normalization for β-actin expression.

*Isolation of nuclear extract from FRO cells.* Cells were harvested by 0.02% EDTA/PBS, washed with ice-cold PBS and resuspended in ice-cold hypotonic buffer [25 mM Tris (pH 7.5), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 5 mM DTT, 0.2 mM PMSF and 0.5 µg/ml leupeptin]. Cells then were minced with 10 strokes in an ice-cold glass Dounce homogenizer (Kimble Kontes, Vineland, NJ, USA), kept for 15 min on ice and centrifuged at 10 000 rpm for 10 min at 4°C. The supernatant was removed and the pellet was resuspended in ice-cold nuclear extract buffer [25 mM Tris (pH 7.5), 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 1 mM DTT, 25% Sucrose, 0.2 mM PMSF and 0.5 µg/ml leupeptin] and incubated on ice for 1 h. Samples were centrifuged at 13 000 rpm for 30 min at 4°C and supernatants, containing the nuclear fraction were saved at -20°C. Samples (5 µg of protein/sample) were subjected to SDS/PAGE as described in the western blot protocol.

*Fluorescence-activated cell sorting (FACS) analysis.* This analysis was based on the measurement of the DNA content of nuclei labelled with propidium iodide. For cell cycle evaluation, cells were treated as for the proliferation experiments, washed with ice-cold PBS and incubated with propidium iodide (0.05% mg/ml in 3.8 µM sodium citrate, 0.1% Triton X-100) for 15 min at room temperature in the dark. Cells then were subjected to FACS and analysis of the cell cycle was evaluated using FACSCalibur flow cytometer (Becton-Dickinson Biosciences, San Jose, CA, USA). Data were analyzed using the Cell Quest Pro software package (BD Biosciences).

*Apoptosis assay.* The cells were treated as for the proliferation experiments, harvested by 0.02% EDTA/PBS, washed twice with ice-cold PBS and then stained with a phycoerythrin-conjugated anti-caspase-3 antibody (1:20) according to the manufacturers instructions (BD Pharmingen, Oxford, UK). Cells were then analysed using the FACScan flow cytometer (BD Biosciences).

*Microscopy.* FRO cells were grown on glass coverslips and treated with SPC (10 $\mu$ M/24h) or vehicle in low FBS containing medium. After fixation with absolute methanol, cells were stained with the cytoskeleton dye phalloidin, and the nuclei were stained by the (DAPI) nucleic acid dye. For some experiments cells were treated with vehicle or with SPC in Hepes-buffered salt solution (HBSS; 118 mM NaCl, 4.6 mM KCl, 1 mM CaCl<sub>2</sub>, 10 mM glucose and 20 mM Hepes, pH 7.4), or in low sodium (10 mM) or high potassium (130 mM) containing HBSS, and monitored microscopically. Fluorescence and light microscopic monitoring were carried out using the Axiovert 35 (Carl Zeiss, Oberkochen, Germany) fluorescence microscope and analysed by the “Scion” image-processing program (Scion Corporation, MD, USA). For confocal microscopy the Leica TCS SP MP microscope (Leica, Heidelberg, Germany) equipped with an Argon-Krypton laser (Omnichrome, Melles Griot, Carlsbad, CA, USA) was used.

*Fast Activated Cell-based ELISA (FACE) AKT.* Phosphorylation of Akt was determined by seeding cells (10 000/well) in 96-well plates for 24 h, after which the cells were stimulated with different concentrations of SPC for 10 min. The cells were fixed with 4% paraformaldehyde in PBS for 20 min. The colorimetric assay was performed with an

antibody recognizing Ser<sup>473</sup>-phosphorylated or total Akt according to the instructions provided by the manufacturer, and the absorbance was read at 450 nm.

*Cell adhesion assay.* 10 000 FRO cells were plated on collagen I (5 µg/cm<sup>2</sup>) coated 96-well plates for 24 h and after stimulation with SPC or vehicle for 24 h cells were washed with PBS and fixed with 70% ethanol and then stained with crystal violet (0.1% in 20% methanol) for 10 min. After washing with water, the wells were allowed to dry. The crystal violet was dissolved in 10% acetic acid, and the absorbance was measured at 570 nm.

*Statistics.* Results are expressed as means ± SEM. Analysis of statistical significance was performed using Student's *t* test for paired observations. Three or more means were tested using one-way ANOVA and Dunnett's post hoc test. Curve fitting and statistical analyses were made using the Prism 3.03 program (GraphPad, San Diego, CA, USA). A P-value less than 0.05 was considered significant.

## Results

*Effects of SPC on thyroid cancer cell proliferation.* Previous studies have shown that sphingolipids can either enhance or attenuate thyroid cell proliferation (Törnquist and Ekokoski, 1994; Törnquist et al., 1997; Balthasar et al., 2006). In FRO, NPA and ML-1 thyroid cancer cells, SPC decreased the incorporation of <sup>3</sup>H-thymidine in a concentration-dependent manner (Fig. 1A). A similar SPC-evoked decrease in proliferation of FRO cells was seen with the MTT assay (Fig. 1B). As some FRO cells detached after SPC

treatment, we verified the results by counting all cells in a cell dish. In these experiments we observed an increase in the cell number in control dishes, whereas no increase was observed in cells incubated with 10  $\mu$ M SPC for 24 h (Fig. 1C). As it was shown previously that some effects evoked by SPC are mediated by the  $G_i$  protein (Nikmo et al., 1999), we preincubated the cells with pertussis toxin (Ptx, 50 ng/ml for 24 h) prior to stimulation with SPC. Ptx *per se* decreased FRO cell proliferation by  $32 \pm 5.4\%$  ( $p < 0.05$ ), and was unable to block the effect of SPC on the  $^3\text{H}$ -thymidine incorporation (Table 1). In order to clarify the mechanism of action of the antiproliferative effect of SPC, we preincubated the cells for 1 h with the PLC inhibitor U73122 (5  $\mu$ M) and its negative analogue U73343 (5  $\mu$ M), the PKC inhibitor GF 109203X (10  $\mu$ M), the PKC inhibitor calphostin C (100nM), the PKC inhibitor rottlerin (10  $\mu$ M), the p38 kinase inhibitor SB 203580 (5  $\mu$ M), or the jun kinase inhibitor SP 600125 (1-10  $\mu$ M). Some of these compounds *per se* decreased substantially FRO cell proliferation, and all were, in addition, unable to attenuate the SPC-induced effect on cell proliferation (Table 1). We also investigated whether SPC could modulate MAP/ERK1/2 kinase phosphorylation in FRO cells. SPC did not modulate the phosphorylation of this kinase, when the cells were stimulated for short periods ( $2\text{-}4\% \pm 1.5\%$  in 10, 30 and 60 min) with 10  $\mu$ M SPC (Fig. 1D). However, when the cells were incubated with 10  $\mu$ M SPC for 24 h, a marked decrease in the amount of phosphorylated MAP/ERK1/2 kinase ( $66\% \pm 9\%$ ,  $P < 0.05$ ) was observed (Fig. 1E). Furthermore, as SPC evokes calcium entry in FRO cells (Afrasiabi et al., 2006), we investigated whether the effect of SPC on proliferation was mediated by the calcium response. Preincubating the cells with 10 $\mu$ M of the calcium chelator BAPTA

AM resulted in a total block of cell proliferation, thus precluding further experiments (Table 1).

*Effects of SPC on FRO cell adhesion, migration and morphology.* Our previous investigations have showed that the related sphingolipid S1P modulated thyroid cancer cells in a complex fashion (Balthasar et al., 2006). SPC did not affect the adhesion of FRO cells to Collagen I (data not shown). When we studied the migration of FRO cells we noticed that SPC in a concentration-dependent manner reduced the migration of FRO cells when lipid-stripped FBS was used as a chemoattractant. The inhibitory concentrations were much lower compared to those needed to attenuate the proliferation (Fig. 2A).

The above results suggested a pathophysiological effect of SPC on FRO cells. Pathophysiological changes can often result in profound morphological alterations in the cells. Therefore, we investigated the effects of SPC on FRO cell morphology. Treatment with SPC resulted in a rapid (within 10 min) rounding of the cells (Fig. 2B). This rounding was dependent on extracellular sodium, as incubation of the cells in an extracellular solution containing low sodium concentrations abolished the effect of SPC (Fig. 2B). Increasing extracellular potassium to 130 mM did not inhibit the effect of SPC on cell rounding (Fig. 2B). As cell rounding is a hallmark of apoptosis (Bortner et al., 1997), we stained cells with the nucleic acid stain DAPI for detection of apoptotic cells. Although treatment of the cells with SPC evoked some changes in both the nucleus and the cytoskeleton (Fig. 2C), no typical apoptotic changes (e.g. apoptotic bodies) were detected. Furthermore, SPC did not evoke caspase-3 activation (Fig. 2D). In addition,

when cells treated with SPC for 24 h were washed and replated, the cells continued to grow with almost the same efficiency as control cells (Fig. 2E). Thus, although SPC attenuated both the proliferation and the migration of FRO cells, it did not induce apoptosis of the cells.

*Effects of SPC on the cell cycle and Akt phosphorylation.* To further understand by which mechanism SPC blocked cell proliferation, we next determined the cell cycle profile of cells after the treatment with SPC. Interestingly, SPC induced a significant change in the distribution of the cells: a marked increase of the cell population in the G2 phase and a decrease in the S phase of the cell cycle (Fig. 3A and Table 2). We were also unable to detect any apoptotic cells in this assay. In sharp contrast to the effect of SPC, staurosporin (1  $\mu$ M) evoked a massive apoptosis of the cells (Fig. 3A).

Both proliferation and migration of cells are commonly evoked by activation of the PI3K/Akt pathway (Menu et al., 2004), and we have shown previously that S1P enhanced thyroid cell migration by a mechanism dependent on Akt kinase (Balthasar et al, 2006). We thus investigated whether SPC could modulate Akt phosphorylation, and as can be seen in Fig. 3B, SPC did indeed attenuate Akt phosphorylation. Interestingly, S1P did not evoke an inactivation of Akt in FRO cells (data not shown). To further investigate the importance of Akt, we next studied the effect of wortmannin, an inhibitor of PI3kinase, on the proliferation and migration of FRO cells. Both the proliferation and the migration were inhibited by wortmannin (Fig. 4A&B). In addition, wortmannin attenuated MAP kinase phosphorylation ( $72\% \pm 8\%$ ,  $P < 0.05$ ) in FRO cells (Fig. 4C). However, the distribution of the cells in the cell cycle was different from that seen with

SPC, as wortmannin evoked an arrest of the cells in the G1 phase of the cell cycle (Fig. 4D and Table 2).

To further clarify the mechanism by which SPC induced cell cycle changes, we examined the possible roles of p53 and p21, two oncogenes linked to the regulation of the cell cycle (Damia and Broggin, 2004), and also cyclin B1 and Cdc2, which are of importance in the regulation of the cell cycle in the G1 and the G2 phases. We noted that the phosphorylation of p53 was not changed in cells stimulated with either SPC or wortmannin. However, the level of p21<sup>waf1/cip1</sup> was significantly increased, compared with control cells. Additionally, treatment of the cells with SPC decreased both the levels of cyclin B1 and that of Cdc2. Similar results were obtained with wortmannin, although the effects appeared less pronounced than those of SPC. We examined the phosphorylation of Cdc2 at the residue Tyr-15 and Thr-161 and found that the phosphorylation of Cdc2 at Tyr-15 was inhibited by SPC and wortmannin while the phosphorylation of Cdc2 at Thr-161 remained unchanged by these two agents (Fig. 5 and Table 3). As p53 functions in the nucleus to stimulate p21<sup>waf1/cip1</sup> transcription (Damia and Broggin, 2004), we further assessed the relative p53 protein levels in the nuclear fractions of the cells. No significant changes were detected in the amount of p53 in the nuclear fraction after stimulating the cells with SPC ( $0.53 \pm 0.04$ ) or with wortmannin ( $0.52 \pm 0.1$ ), compared with control cells ( $0.56 \pm 0.06$ ).

## Discussion

In the present study we show that SPC is an inhibitor of both thyroid cancer cell proliferation and migration. Our results suggest that the effect of SPC was due to an

arrest of the cells in the G2-M phase of the cell cycle. This arrest was probably the result of a decrease in both Cdc2 and cyclin B1, and an increase of the inhibitory protein p21<sup>waf1/cip1</sup>. Part of the inhibitory effects is probably mediated through an inhibition of the PI3K/Akt and MAP kinase pathways, as SPC decreased the phosphorylation of both Akt and MAP kinase in the cells. To our knowledge, this is the first report to elucidate a mechanism by which SPC inhibits cellular proliferation and migration.

Earlier investigations have shown that SPC is a potent mitogen in several cell types, and that SPC stimulates migration of cells (Zu Heringdorf et al., 2002). In rat thyroid FRTL-5 cells, SPC had a small, but significant stimulatory effect on DNA synthesis (Björklund et al., 2005). In rat thyroid PC Cl3 cells, SPC was without an effect on the proliferation and only modestly attenuated migration of these cells (Balthasar and Törnquist, unpublished observations). The stimulatory mechanism of action of SPC on proliferation has been attributed to stimulation of the MAP kinase pathway (Seufferlein and Rozengurt, 1995). In some cell types, in particular cancer cells, SPC can inhibit proliferation (Xu et al., 1995; Yamada et al., 1997). Some investigations have suggested that this effect is mediated by a prolonged stimulation of MAP kinase (Clark et al., 2004). A 24 h incubation with SPC in our cells resulted in a marked decrease in the phosphorylation of MAP kinase. An IL22-evoked decrease in MAP kinase phosphorylation has also been correlated with attenuated proliferation in EMT6 breast cancer cells (Weber et al., 2006). Thus, the decrease in phosphorylation of MAP kinase is probably, in part, explaining the SPC-evoked decrease in proliferation.

Previous investigations have shown that activation of Akt in proliferating cells may suppress the expression of p21<sup>waf1/cip1</sup> (Liang and Slingerland, 2003), and that Akt is

of significance in stimulating the G2-M transition (Lee et al., 2005, Katayama et al., 2005). Thus, one mechanism by which SPC exerts its effect could be through inhibition of Akt, resulting in an upregulation of p21<sup>waf1/cip1</sup> and arrest of the cells in the G2-M phase. Wortmannin showed that inhibition of PI3K indeed upregulated p21<sup>waf1/cip1</sup>, but less efficiently in comparison to SPC. The effects on Cdc2 and cyclin B1 were of the same magnitude for both SPC and wortmannin. However, as wortmannin arrested the cells in the G1 phase of the cell cycle, it is clear that SPC must inhibit proliferation also through some other, yet unidentified pathways. Furthermore, the decreased phosphorylation of Akt could, at least in part, explain the inhibitory effect of SPC on migration. Our recent results with ML-1 thyroid cancer cells have shown that the S1P-evoked migration of these cells is crucially dependent on phosphorylation of Akt, and is totally inhibited by wortmannin (Balthasar et al., 2006).

Although p21<sup>waf1/cip1</sup> is an important inducer of cell cycle arrest in the G1 phase, it is also active in G2/M (Damia and Broggin, 2004). SPC evoked a dephosphorylation of Cdc2 at Tyr-15, which usually is a prerequisite for activation of a Cdc2 and cyclin B complex, and progression through the cell cycle. As p21<sup>waf1/cip1</sup> can associate with the activated, Tyr-15 dephosphorylated form of Cdc2 (Barboule et al., 1999), the formation of a Cdc2 and cyclin B1 complex was probably efficiently inhibited in our cells. In our studies SPC did not have any measurable effects on the phosphorylation of Thr-161 on Cdc2. Phosphorylation of Thr-161 is usually associated with enhanced activity of Cdc2 (Smits et al., 2000). Thus, the increase in p21<sup>waf1/cip1</sup> and the marked decrease in the amount cyclin B1 probably effectively blocked cell cycle progression. However, we

cannot exclude that the decrease in total Cdc2 also could indicate that the relative phosphorylation of Tyr161 was increased, thus hampering cell cycle progression.

We were not able to see an effect of SPC on p53, the perhaps most important regulator of cell cycle progression (Taylor and Stark, 2001). Other studies have shown that FRO cells express very low levels of p53, compared to other thyroid cancer cell lines (Fagin et al., 1993). Thus the SPC-evoked upregulation of p21<sup>waf1/cip1</sup> probably occurs by a p53-independent mechanism. Similar results have also been obtained in other studies (Agrawal et al., 2002). Several signalling mediators (e.g. PLC, p38 and JNK) appeared not to be involved in the SPC-evoked inhibition of proliferation. As some of the inhibitors by themselves decreased proliferation, it is impossible to make a clear-cut conclusion on the physiological importance of these signalling modulators. Furthermore, SPC evoked a substantial rounding of the cells, which was dependent on sodium fluxes. Although cell rounding is a hallmark of apoptosis, cell rounding or shrinkage *per se* is not sufficient to evoke apoptosis (Bortner and Cidlowski, 2003). Previous investigations have shown that a decrease in intracellular potassium concentrations is important for the initiation of apoptosis (Hughes et al., 1997). The lack of an effect of increasing extracellular potassium on SPC-evoked cell rounding suggests that in our cells, no dramatic changes in intracellular potassium concentrations occurred, and apoptosis was not initiated.

Detailed investigations on the mechanisms of action of SPC are presently hampered by the absence of specific receptors for SPC. Previous studies have shown that SPC is a weak agonist for the well-known S1P receptors S1P<sub>1-3</sub> (Clair et al., 2003), and a high affinity agonist in addition to S1P for GPR12 (Ignatov et al., 2003). We have

previously shown that FRO cells express S1P<sub>2</sub> (Balthasar et al., 2006), the receptor subtype mediating the inhibitory effect of S1P on migration and proliferation (Ikeda et al., 2003; Goparaju et al., 2005; Björklund et al., 2005). However, the inhibitory effect of SPC on the proliferation of FRO cells is much more pronounced, compared with that of S1P (Balthasar et al., 2006). In addition, S1P did not evoke an inactivation of Akt (Balthasar and Törnquist, unpublished observations). Furthermore, we have recently shown that SPC evokes intracellular calcium signals in FRO cells through a mechanism distinctly different from that of S1P (Afrasiabi et al., 2006). It is, of course, impossible to totally exclude that some of our effects seen with SPC are mediated e.g. through the S1P<sub>2</sub> receptor. We do, however, think that our results with SPC differ from that obtained with S1P sufficiently to warrant the conclusion that the observed effects are specific for SPC. We have shown previously that FRO cells express GPR4 (Afrasiabi et al., 2006), a receptor shown to mediate SPC-evoked proliferation and migration of human umbilical vein endothelial cells (Kim et al., 2005). In addition, the FRO cells express the OGR1 receptor (Afrasiabi et al., 2006). Although this receptor apparently is a receptor for protons, some antagonistic effects of SPC on the function of OGR1 have been reported (Mogi et al., 2005). However, the overall lack of suitable receptor-specific antagonists and agonists make investigations on the importance of these lipid receptors complicated.

In conclusion, we have in the present report shown that SPC inhibits both the proliferation and migration of human thyroid FRO cancer cells. As SPC is a naturally occurring lipid in serum, it may be an important signalling molecule regulating the function of thyroid cancer cells.

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### Abbreviations

SPC, sphingosylphosphorylcholine; S1P, sphingosine-1-phosphate; Ptx, pertussis toxin; PLC, phospholipase C; PKC, protein kinase C; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; FBS, foetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; GF 109203X, 2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide; U73122, 1-(6-((17 $\beta$ -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-H-pyrrole-2,5-dione; U73343, 1-(6-((17 $\beta$ -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione; PI3K, phosphatidylinositol 3-kinase; CDKs, Cyclin-dependent kinases.

Table 1. <sup>3</sup>H-thymidine incorporation in FRO cells after 24 h exposure to medium containing different inhibitors and 10 μM of SPC.

Inhibitor	C	SPC	C+inhibitor	SPC+inhibitor
Ptx	100 ± 1.8	4.9 ± 2.7*	68.0 ± 2.6*	7.9 ± 0.8*
U 73122	100 ± 2.4	6.1 ± 0.6*	99.0 ± 2.5	10.0 ± 1.5*
U 73343	100 ± 2.4	6.1 ± 0.6*	97.0 ± 1.0	7.0 ± 1.2*
GF 109203X	100 ± 5.2	3.1 ± 0.9*	8.5 ± 1.6*	3.5 ± 0.5*
Calphostin-C	100 ± 6.9	5.0 ± 1.9*	3.6 ± 1.7*	1.5 ± 0.5
Rottlerin	100 ± 0.9	6.9 ± 1.2*	8.0 ± 0.2*	7.9 ± 0.7
SB 203580	100 ± 4.0	13 ± 0.8*	14.0 ± 2.0*	5.0 ± 0.7*
SP 600125	100 ± 2.5	8.8 ± 0.6	8.0 ± 5.0*	4.5 ± 3.0*
BAPTA AM	100 ± 4.3	10.6 ± 1.7	9.9 ± 0.2	8.0 ± 2.2

Cells were preincubated for 1 h with respective inhibitor prior to the treatment with vehicle (C) or 10 μM SPC (SPC), and the <sup>3</sup>H-thymidine incorporation was measured after 24 h as described in the Methods section. Values given are the mean ± SEM of three separate experiments. Data are expressed as percent compared to the 100% of the control.

\*, P < 0.05 compared with respective control value.

Table 2. Effects of SPC and wortmannin on the cell cycle of FRO cells.

Cell cycle phase	C	SPC	W
G0-G1	54.6 ± 2.3	50.7 ± 3.2	82.1 ± 2.1*
S	35.0 ± 2.1	19.7 ± 5.2*	12.9 ± 3.0*
G2-M	10.5 ± 0.2	29.6 ± 4.8*	5.0 ± 1.1*

The cells were treated with vehicle (C), 10  $\mu$ M SPC, or 10  $\mu$ M wortmannin (W). After 24 h the cells were collected and analyzed for the distribution of cells in the cell cycle using FACS analysis. The data gives the mean  $\pm$  SEM of three separate experiments. \*, P < 0.05 compared with respective control value.

Table 3. Summary of densitometric analysis of the levels and activity of cell cycle regulatory proteins in FRO cells.

Antibody	C	SPC	W
p53	1.89 ± 0.06	1.84 ± 0.04	1.93 ± 0.07
p-p53	1.77 ± 0.08	1.76 ± 0.06	1.68 ± 0.09
p21	1.49 ± 0.04	2.06 ± 0.07*	1.85 ± 0.03*
cyclin B1	1.93 ± 0.08	0.44 ± 0.03*	0.48 ± 0.08*
Cdc2	2.06 ± 0.16	1.54 ± 0.04*	1.65 ± 0.03*
pCdc2(Tyr15)	0.98 ± 0.05	0.39 ± 0.05*	0.35 ± 0.01*
pCdc2(Thr161)	0.28 ± 0.01	0.26 ± 0.01	0.28 ± 0.01

Cells were treated with vehicle (C), 10  $\mu$ M SPC (SPC), or 10  $\mu$ M wortmannin (W) for 24 h and western blot analysis were performed. Densitometric results were corrected by normalization for  $\beta$ -actin expression. Values given are the mean  $\pm$  SEM of three independent experiments. \*,  $P < 0.05$  compared with respective control value.

## Legends to the figures

*Figure 1:* Antiproliferative effects of SPC in thyroid cancer cells. A. FRO, NPA and ML-1 cells were grown in 35-mm dishes under conditions of low (5%) serum and after treatment with different doses of SPC for 24 h;  $^3\text{H}$ -thymidine incorporation assay was performed as described in the methods section. B. In the MTT assay, FRO cells were grown on 96-wells plates and treated with different doses of SPC and the formazan formation was determined by measuring the absorbance at 490 nm as described in the methods section. C. SPC reduces FRO cell number. Cell counting was performed after a 24 h-stimulation with vehicle (C) or 10  $\mu\text{M}$  of SPC. The bars give the mean  $\pm$  SEM of 4-6 experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). D. Effects of SPC on the levels of MAP kinase phosphorylation in FRO thyroid cancer cells. Cells were treated with vehicle (0), or 10  $\mu\text{M}$  SPC in 5% lipid-stripped FBS and cellular proteins were extracted after 10, 30, and 60 min. E. Cells were treated with vehicle in medium containing 5% lipid-stripped FBS (C-), with vehicle in medium containing 10% FBS (C+), or 10  $\mu\text{M}$  SPC in 5% lipid-stripped FBS and cellular proteins were extracted after 24 h. A total of 15  $\mu\text{g}$  cell extract protein isolated from the treated and the untreated cells was subjected to SDS-PAGE and immunoblotted with antibodies against phospho-p44/42 MAP kinase (Thr-202/Tyr-204), and p44/42 MAP kinase. Filters were reblotted with an anti- $\beta$ -actin antibody (lower panel) to normalize for protein loading. Western blot data presented are representative of three separate experiments.

*Figure 2:* Effects of SPC on FRO cells migration and cell morphology. A. Cells were grown on Transwell inserts which were coated with collagen I as described in the

methods section and after treatment with vehicle or with different doses of SPC, migrated cells toward lipid-stripped FBS were counted. Each experiment was performed 3-6 times. Data are expressed as percent migrated cells compared to untreated controls (\*,  $P < 0.05$ ).

B. FRO cells were grown as for the proliferation experiment on glass coverslips in 35-mm culture dishes and treated with vehicle (C) or 10  $\mu\text{M}$  SPC (SPC) in HBSS, or in buffer containing 10 mM NaCl ( $-\text{Na}^+$ ) or 130 mM KCl ( $+\text{K}^+$ ). The cells were monitored microscopically prior to (0) and after 10 min (10) of stimulation, and images were recorded with a CCD camera. Each experiment was performed 3-6 times.

C. FRO cells were grown on 35-mm dishes and treated as for the proliferation experiment and then stained with the nucleic acid dye DAPI (blue) and the cytoskeleton dye phalloidine (green) as described in the methods section. Confocal microscopic images were recorded with a CCD camera. C, vehicle treated cells; SPC, cells treated with 10  $\mu\text{M}$  SPC for 24 h. Each experiment was repeated 4-6 times.

D. Cells were treated with vehicle or with 10  $\mu\text{M}$  of SPC, and after 24 h stained with a phycoerythrin-conjugated anti-caspase-3 antibody (1:20) according to the manufacturers instructions and analysed using a FACScan flow cytometer.

E. Cells were treated with vehicle or with 10  $\mu\text{M}$  of SPC, and after 24 h, cells were washed twice with PBS, counted and then replated and the  $^3\text{H}$ -thymidine incorporation assay was performed after 24 h and 48 h, respectively, as described in the methods section. Data is expressed as mean count per minute (CPM) per well. The bars give the mean  $\pm$  SEM of 4-6 experiments (\*,  $P < 0.05$ ).

*Figure 3: SPC induced cell cycle changes and inhibition of Akt phosphorylation on Ser*  
<sup>473</sup>. A. FRO cells were grown on 35-mm plates and treated with vehicle (C), or with 10

$\mu\text{M}$  SPC for 24 h, or with 1  $\mu\text{M}$  staurosporin for 12 h (St) and then incubated with propidium iodide and analysed by FACS for cell cycle measurements as described in the methods section. On the x-axis, the arrows show cells in G1-phase (left arrow) and cells in G2-phase (right arrow) of the cell cycle. Axes were scaled differently to show sub-G1 population of cells. Each experiment was performed 4-6 times. B. FRO cells (10 000 cells/well) were treated with vehicle (0) or with different concentrations of SPC for 10 min. Akt phosphorylation was measured using an antibody against phosphorylated Akt-Ser<sup>473</sup> and a commercial kit following the manufacturer's instructions and the absorbance at 450 nm was recorded. The bars give the mean  $\pm$  SEM of 4-6 experiments (\*,  $P < 0.05$ ).

*Figure 4:* Blockade of the PI3 kinase/ Akt pathway inhibits the growth and the migration of FRO thyroid cancer cells. A. Cells plated and grown on 35-mm plates were treated with vehicle or with 10  $\mu\text{M}$  wortmannin for 24 h and the <sup>3</sup>H-thymidine incorporation assay was performed as described in the methods section. The bars give the mean  $\pm$  SEM of 4-6 experiments (\*,  $P < 0.05$ ). B. Cells were grown on collagen I-coated Transwell inserts as described in the methods section, and after treating with vehicle or with 10  $\mu\text{M}$  wortmannin, migrated cells were counted. Lipid-stripped FBS was used in the lower chamber as chemoattractant. Each experiment was performed three times. Data are expressed as percent migrated cells compared to the control (\*,  $P < 0.05$ ). C. Effects of wortmannin on the levels of MAP kinase phosphorylation in FRO thyroid cancer cells. Cells were treated with vehicle in medium containing 5% lipid-stripped FBS (C-), with vehicle in medium containing 10% FBS (C+), or 10  $\mu\text{M}$  wortmannin (W) and cellular proteins were extracted after 24 h. A total of 15  $\mu\text{g}$  cell extract protein isolated from the

treated and the untreated cells was subjected to SDS-PAGE and immunoblotted with antibodies against phospho-p44/42 MAP kinase (Thr-202/Tyr-204), and p44/42 MAP kinase. Filters were reblotted with an anti- $\beta$ -actin antibody (lower panel) to normalize for protein loading. Western blot data presented are representative of three separate experiments. D. Cell cycle changes induced by wortmannin in FRO cells. Cells were treated with vehicle (C), or with 10  $\mu$ M wortmannin for 24 h (W) and subjected for FACS analysis as described in the methods section. On the x-axis, the arrows show cells in G1-phase (left arrow) and cells in G2-phase (right arrow) of the cell cycle. Axes were scaled differently to show sub-G1 population of cells. Data presented are representative of three separate experiments.

*Figure 5:* Effects of SPC and wortmannin on the levels and activity of cell cycle regulatory proteins in FRO thyroid cancer cells. Cells were treated with vehicle (C), 10  $\mu$ M SPC or 10  $\mu$ M wortmannin and cellular proteins were extracted after 24 h. A total of 15  $\mu$ g cell extract protein isolated from the treated and the untreated cells was subjected to SDS-PAGE and immunoblotted with antibodies against p53, phospho-p53 (Ser 392), p21, cycline B1, Cdc2, phospho-Cdc2 (Tyr 15) and phospho-Cdc2 (Thr 161). Filters were reblotted with an anti- $\beta$ -actin antibody (lower panel) to normalize for protein loading. Western blot data presented are representative of three separate experiments.









