FTY720 (Fingolimod) attenuates basal and sphingosine-1-phosphate-evoked thyroid cancer cell invasion

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Abstract

The bioactive lipid sphingosine-1-phosphate (S1P) is a potent inducer of ML-1 thyroid cancer cell migration and invasion. It evokes migration and invasion by activating S1P receptor 1 and 3 (S1P_{1,3}) and downstream signaling intermediates as well as through cross-communication with vascular endothelial growth factor receptor 2 (VEGFR2). However, very little is known about the role of S1P receptors in thyroid cancer. Furthermore, the currently used treatments for thyroid cancer have proven to be rather unsuccessful. Thus, due to the insufficiency of the available treatments for thyroid cancer, novel and targeted therapies are needed. The S1P receptor functional antagonist FTY720, an immunosuppressive drug currently used for treatment of multiple sclerosis, has shown promising effects as an inhibitor of cancer cell proliferation and invasion. In this study, we investigated the effect of FTY720 on invasion and proliferation of several thyroid cancer cell lines. We present evidence that FTY720 attenuated basal as well as S1P-evoked invasion of these cell lines. Furthermore, FTY720 potently downregulated S1P₁, protein kinase C α (PKC α), PKC β I, and VEGFR2. It also attenuated S1P-evoked phosphorylation of ERK1/2. Our results also showed that FTY720 attenuated S1P-induced MMP2 intracellular expression, S1P-induced secretion of MMP2 and MMP9, and decreased basal MMP2 and MMP9 activity. Moreover, in FTY720-treated cells, proliferation was attenuated, p21 and p27 were upregulated, and the cells were arrested in the G1 phase of the cell cycle. FTY720 attenuated cancer cell proliferation in the chick embryo chorioallantoic membrane assay. Thus, we suggest that FTY720 could be beneficial in the treatment of thyroid cancer.

Key Words

- thyroid
- cancer
- sphingosine-1-phosphate
- invasion
- proliferation

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Introduction

Sphingosine-1-phosphate (S1P) is a member of the sphingomyelin family of bioactive lipids regulating several central cellular processes. It is generated in cells from sphingosine by the action of sphingosine kinase 1 (SK1) and is transported out from the cells by the aid of several transport mechanisms. However, a role for S1P as an

intracellular messenger has become evident during the past few years (Alvarez *et al.* 2007, Strub *et al.* 2010, Alshaker *et al.* 2013, Pyne & Pyne 2013, Takabe & Spiegel 2014).

Extracellular S1P elicits its effects through binding to one of its five highly specific G-protein-coupled receptors, termed S1P_{1–5}, and by activating different G-proteins.

The final outcome of S1P treatment, however, is dependent on which receptor and G-protein it activates. As most cell types express receptors for S1P, this metabolite is unambiguously an important regulator of cell function during normal development and normal physiology. In addition, a substantial amount of evidence has implicated S1P in the progression of several types of cancers by enhancing many central events such as proliferation, angiogenesis, migration, and invasion (Alvarez *et al.* 2007, Strub *et al.* 2010, Alshaker *et al.* 2013, Pyne & Pyne 2013, Takabe & Spiegel 2014).

The importance of sphingolipids in thyroid cancer has not been studied in great detail. We have shown that S1P is without an effect on the proliferation and invasion of normal thyroid cells but induces a marked migration and invasion of ML-1 follicular thyroid cancer cells. This effect is mediated by S1P_{1/3}, G_{i/o}, PI3-kinase, protein kinase C (PKC), and ERK1/2 kinase (Balthasar *et al.* 2006, Bergelin *et al.* 2010). Furthermore, we have identified hypoxia-induced factor-1 α (HIF-1 α) (Kalhori *et al.* 2013) and matrix metalloproteinases 2 and 9 (MMP2 and MMP9) (Kalhori & Törnquist 2015) as mediators of the S1P-evoked migration and invasion of ML-1 cells. Moreover, SK1 enhances migration and invasion through an autocrine mechanism in these cells (Bergelin *et al.* 2009, 2010). Thus, in thyroid cancer cells, S1P-evoked signaling induces an aggressive and invasive phenotype. This is strengthened by observations that SK1 is overexpressed in thyroid cancer and correlates with a high degree of thyroid malignancy and poor prognosis (Guan *et al.* 2011).

In almost all forms of thyroid cancer, vascular endothelial growth factor receptor 2 (VEGFR2) and the secretion of VEGF are upregulated, enhancing the development of metastasis through an autocrine mechanism (Turner *et al.* 2003, Vieira *et al.* 2005, Kim *et al.* 2006). Our investigations have shown a complex cross talk between VEGFR2 and S1P receptors in ML-1 cells. This communication is of crucial importance for migration (Balthasar *et al.* 2008, Bergelin *et al.* 2010).

Fingolimod (FTY720) is an immunosuppressive drug approved by both the FDA and the EMA for treatment of multiple sclerosis (MS) and is presently the only available S1P receptor-modulating drug in clinical use. It has affinity for all S1P receptors, except for S1P₂ (but see Sobel *et al.* 2015). Exogenous FTY720 is taken up by cells, phosphorylated by SK2 (Paugh *et al.* 2003), and finally secreted into the extracellular space, where it binds to S1P receptors. This results in internalization and proteasomal degradation of the S1P receptors, events highlighting the inhibitory effects of FTY720 (Oo *et al.* 2007). In addition

to its immunosuppressive effects in the treatment of MS, FTY720 has shown promising effects as an inhibitor of cancer cell migration and proliferation. This effect is due to, for example, inhibition of several key players in these cellular events, as well as by acting as an inhibitor of SK1 (Alshaker 2013, Kunkel *et al.* 2013, Pyne & Pyne 2013, Selvam & Ogretmen 2013).

The incidence of thyroid cancer has increased significantly worldwide. The prognosis for many forms of thyroid cancer is very good. However, there are certain aggressive forms with extremely poor and unfavorable prognosis. The available treatments for these forms have shown poor overall effects. Thus, to overcome the difficulties in treating thyroid cancer, new approaches are needed (Haugen & Sherman 2013, Perri *et al.* 2014).

In this study, we have investigated the effect of FTY720 on the invasion and proliferation of thyroid cancer cells *in vitro*. Our results show that FTY720 efficiently inhibits both basal and S1P-evoked invasion by downregulating signaling intermediates of crucial importance for ML-1 cell migration. In addition, FTY720 attenuates the proliferation of thyroid cancer cells. Given the fact that FTY720 is already in clinical use for treatment of MS, we believe that it could be evaluated and considered for new medical indications. Thus, we suggest that FTY720 could be beneficial in the treatment of thyroid cancer.

Materials

Reagents

Dulbecco's modified Eagle's medium (DMEM), fatty acid-free bovine serum albumin (BSA), sodium pyruvate solution (1 mM), propidium iodide (PI), Bafylomycin, MG132, ³H-thymidine, and FTY720 were purchased from Sigma-Aldrich. RMPI 1640 medium (without L-glutamine) was from Lonza (Basel, Switzerland). Fetal bovine serum (FBS), 2.5% trypsin, L-glutamine, penicillin/streptomycin, and F-12 (Ham's nutrient medium) were from GIBCO. S1P was from Biomol (Plymouth, PA, USA). The CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit was from Promega. S1P₁, S1P₃, mouse VEGFR-2, PKC α and PKC β I, and Hsc70 primary antibodies were from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad Laboratories. Antibodies against phospho- and total ERK1/2, HIF-1 α (WB), β -actin, SK1, MMP2, p21, p27, and anti-rat and anti-mouse IgG were from Cell Signalling Technology. MMP9 antibodies were from Abcam. Cell culture plastic ware and human collagen type IV were

from BD Biosciences. The Trans-well inserts for migration assays were from Corning. The nitrocellulose membrane was from PerkinElmer. The BCA (bicinchoninic acid) Protein Assay Reagent kit was from Pierce Biotechnology.

Methods

Cell lines

The follicular ML-1 thyroid cancer cells, provided by Dr Johann Schönberger (University of Regensburg, Germany), were cultured in DMEM supplemented with 2mM L-glutamine, 10% FBS and 100IU/mL penicillin–streptomycin (Schonberger *et al.* 2000). The FTC-133 follicular thyroid cancer cells were obtained from Banca Biologica e Cell Factory, National Institute for Cancer Research (Genova, Italy). The cells were grown in DMEM and Ham's F12 medium (1:1) supplemented with 10% FBS and 2mM L-glutamine. The C643 anaplastic thyroid cancer cells, provided by Dr Nils-Erik Heldin (Karolinska Institute, Stockholm, Sweden), were cultured in DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin–streptomycin. Normal human thyroid N-Thyr-Ori 3-1 cells (Culture Collections, Public Health England, Porton Down, Salisbury, UK) were grown in RPMI 1640 medium supplemented with 2mM glutamine, 10% FBS, and 1% penicillin–streptomycin. All cell lines were maintained in a humidified incubator at 37°C and 5% CO₂. The cell lines not obtained from cell banks (the ML-1 and C643 cells) have been analyzed by DNA profiling by us (the ML-1 cells) and in the paper by Schweppe and coworkers (Schweppe *et al.* 2008). The C643 cells were obtained from the original source (Heldin), the same as Schweppe and coworkers (Schweppe *et al.* 2008). The FTC-133 cells were also analyzed by Schweppe and coworkers (Schweppe *et al.* 2008). All cell lines are authentic thyroid cancer cell lines. The cell passage never exceeded passage 28.

Western blot

Cells were treated as indicated, washed 3 times with ice-cold PBS, and lysed in cell lysis buffer (10mM Tris (pH 7.7), 150mM NaCl, 7mM EDTA, 0.5% NP-40, 0.2mM phenylmethylsulfonyl fluoride (PMSF), and 0.5%g/mL leupeptin). Thereafter, the lysates were centrifuged at 15,000g for 15 min at 4°C, after which the supernatants were collected, protein concentration was determined using the BCA Protein Assay Kit, and Laemmli sample buffer was added to the samples. The samples were

subjected to SDS/PAGE (8–12% polyacrylamide); thereafter, proteins were transferred onto nitrocellulose membrane by Western blotting. Next, membranes were incubated with primary antibodies targeted against specific proteins overnight, followed by incubation with secondary antibodies for 1 h at room temperature. Finally, the proteins were detected by enhanced chemiluminescence, and densitometric analysis was performed using ImageJ software (<http://rsb.info.nih.gov/ij/>). Results were normalized against β -actin expression in each sample. In experiments where collected medium was subjected to SDS/PAGE, the results were analyzed against total protein concentrations in respective samples.

Proliferation assay

CellTiter assay A total of 10,000 cells/well were cultured in a 96-well plate for 24 h. Next, complete medium was changed to serum-free medium (SFM) supplemented with 0.2% fatty acid-free BSA for another 24 h, after which cells were treated with FTY720 as indicated. Proliferation was measured using the CellTiter 96 Aqueous One Solution cell proliferation assay kit, according to the manufacturer's instructions.

³H-thymidine incorporation assay For this assay, 75,000 cells were cultured for 24 h on 35 mM as described previously. After being serum starved for 24 h, the cells were treated as indicated. Four hours before ending the experiments, cells were incubated with ³H-thymidine (0.4 μ Ci/mL). Then, the cells were washed 3 times with ice-cold PBS. After that, the cells were incubated with 5% trichloroacetic acid (TCA) for 10 min followed by incubation with 0.1 N NaOH for another 10 min. The radioactivity was measured by using a Wallac 1410 liquid scintillation counter (Wallac OY, Turku, Finland).

Fluorescence-activated cell sorting Cells were grown in 10 cm plates until 70% confluency was reached. Next, complete medium was changed to SFM overnight, and cells were treated with 1 μ M FTY720 for 24 h. After being washed with ice-cold PBS and detached, 500,000 cells were suspended in PI solution (0.05 mg/mL PI, 3.8 μ M sodium citrate, and 0.1% Triton-X-100 in PBS) and incubated for 15 min at room temperature. Finally, the samples were analyzed by flow cytometry using ModFit LT 4.1 software (Verity Software House, Inc., Topsham, ME, USA).

Figure 1

The effect of FTY720 on invasion of thyroid cancer cells. (A) Lack of an effect of 10 μ M FTY720 on invasion (17 h) of N-thy-Ori cells. (B–D) Invasion of anaplastic C643 cells (6 h), follicular ML-1 cells (6 h), and follicular FTC-133 (20 h) thyroid cancer cells is blocked by 10 μ M FTY720. (E–F) FTY720 attenuates S1P-evoked invasion of ML-1 cells. Cells were preincubated for 1 h with 1 μ M FTY720 (E) or 10 μ M FTY720 (F) and allowed to invade toward 100 nM S1P and 10% LS-FBS for 4–6 h. The scale bar in the photos are 50 μ m. (G) FTY720 (10 μ M and 1 h preincubation) attenuates S1P-evoked invasion of FTC-133 cells (20 h). (H) Cells were preincubated for 1 h with 1 μ M p-FTY720 and allowed to invade toward 100 nM S1P and 10% LS-FBS for 4 h. p-FTY720 was present in both the upper and lower chambers during the entire experiments. The results are the mean $\pm$ S.E.M., $n \geq 3$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate a statistically significant difference between FTY720 treatment and respective control. ° indicates a statistically significant difference between S1P treatment and FTY720 + S1P. # indicates a statistically significant difference between FTY720 treatment and FTY720 + S1P.

Invasion assay Invasion experiments were performed on 6.5 mm-diameter Trans-well chambers with 8 μ m pore size. The membranes were coated with 5 g/cm² collagen IV overnight and reconstituted with SFM for 1 h at 37°C prior to the experiment. After being serum starved overnight, cells were preincubated for 1 h with 1 or 10 μ M FTY720. A cell suspension containing 100,000 cells was added to the upper wells, and SFM with or without S1P and 10% lipid-stripped FBS (LS-FBS) was added to the lower wells. In experiments with FTY720, FTY720 was present in both chambers during the entire experiment. The cells were allowed to migrate in the incubator as indicated. Next, uninvaded cells from the topside of the membrane were wiped off with a cotton swab. The migrated cells were fixed

in 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. The cells were counted with 40 $\times$ magnification in eight microscopic fields in a straight line bisecting the membrane.

Zymography Cells were stimulated as indicated, conditioned medium was collected, and cells were lysed as described previously. Next, equal volumes of sample medium were mixed with loading buffer (0.1 M Tris-phosphate buffer (pH 6.8) containing 20% glycerol, 6% SDS, 0.04% bromophenol blue). The samples were electrophoresed on 10% SDS-polyacrylamide gels containing 1 mg/mL gelatin. Gels were then incubated

Figure 2

FTY720 causes lysosomal degradation of S1P₁. (A–B) The expression of S1P₁ is downregulated by FTY720 after 5 h in a concentration- (A) and time-dependent (B) manner. (C) The lysosomal inhibitor Bafilomycin (100 nM, 1 h preincubation) attenuates downregulation of S1P₁ evoked by 1 μM FTY720. The cells were incubated with FTY720 for 24 h. (D) Lack of an effect of the proteasome inhibitor MG132 (10 μM, 1 h preincubation) on the FTY720-evoked (1 μM, 24 h) downregulation of S1P₁. The upper panel shows Western blot pictures and the lower panel shows densitometric analyses. The arrows in C and D indicate the S1P₁ protein. The results are the mean ± S.E.M., $n \geq 3$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate a statistically significant difference between FTY720 treatment and respective control. °indicates a statistically significant difference between FTY720 treatment and FTY720 + inhibitor. #indicates a statistically significant difference between inhibitor treatment and FTY720 + inhibitor.

in zymo buffer I (50 mM Tris-HCl containing 2.5% Tween 80 and 0.02 NaN₃ (pH 7.5)) for 30 min, followed by incubation for another 30 min in the zymo buffer II (zymo buffer I in which 1 μM ZnCl₂ and 5 mM CaCl₂ were added). Finally, to allow gelatinolytic activity, gels were incubated in zymo buffer III containing 50 mM Tris-HCl, 5 mM CaCl₂, 1 μM ZnCl₂, and 0.02% NaN₃ (pH 7.5) overnight at 37°C. To detect bands representing gelatinolytic activity, gels were stained with Coomassie Brilliant Blue R250 for 1–2 h and destained for 30–60 min. Gels were then packed in plastic bags and scanned. Clear bands showing gelatinolytic activity were quantified by ImageJ software. MMP activity was normalized with the respective total protein concentrations in the plates.

Chick embryo chorioallantoic membrane model The chorioallantoic membrane (CAM) model has been described elsewhere (Ossowski & Reich 1980, Björk *et al.* 2015). In brief, fertilized chicken eggs were placed with sharp edge down in a humidified egg incubator (37°C) and incubated for 3 days in the rocking mode. On egg

development day (EDD) 4, 2-mm holes were made on the sharp edge of the eggs. The holes were covered with tape and the eggs were incubated stationary for 3 days, sharp edge up. On EDD 7, the holes were enlarged to approximately 2 cm in diameter. On EDD 8, ML-1 cells were harvested, suspended in DMEM medium without supplements (1×10^6 cells per 10 μL medium), and mixed 1:1 with ice-cold Matrigel. Then, 20 μL of the cell suspension was pipetted onto the CAM within the perimeter of a silicone ring. The holes were sealed with sealing film, whereafter the eggs were placed back into the incubator. The ML-1 tumors were treated on EDD 9–11 with the control solution (100 μL PBS with 0.05% DMSO) or with FTY720 (100 μL PBS with 1 μg FTY720 and 0.05% DMSO), respectively. The eggs were photographed and the tumor areas from EDD 9 and EDD 12 were measured using ImageJ software. Relative tumor areas were calculated by dividing the tumor area by the silicone ring area of each sample. The obtained values were used to calculate the percentage change in relative tumor area from EDD 9 to EDD 12. The normalized percentage change values were used for statistical analysis.

Figure 3

The effect of FTY720 on signaling intermediates in ML-1 cells. (A) 10 μM FTY720 attenuates the expression of PKCα in a time-dependent manner and PKCβ1 expression after 24 h. (B) FTY720 (1 h preincubation and 1 μM) attenuates phosphorylation of ERK1/2 evoked by S1P (100 nM and 30 min). (C) Lack of a significant effect of FTY720 (10 μM) on HIF-1α expression. (D–E) FTY720 inhibits the expression of VEGFR2 in a concentration- (FTY720, 5 h, and 0.1–10 μM) and time- dependent (FTY720, 10 μM and 1–24 h) fashion. The upper panel shows Western blot pictures and the lower panel shows densitometric analyses. The results are the mean ± S.E.M., $n \geq 3$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate a statistically significant difference between FTY720 treatment and respective control. #Indicates a statistically significant difference between S1P treatment and FTY720 + S1P. *Indicates a statistically significant difference between FTY720 treatment and FTY720 + S1P.

Scratch-wound experiment FTC133 cells were seeded onto 96-well plates at densities of 1×10^5 and 1.5×10^5 cells per well, respectively. The cells were grown for 24 h, whereafter the wounds were created by employing a wound maker tool (WoundMaker, Essen Bioscience, Ann Arbor, MI, USA). Then, the cells were washed twice with medium without supplements. Thereafter, the medium wash changed to DMEM and Ham's F12 medium (1:1) supplemented with 5% LS-FBS and 1% penicillin-streptomycin and mitomycin C (1 μg/mL) containing either the control solution (DMSO) or FTY720. The imaging was conducted with an automated live-cell imaging apparatus (Cell-IQ, CM Technologies, Tampere, Finland). The wound area analysis was conducted with the Cell-IQ Analyzer software.

Statistics

All numerical data are presented as the mean ± S.E.M. for at least three independent measurements. Statistical analysis

of two means was performed with Student's *t*-test. Three or more means were analyzed with one-way ANOVA and Bonferroni's *post hoc* test. A probability value of less than 0.05 was considered statistically significant.

Results

The effect of FTY720 on invasion of thyroid cancer cells

Our previous investigations had shown that S1P is a potent enhancer of ML-1 follicular thyroid cancer cell migration via S1P_{1,3}, but not of normal thyroid cells (Balthasar *et al.* 2006, Bergelin *et al.* 2009, 2010). Furthermore, FTY720 has been shown to inhibit migration of both noncancerous (Matloubian *et al.* 2004, Lo *et al.* 2005, Mousseau *et al.* 2012) and cancerous cells (Tonelli *et al.* 2010, Lim *et al.* 2011). Thus, the aim of this study was to investigate the effect of FTY720 on invasion of thyroid cancer cells, and for this purpose, we tested several thyroid cell lines.

Figure 4

FTY720 attenuates MMP2 and MMP9 secretion. (A) Cells were stimulated with S1P (100 nM for 48 h) in the presence or absence of 10 μ M FTY720 (1 h preincubation) and the expression of MMP2 was measured. (B) FTY720 (10 μ M, 1 h preincubation) attenuates S1P-evoked (100 nM, 4 h) MMP2 and MMP9 secretion. In (A–B), FTY720 was present during the entire experiment. (C) Incubating the cells with 10 μ M FTY720 for 24 h decreased basal MMP2 and MMP9 activity. The upper panel shows Western blot or zymography pictures and the lower panel shows densitometric analyses. The amount of secreted MMPs was normalized against total protein concentrations in respective samples. The results are the mean $\pm$ S.E.M., $n \geq 3$. * $P < 0.05$ and ** $P < 0.01$ indicate a statistically significant difference between FTY720 or S1P treatment and respective control.

As can be seen in Fig. 1A, the invasion of normal thyroid cells was not affected by FTY720, but the basal invasion of all thyroid cancer cells tested was decreased after incubation with 10 μ M FTY720 (Fig. 1B–D). Moreover, invasion of both ML-1 cells (Fig. 1E and F) and FTC-133 cells (Fig. 1G) evoked by 100 nM S1P was attenuated by FTY720. Next, we investigated if the phosphorylated form of FTY720 (p-FTY720) could show similar effects in ML-1 cells. Similar to FTY720, p-FTY720 (1 μ M and 1 h preincubation) was able to attenuate the S1P-evoked invasion of ML-1 cells (Fig. 1H). Taken together, FTY720 inhibits both basal and S1P-induced invasion of thyroid cancer cells.

FTY720 causes lysosomal degradation of S1P₁

Previous studies have shown that FTY720 has the ability to downregulate and degrade S1P_{1,3} in the proteasome (Oo et al. 2007, Mousseau et al. 2012). As FTY720 was able to inhibit invasion in our experiments, we thus tested whether FTY720 could affect the expression of S1P_{1,3} in

ML-1 cells. We treated the cells with FTY720 (0.1, 1, 3, 5, and 10 μ M) for different times (1, 3, 6, and 24 h), and could show that FTY720 was able to downregulate S1P₁ in a concentration- and time-dependent manner (Fig. 2A–B). However, to downregulate S1P₃, the cells had to be incubated with 10 μ M FTY720 (the highest concentration tested) for 24 h (results not shown). Next, we asked if FTY720 caused degradation of S1P₁; therefore, we tested the effect of the lysosome inhibitor Bafilomycin and the proteasome inhibitor MG132. Bafilomycin (100 nM, 1 h preincubation) was able to abolish the downregulation of S1P₁ evoked by 1 μ M FTY720 (Fig. 2C). Interestingly, MG132 increased the expression of S1P₁. However, MG132 was not able to abolish the response caused by 1 μ M FTY720 (Fig. 2D). To further confirm our results on the role of proteasome in degradation of S1P₁ caused by FTY720, we performed ubiquitination studies. In these studies, we were not able to detect any ubiquitination of the receptor (results not shown). Collectively, FTY720 downregulates S1P₁ by inducing lysosomal degradation. In contrast to its rapid effects at low concentrations on

Figure 5

FTY720 inhibits thyroid cell proliferation. FTY720 attenuated in a concentration-dependent manner the proliferation of (A) N-Thy-Ori thyroid cells, (B) anaplastic C643 cancer cells, (C) follicular ML-1 cancer cells, and (D) follicular FTC-133 cancer cells using the CellTiter analysis methods. The cells were incubated with FTY720 for 24 h. (E–F) FTY720 blocked proliferation of follicular ML-1 and FTC-133 cells investigated with the 3 H-thymidine incorporation assay. (G–H) Incubating with 1 μ M FTY720 for 24 h accumulated ML-1 and FTC-133 cells in the G1 phase and decreased cells in the S phase of the cell cycle. (I) Incubating ML-1 cells with 1 μ M FTY720 for 24 h upregulated the expression of p21 and p27. (J) ML-1 cells transfected with control siRNA or p21/p27 siRNA. The representative blots show the expression of p21 and p27 compared to the control. Hsc70 was used as loading control. (K) Treatment with p21 + p27 siRNA attenuates the effect of FTY720 on the proliferation of ML-1 cells. Proliferation was measured using the CellTiter proliferation assay kit. The results are the mean $\pm$ S.E.M., $n \geq 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ indicate a statistically significant difference between FTY720 treatment and respective control.

S1P₁, FTY720 requires longer incubations and higher concentrations to be able to downregulate S1P₃.

The effects of FTY720 on signaling intermediates in ML-1 cells

Previously, we showed that the S1P-induced migration of ML-1 cells is mediated by PKC isoforms, ERK1/2, SK1 (Balthasar et al. 2008, Bergelin et al. 2010), and HIF-1 α (Kalhori et al. 2013). Moreover, a cross talk between S1P₁ and VEGFR2 of crucial importance for migration exists in ML-1 cells. Hence, we investigated the effect of FTY720 (0.1, 1, and 10 μ M) for different times (1, 3, 6, and 24 h) on these signaling intermediates. Our results showed that 1 μ M FTY720 had no effect on the expression of neither PKC α nor PKC β I (results not

shown). However, after incubating the cells with 10 μ M FTY720, the expression of PKC α was downregulated in a time-dependent manner, whereas the expression of PKC β I was significantly downregulated after 24 h only (Fig. 3A). Interestingly, FTY720 was without an effect on both the total amount of ERK1/2 and the basal phosphorylation of ERK1/2 (results not shown). However, FTY720 attenuated the S1P-evoked phosphorylation of ERK1/2 (Fig. 3B). We also investigated the expression of HIF-1 α but were not able to show a statistically significant decrease of HIF-1 α expression after incubation with 10 μ M FTY720 (Fig. 3C). An important observation was that FTY720 decreased the expression of VEGFR2 in a time- and concentration-dependent fashion (Fig. 3D–E).

We have previously shown that SK1 is of importance in regulating ML-1 cell migration (Bergelin et al. 2009),

Figure 6

FTY720 inhibits the growth of ML-1 cells in a chick embryo CAM model and blocks the migration of FTC133 cells in a scratch-wound experiment. (A) Representative images of control and FTY720-treated ML-1 cell tumors in the CAM. (B) Quantification of the relative change in ML-1 cell tumor area. The results are mean $\pm$ S.E.M., $n = 13$ eggs (control), 14 eggs (FTY720 treated), $*P < 0.05$. (C) Representative images of FTC133 cells in a scratch-wound experiment. (D) Quantification of the wound closure at the 48-h time point. The results are mean $\pm$ S.E.M., $n = 4$, $*P < 0.05$.

and previously, Tonelli and coworkers showed that FTY720 downregulated SK1 (Tonelli *et al.* 2010). We were, however, not able to show any effects of FTY720 on the levels of SK1 in ML-1 cells (results not shown). Taken together, FTY720 inhibits signaling intermediates of crucial importance for ML-1 cell invasion.

FTY720 modulates MMP2 and MMP9

Previous studies have shown that FTY720 inhibits MMP2 and MMP9 in human glioblastoma cell lines (Zhang *et al.* 2014). In addition, we showed earlier that S1P via S1P_{1,3} induced the secretion of MMP2 and MMP9 in ML-1 cells, and that these MMPs are of importance for S1P-induced invasion (Kalhori & Törnquist 2015). In accordance with previous results, FTY720 attenuated the S1P-evoked upregulation of MMP2 after 48 h (Fig. 4A), and the S1P-evoked secretion of both MMP2 and MMP9 (Fig. 4B). Interestingly, FTY720 per se did not decrease the secretion of MMP2 or MMP9 (Fig. 4B). However, an important observation was that when we exposed ML-1 cells to 10 μ M FTY720 for 24 h, FTY720 decreased the activity of the secreted MMP2 and MMP9 (Fig. 4C). In conclusion, FTY720 attenuates S1P-evoked secretion of MMP2 and

MMP9 as well as MMP2 expression. In addition, FTY720 decreases MMP2 and MMP9 activity.

FTY720 inhibits thyroid cancer cell proliferation

As FTY720 decreased the invasion of the thyroid cancer cells, we investigated the effect of different concentrations of FTY720 on the proliferation of normal thyroid cells and several thyroid cancer cell lines. Results obtained using the CellTiter assay showed that FTY720 was able to attenuate proliferation of normal N-Thyr-Ori 3-1 and anaplastic C643 cells in a concentration-dependent manner (Fig. 5A–B). By performing both CellTiter and ³H-thymidine incorporation assays, we could show that FTY720 is able to inhibit proliferation of ML-1 and FTC-133 cells in a concentration-dependent fashion (Fig. 5C–D, CellTiter; Fig. 5E–F, ³H-thymidine incorporation). Next, by performing FACS analysis, we found that FTY720 inhibited proliferation of ML-1 and FTC-133 cells by accumulating cells in the G1 phase and decreasing the amount of cells in the S phase of the cell cycle (Fig. 5G–H). Next, we investigated the expression of proteins involved in cell cycle regulation and found that incubating the cells with 1 μ M FTY720 for 24 h induced

an upregulation of p21 and p27 in ML-1 cells (Fig. 5I). To investigate the significance of p21 and p27 in proliferation, ML-1 cells were transfected with siRNA against p21 and p27. The results showed that the basal proliferation was significantly enhanced and the 3 μ M FTY720-induced inhibition of proliferation was attenuated (Fig. 5J–K).

To increase the efficiency of therapeutic drugs, combined therapy is becoming an attractive tool in the treatment of different diseases. Therefore, we tested both FTY720 and p-FTY720 (1 μ M) in combination with doxorubicin (0.1, 0.5, 1, and 5 μ M) to investigate whether an additive or synergistic inhibitory effect on proliferation could be obtained. However, we were not able to see any additive or synergetic effects of these compounds (results not shown). Taken together, FTY720 inhibits proliferation of thyroid cancer cell lines. In ML-1 cells, this inhibition is due to an increase in p21 and p27, and an arrest in the G1 phase of the cell cycle.

FTY720 inhibits thyroid cancer cell growth in the chick CAM model and cell migration in the scratch-wound assay

To assess the antiproliferative effect of FTY720 *in vivo*, we employed the chick CAM model (Ribatti 2014). ML-1 cells were implanted in to the CAM and allowed to develop into a tumor. The growth of these tumors was slightly but significantly attenuated by FTY720 treatment (Fig. 6A–B). The effect of FTY720 on FTC133 cell migration in a scratch-wound model was investigated, and the results showed a significant attenuation in wound healing upon 3 μ M FTY720 treatment (Fig. 6C–D).

Discussion

Currently, not many studies address the importance of sphingolipid signaling in thyroid cancer progression, although we have shown that S1P and SK1 are important players in regulating many essential cellular events in different types of thyroid cancer cells (Balthasar et al. 2006, Bergelin et al. 2009, 2010, Kalhori et al. 2013, Törnquist 2013, Kalhori & Törnquist 2015). In addition, our *in vitro* results have been corroborated by investigations on human thyroid samples (Guan et al. 2011). In this study, we have investigated a novel approach to curtail the proliferation and invasion of thyroid cancer cells. We evaluated the effect of FTY720, a drug that is approved by both the FDA and the EMA to be clinically used for treatment of MS on thyroid cancer cell proliferation and invasion.

Our results showed that FTY720 did not affect the invasion of normal thyroid cells; however, the basal invasion of all thyroid cancer cell lines tested and the S1P-evoked invasion of ML-1 and FTC-133 cells were attenuated by FTY720. Furthermore, p-FTY720 was able to block the S1P-induced invasion of ML-1 cells. FTY720 also potentially attenuated migration of FTC-133 cells in a scratch-wound assay. Our results are in line with previously published data, showing that FTY720 is able to inhibit migration of noncancerous cells (Mousseau et al. 2012) as well as several cancer cell types, including breast cancer cells (Tonelli et al. 2010, Lim et al. 2011), liver cancer cells (Li et al. 2012), and prostate cancer cells (Tonelli et al. 2010). Thus, we suggest that FTY720 acts as an anti-invasive drug in thyroid cancer cells.

Results published earlier by Mousseau and coworkers showed that FTY720, at low concentrations (100 nM), caused both internalization and downregulation of S1PR_{1,3} in vascular smooth muscle cells (Mousseau et al. 2012). Our study showed that FTY720 clearly downregulated S1P₁ in a concentration- and time-dependent manner. Surprisingly, low concentration of FTY720 could not downregulate S1P₃ in ML-1 cells. However, we were able to see decreased expression of S1P₃ after incubating the cells with 10 μ M FTY720 for 24 h.

Oo and coworkers showed that FTY720 caused proteasomal degradation of S1PR_{1,3} (Oo et al. 2007). However, our findings suggested that FTY720 induced degradation of S1P₁ in the lysosome, but the normal maintenance of S1P₁ in ML-1 cells occurs through proteasomal degradation. The observation that FTY720 is not able to degrade S1P₁ in the proteasome was strengthened by the fact that we could not detect any ubiquitination of S1P₁ in our investigations. Thus, based on our results, we conclude that FTY720 is a potent inhibitor of S1P₁ and that it causes lysosomal degradation of the receptor. High concentrations of FTY720 are required to downregulate S1P₃, highlighting the importance of cell specificity, and concentrations used in different studies.

S1P induces migration and invasion of ML-1 cells through several mediators, such as ERK1/2, PKC α and PKC β I, and HIF-1 α . Furthermore, S1P communicates with VEGFR2, which is essential for migration (Balthasar et al. 2006, Bergelin et al. 2009, Kalhori et al. 2013, Kalhori & Törnquist 2015). In this study, we were able to show that FTY720 downregulated both PKC α and PKC β I, but we were not able to see a statistically significant decrease in HIF-1 α expression. Moreover, FTY720 was also able to attenuate S1P-evoked phosphorylation of ERK1/2. Similarly, Zhang and coworkers could show that FTY720

inhibited ERK1/2 in human glioblastoma cell lines (Zhang *et al.* 2014). Interestingly, Tonelli and coworkers showed that FTY720, albeit at lower concentrations, was able to downregulate SK1 expression (Tonelli *et al.* 2010). We were, however, not able to see this in our study. Furthermore, FTY720 inhibited the expression of VEGFR2 in a time- and concentration-dependent fashion.

We have previously shown that S1P enhances MMP2/9 secretion and the expression of intracellular MMP2 in ML-1 cells (Kalhori & Törnquist 2015). In this investigation, we show that these effects of S1P can be attenuated by FTY720. Furthermore, although FTY720 per se did not attenuate the secretion of MMP2/9, the activity of MMP2/9 in collected media was attenuated by FTY720. Our results are in line with the results of Zhang and coworkers, who demonstrated that FTY720 inhibited MMP2 and MMP9 expression and activity in human glioblastoma cell lines (Zhang *et al.* 2014). Taken together, FTY720 is able to downregulate signaling intermediates of importance for ML-1 cell invasion and migration.

FTY720 has been proven to inhibit proliferation of both normal and cancerous cells (Mousseau *et al.* 2012, Permpongkosol *et al.* 2002). In our study, FTY720 was able to block proliferation significantly in a concentration-dependent fashion with two methods tested. Of these, the ³H-thymidine incorporation assay proved to be a more sensitive method. Furthermore, using the CAM model, we could show that FTY720 slightly, but significantly, attenuated ML-1 tumor growth *in ovo*. In a previous report, Permpongkosol and coworkers showed that FTY720 inhibited proliferation of human prostate carcinoma DU145 cells by inducing apoptosis and arresting cells in the G1 phase of the cell cycle (Permpongkosol *et al.* (2002). In our study, we could also see an accumulation of both ML-1 cells and FTC-133 cells in the G1 phase. Furthermore, we observed an upregulation of p21 and p27, two important regulators of the G1 phase. Nevertheless, we were not able to strengthen the effect of FTY720 on proliferation by cotreatment with doxorubicin.

In conclusion, we suggest that FTY720, which is currently in clinical use for the treatment of MS, is an inhibitor of thyroid cancer cell proliferation and invasion. Perri and coworkers and Manfredi and coworkers described in recent reviews, and Dicitore and coworkers in a recent report, that there are, in fact, several small-molecule inhibitors targeted to thyroid cancer, albeit they have mostly been proven to have small overall effects (Perri *et al.* 2014, Manfredi *et al.* 2015, Dicitore *et al.* 2016). Thus, novel approaches to curtail and stop the progression of aggressive thyroid cancers to a metastatic

and terminally fatal disease are urgently warranted. Therefore, we suggest that FTY720 could be beneficial in the treatment of thyroid cancer.

Declaration of statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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