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Phosphorylation of the Immunomodulator FTY720 Inhibits Programmed Cell Death of Fibroblasts Via the $S1P_3$ Receptor Subtype and Bcl-2 Activation

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Key Words
FTY720 • $S1P_3$ • Fibroblasts • Bcl-2 • Mitochondria

Abstract
Background: FTY720, a synthetic compound produced by modification of a metabolite from Isaria sinclairii, is known as a unique immunosuppressive agent that exerts its activity by inhibiting lymphocyte egress from secondary lymphoid tissues. FTY720 is phosphorylated in vivo by sphingosine kinase 2 to FTY720-phosphate (FTY720-P), which acts as a potent sphingosine-1-phosphate (S1P) receptor agonist. Despite its homology to S1P, which exerts antiapoptotic actions in different cells, FTY720 has also been reported to be able to induce apoptosis in a variety of cells. Methods: Therefore, we investigated the action of both, FTY720 and its phosphorylated version FTY720-P, on apoptosis. Moreover, signalling pathways of apoptosis in response to FTY720 and FTY720-P were examined. Results and Conclusions: Although FTY720 acts apoptotic at micromolar concentrations in human fibroblasts the phosphorylated compound FTY720-P possesses a pronounced antiapoptotic effect counteracting FTY720-induced programmed cell death. Interestingly, none of the classical antiapoptotic pathways like MAP kinases, Akt or mTOR play a role in the protective role of FTY720-P. Most important, we identified that the $S1P_3$ receptor subtype is involved in the antiapoptotic action of FTY720-P leading to an increased phosphorylation of Bcl-2 and changes in the mitochondrial membrane potential.

Introduction

FTY720 is a novel immunomodulator, which is currently undergoing phase III studies for treatment of relapsing multiple sclerosis [1]. Its mechanism of action is unique compared to other used immunosuppressants as it reduces the number of circulating lymphocytes within a few hours and causes an accumulation of lymphocytes in lymph nodes [2-5]. FTY720 is a prodrug that is mainly phosphorylated by sphingosine kinase type 2 (SphK2) to the biological active metabolite FTY720-phosphate (FTY720-P), an analogue of sphingosine-1-phosphate (S1P) [2]. The relevance of S1P in cellular processes has been emphasized by identifying its function as a lig-

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and on a family of G-protein coupled receptors (GPCRs) from which five subtypes, namely $S1P_1$-$S1P_5$, exist [6]. The importance of these GPCRs in physiological and pathophysiological conditions has been clearly demonstrated by gene deletion studies and reverse pharmacology. Indeed, each receptor subtype signals through at least two different G proteins activating various downstream signalling cascades which have been reviewed elsewhere [6].

Most interestingly, there is evidence that S1P possesses a substantial role in the regulation of lymphocyte circulation [7]. The S1P gradient between lymphoid organs with low S1P-levels and lymph and blood with high S1P concentrations determine the chemotactic egress of lymphocytes from lymphoid tissues toward the blood or the lymph [2-4]. A key role of the $S1P_1$ receptor subtype has been demonstrated in this migratory response [8]. It has been clearly demonstrated that FTY720-P, which is an agonist of all S1P receptor subtypes, except $S1P_2$, induces a prolonged internalization of the $S1P_1$ thereby interrupting the S1P-$S1P_1$ receptor axis necessary for the egress of thymocytes and lymphocytes from secondary lymphoid tissues [9].

It is of interest that S1P and FTY720 have been shown to regulate apoptosis in a divergent manner. Apoptosis of cells can be induced by receptor mediated stimuli (extrinsic pathway). Typical death receptors are Fas or tumor necrosis factor (TNF) receptor. The receptor-induced pathway leads to the recruitment of caspases to the DISC (death inducing signaling complex) which leads to activation of other caspases that degrade cellular targets [10]. Another pathway for triggering apoptosis involving mitochondria is also well documented. A variety of extra- and intracellular stresses can activate this intrinsic pathway. Loss of mitochondrial membrane potential can lead to cytochrome c release from mitochondria into the cytosol. Cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1) which triggers the formation of the apoptosome that catalyses activation of caspases. It is well known that the B-cell lymphoma-2 (Bcl-2) protein family consists of pro- and antiapoptotic proteins which can interact with each other and trigger several downstream signalling cascades. The proapoptotic protein Bax can form a complex with the voltage dependent anion channel (VDAC) in mitochondria that leads to release of cytochrome c and other proapoptotic factors [11]. On the other hand, the antiapoptotic Bcl-2 is able to inactivate Bax and therefore inhibits cytochrome c release and activation of downstream processes that lead to cell death [12].

It has been indicated that S1P interrupts apoptosis by activation of distinct pathways including the MAP kinase pathway [13], Akt along with the mammalian target of rapamycin (mTOR) pathway [14] and the intrinsic pathway via modulation of Bcl-2 family proteins [15]. Depending on the cell type, $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ have been identified to account for its cytoprotective effects [16-19].

In contrast, several studies indicate that FTY720 induces apoptosis in a variety of human cancer cell lines. Antitumor effects have been shown in hepatocellular carcinoma cells [20], large granular t-lymphocytes, multiple melanoma cells [21], Jurkat T cells [22, 23], and cell lines of the prostate [24-26], kidney [27], pancreas [28], and breast [29]. For these cytotoxic effects, S1P receptor independent mechanisms have been proposed [30]. Several pathways have been identified to be involved in FTY720 triggered apoptosis in diverse cancer cell lines. Interference with mitogen-activated protein (MAP) kinases [22, 31, 32], protein kinase B (Akt) [21, 23, 28, 33], focal adhesion kinase [24], Rho-GTPase [34], Bcl-xL [21] and changes in mitochondrial membrane potential caused by FTY720 have been published [35]. Controversially, it has also been shown that FTY720 is capable of protecting oligodendrocytes from cell death [36].

In this study we present data that clearly indicate FTY720-P as a potent cytoprotective substance in human fibroblasts which is in direct contrast to its parent form. Furthermore, we hereby provide evidence of the connection of activation of $S1P_3$, stabilisation of mitochondrial membrane potential and phosphorylation of Bcl-2 by FTY720-P in primary human fibroblasts.

Materials and Methods

Materials

FTY720 was purchased from Biozol (Eching, Germany). FTY720-(S)-phosphate was from Toronto Research Chemicals (North York, Canada). Pertussis toxin (PTX) and suramin, sodium salt were purchased from Calbiochem/Merck Biosciences (Darmstadt, Germany) and stored at -20°C at a concentration of 0.03 M. Trypsin, PBS without Ca^{2+}/Mg^{2+}, Igepal, Na-desoxycholate, sodium dodecyl sulphate (SDS), phenylmethylsulfonyl fluoride, leupeptin, aprotinin, pepstatin, ethylenediamine tetraacetic acid (EDTA), sodium fluoride, sodium orthovanadate, sodium chloride, ethanol, dimethyl sulfoxide, propidiumiodide (PI), HEPES, calcium chloride, Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, SphK2 specific small interfering RNA (siRNA), Nanoparticle siRNA transfection system, PD98059, U0126, 9-fluorenylmethyl chloroformiate (FMOC-Cl), Actinomycin D and tumor necrosis factor (TNF-α) were purchased from Sigma-Aldrich

(Taufkirchen, Germany). Bovine serum albumin (BSA), fetal calf serum (FCS) and *L*-glutamine solution were from Seromed Biochrom (Berlin, Germany). Rapamycin, monoclonal rabbit anti-phospho-Akt (Ser^{473}) antibody, polyclonal rabbit anti-Phospho-mTOR (Ser^{2448}) antibody, monoclonal mouse anti-Phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibody, anti-rabbit IgG, anti-mouse IgG, SDS-sample buffer, dithiothreitol, LumiGlo® reagent and peroxide as well as protein marker were obtained from New England Biolabs (Beverly, MA). Polyclonal rabbit anti-p-Bcl-2 antibody, monoclonal mouse anti-Bcl-2 antibody and control-siRNA B were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The β-actin antibody (rabbit, polyclonal) was obtained from Abcam (Cambridge, UK). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore (Schwalbach, Germany). PCR-primers were synthesized by Tib Molbiol (Berlin, Germany). RNeasy Mini Kit and Quia Shredders were purchased from Quiagen (Hilden, Germany). FermentasAid™ First strand cDNA synthesis kit was obtained from Fermentas (St. Leon-Roth, Germany). Annexin V-fluorescein isothiocyanate (Annexin V-FITC) was obtained from Axxora Deutschland GmbH (loerrach, Germany). FacsClean, Facsflow, FacsRinse were from BD Biosciences (Heidelberg, Germany). LightCycler480 SYBRGreen I MasterMix was purchased from Roche Diagnostics (Mannheim, Germany). VPC23019 was purchased from Avanti Polar Lipids (Alabaster, USA). JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanineiodide) was obtained from Invitrogen (Karlsruhe, Germany).

Isolation of primary human fibroblasts

To isolate human fibroblasts, juvenile foreskin from surgery was incubated at 37 °C and 5 % CO_2 for 2.5 h in a solution of 0.25 % trypsin and 0.2 % EDTA. Trypsinisation was terminated by the addition of DMEM containing 10 % FCS. Afterwards, cells were washed with phosphate-buffered saline (PBS) and centrifuged at 250×g for 5 min. The pellet was re-suspended in fibroblast growth medium that was prepared from DMEM by the addition of 7.5 % FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Fibroblasts were pooled from at least three donors and cultured at 37 °C and 5 % CO_2. Only cells of the second to fourth passage were used for the experiments.

Isolation of $S1P_3^{(-/-)}$ and wild-type fibroblasts

All animal experimentation conforms to protocols approved by the institutional animal care committee. Wild-type and $S1P_3$ knockout mice were generated as recently described [37]. To isolate murine fibroblasts, skin was incubated at 37 °C for 2.5 h in a solution of 0.25 % trypsin and 0.2 % EDTA. Trypsinisation was completed by the addition of DMEM containing 10 % FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin (murine fibroblasts growth medium). Isolated fibroblasts were washed with PBS and centrifuged at 250×g for 5 min. The pellet was re-suspended in murine fibroblasts growth medium and cultured at 37 °C and 5 % CO_2. Cells were genotyped by polymerase chain reaction. The following primers were used: 5'-CAC AGC AAG CAG ACC TCC AGA-3', 5'-TGG TGT GCG GCT GTC TAG TCA A-3' and 5'-ATC GAT ACC GTC GAT CGA CCT-3'.

Measurement of apoptosis by flow cytometry

Fibroblasts ($1*10^5$ cells per well) were cultured in DMEM supplemented by penicillin and streptomycin for 24 h. Cells were grown for another 24 h under serum free conditions. After incubation with the indicated substances the cells were treated with TNF-α (20 ng/ml) and actinomycin (0.1 μg/ml) for 24 h. Then cells were washed twice with binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Apoptosis was determined by flow cytometric detection of phosphatidylserine translocation using Annexin V-FITC. Exclusion of the non-vital dye PI was simultaneously measured to discriminate between early apoptotic cells (Annexin V^+/PI^-) and late apoptotic and necrotic cells (Annexin V^+/PI^+). For this, cells were resuspended in binding buffer followed by the addition of Annexin V-FITC (final concentration 0.5 μg/ml). The mixture was incubated for 10 min in the dark at room temperature, washed and resuspended in binding buffer. PI was added (1 μg/ml) and samples were analysed by bivariate flow cytometry. The determined apoptotic rates in percent represent Annexin V^+/PI^- and Annexin V^+/PI^+-fibroblasts.

Abrogation of SphK2 by siRNA

Gene silencing was performed using sequence specific SphK2-siRNA. Fibroblasts were transfected with a final concentration of 40 mM SphK2 or control-siRNA in medium without antibiotics using the Nanoparticle siRNA Transfection System according to the manufacturer's instructions. After a transfection period of 24 h, cells were incubated with fibroblasts basal medium for 24 h. The target sequences for SphK2-siRNA were as follows: 5'-GAG GGU AGU GCC UGA UCA A-3' (sense) and 5'-UUG AUC AGG CAC UAC CCU C-3' (antisense). The silencing efficiency was verified by reverse transcriptase real time PCR.

Quantitative real-time polymerase chain reaction (PCR)

Fibroblasts were cultured in growth medium and then incubated in DMEM containing 2 mmol/L *L*-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (basal medium) for 24 h. Total RNA was collected using the RNeasy Mini Kit. cDNA was generated from total RNA using the FermentasAid™ First strand cDNA synthesis kit according to the instructions of the manufacturer. Quantitative real-time PCR was performed using a LightCycler480 (Roche Diagnostics - Applied Science, Mannheim, Germany) and the LightCycler480 SYBRGreen I MasterMix. CyclophilinA was used as a normalization control for all experiments. For the measurement of SphK2 the following primers were used: SphK2 5'-CCT GGC TGC TAG AGT TG-3' (forward), 5'-CCC TCA TTG ATC AGG CAC-3' (reverse); cyclophilinA 5'-TTT GCT TAA TTC TAC ACA GTA CTT AGA T-3' (forward), 5'-CTA CCC TCA GGT GGT CTT-3' (reverse). Total RNA (5 ng) of at least three different sets of fibroblasts were used to analyse gene expression. Relative mRNA expression was quantified using the comparative threshold cycle method. Data were obtained in at least triplicate and the specific mRNA levels were expressed as the mean ± SEM of relative mRNA expression relative to control cells.

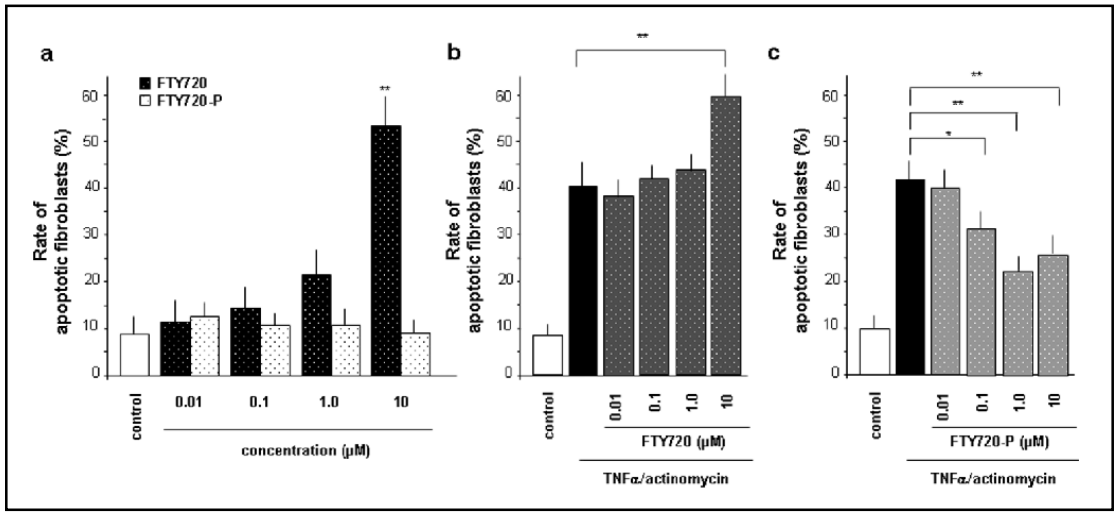

Fig. 1. FTY720-P protects human fibroblasts from apoptosis. Human fibroblasts were incubated with the indicated concentrations of FTY720 or FTY720-P for 1 h (1a). Apoptosis was determined by Annexin V-FITC/PI double staining as described in the "Materials and methods" section. To measure the effects of FTY720 (1b) and FTY720-P (1c) on cytoprotection, cells were incubated with the indicated concentrations for 1 h. Then 100 ng/ml Actinomycin and 20 ng/ml TNF-α were added for 20 h and apoptosis was measured. *P<0.05 and **P<0.001 indicate a statistically significant difference versus vehicle-stimulated cells.

Western blot analysis

Fibroblasts were seeded in six-well plates and cultured for 24 h. After a medium switch cells were treated with the indicated substances. To finish the stimulation, cells were rinsed twice with ice-cold PBS and harvested in lysis buffer (PBS without Ca^{2+}/Mg^{2+}, 1 % Igepal, 0.5 % Na-desoxycholate, 0.1 % SDS), containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 µg/ml pepstatin) and phosphatase inhibitors (1 mM sodium orthovanadate and 50 mM sodium fluoride). Lysates were centrifuged at 14,000xg for 30 min. Probes containing 20 - 40 µg protein were boiled in SDS sample buffer (100 mM Tris/HCl, pH 6.8, 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol, 200 mM dithiothreitol) and separated by 7.5 % SDS-PAGE (Phospho-mTOR), 10 % SDS-PAGE (Phospho-Akt, Phospho-ERK1/2) or 12.5 % SDS-PAGE (Phospho-Bcl-2). Gels were blotted overnight onto PVDF membranes. After blocking with 5 % non-fat dry milk for 1 h at 37 °C, membranes were incubated with the appropriate primary antibodies for 2 h at room temperature, and further incubated with horseradish-peroxidase-conjugated secondary antibodies for 1 h. Bound antibody was detected by chemiluminescence and membranes were developed according to the manufacturer's protocol.

Measurement of mitochondrial membrane potential

To define changes in mitochondrial membrane potential, fibroblasts ($1x10^5$ cells per well) were seeded into 6-well plates and cultured for 24h in basal medium. After stimulation cells were treated with TNF-α and actinomycin for 20h and washed with preheated PBS followed by incubation with the fluorescent dye JC-1 at a concentration of 1.25 µg/ml for 30 min at 37 °C and 5 % CO_2. Next, additional washing procedures were executed and staining was examined using Keyence BZ-8000 microscope (Keyence, Neu-Isenburg, Germany). A red dotted staining of cells encodes for non-apoptotic cells with a higher membrane potential whereas a diffuse green staining encodes for apoptotic cells. Appropriate emission filter settings and controls were included for bleed-through effects.

Measurement of FTY720-P levels

Fibroblasts ($2*10^5$ cells per well) were seeded into 6-well plates and cultured for 24h in basal medium. After stimulation with FTY720 the medium was collected and the adherent cells were scraped off in 1N NaCl solution. Medium and cell samples were extracted performing a double extraction with $CHCl_3$. Samples were vacuum dried and subjected to HPLC analysis as previously described [38].

Statistics

Data are expressed as the mean ± SEM of results from at least three experiments, each run in triplicate. Statistics were performed using Student´s t test. * $P < 0.05$ and ** $P < 0.01$ indicate a statistically significant difference vs. control experiments.

Results

Divergent action of FTY720 and FTY720-P on apoptosis of human fibroblasts

It has been clearly indicated that the sphingolipid S1P possesses cytoprotective properties in human fibroblasts [39]. As the novel immunomodulator FTY720

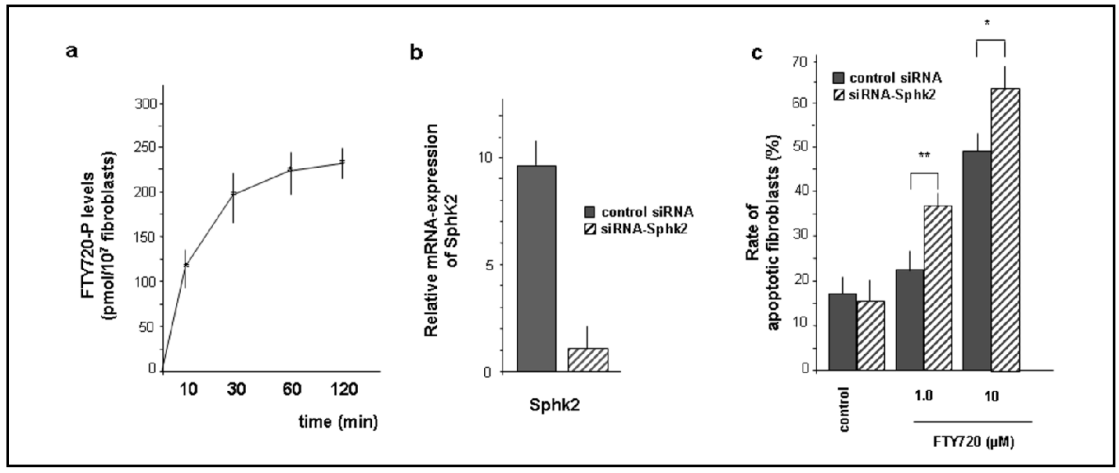

Fig. 2. Inhibition of SphK2 enhances proapoptotic effect of FTY720. To examine whether FTY720 is phosphorylated in human fibroblasts, cells were incubated with FTY720 (1 µM) and lysed at the indicated time periods. The amount of FTY720-P was determined by HPLC-analysis as described in the "Materials and methods" section (2a). To measure the effect of SphK2 siRNA on FTY720-induced apoptosis fibroblasts were transfected with SphK2 siRNA or control siRNA (final concentration 40 µM) for 24 h. Then cells were incubated with FTY720 for 1 h with the indicated concentrations followed by the addition of 100 ng/ml Actinomycin and 20 ng/ml TNF-α for 20 h (2c). Apoptosis was determined by Annexin V-FITC/PI double staining. *P<0.05 and **P<0.001 indicate a statistically significant difference versus vehicle-stimulated cells. Downregulation of SphK2 was confirmed by real-time PCR (2b).

is phosphorylated to its active metabolite FTY720-P, which is an analogue of S1P, it was of interest to examine the role FTY720 and FTY720-P in apoptosis. Measurement of the translocation of phosphatidylserine, an early event in the apoptotic process, and PI-staining indicated that FTY720-P did not increase the rate of apoptotic and necrotic fibroblasts up to a concentration of 10 µM (Fig. 1a). In contrast, FTY720 induced a drastic increase of apoptotic cells at a concentration of 10 µM (Fig. 1a). To investigate an antiapoptotic effect of FTY720-P, fibroblasts were treated with TNF-α/actinomycin, which resulted in a pronounced induction of programmed cell death. Most interestingly, when cells were preincubated with FTY720-P, TNF-α/actinomycin-induced apoptosis was markedly reduced. FTY720-P protected primary human fibroblasts from undergoing apoptosis in a dose-dependent manner. A significant decrease in apoptotic cells occurred at 0.1 µM whereas a maximal effect was detected at 1 µM (Fig. 1c). In contrast to the phosphorylated molecule, FTY720 was not able to protect fibroblasts from TNF-α/actinomycin-induced apoptosis. In congruence with its proapoptotic action, TNF-α/actinomycin-induced cell death was further increased in the presence of 10 µM of FTY720 (Fig. 1b). These results suggest that there exists a discrepancy between the prodrug FTY720 and its active metabolite FTY720-P. To examine whether FTY720 is phosphorylated in human fibroblasts, FTY720-P levels were measured after incubation with its precursor. Indeed, a significant increase of the phosphorylated molecule was measured after an incubation period of 10 min of FTY720 (Fig. 2a). As the formed FTY720-P should counteract a proapoptotic action of FTY720, we measured apoptosis of FTY720 after silencing of SphK2. The successful silencing was confirmed by real time PCR (Fig. 2b). HPLC analysis confirmed that no FTY720-P was formed. Indeed, when the formation of FTY720-P is inhibited a pronounced proapoptotic action of FTY720 is already visible at 1 µM indicating that FTY720 and FTY720-P have opposing effects on apoptosis (Fig. 2c).

3.2 $S1P_3$ receptor subtype mediates antiapoptotic action of FTY720-P

As FTY720-P binds to four of the five S1P receptors, it was of interest to figure out which receptor subtype is involved in the cytoprotective action. Most recently, $S1P_3$ has been identified as the crucial receptor subtype in S1P-mediated cytoprotection of human fibroblasts [17]. Moreover, this receptor is the most abundant subtype in this cell type [40]. Taking together these facts and the ability of FTY720-P to prevent fibroblasts from undergoing apoptosis, it was obvious to test whether the $S1P_3$ receptor subtype is responsible for FTY720-P-mediated antiapoptotic actions. Therefore,

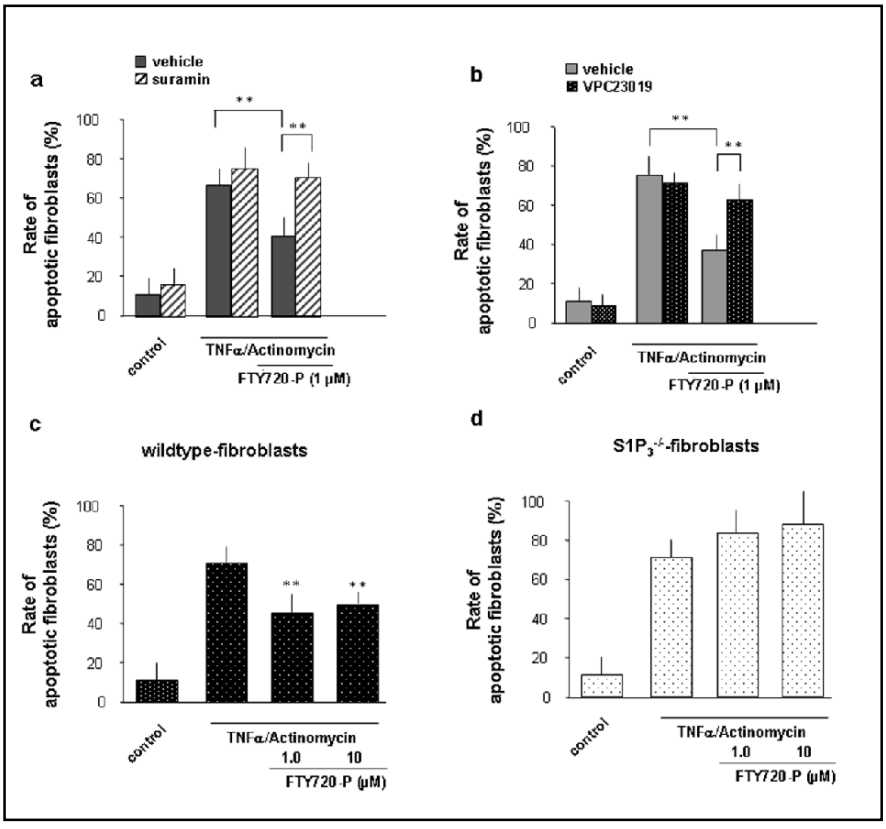

Fig. 3. $S1P_3$ receptor subtype is crucial for FTY720-P induced cytoprotection in fibroblasts. Human fibroblasts were pretreated with suramin (300 μM) for 30 min (3a) or VPC23019 (10 μM) for 30 min (3b). Then, cells were incubated with FTY720-P (1 μM, 1 h) followed by the addition of 100 ng/ml Actinomycin and 20 ng/ml TNFα for 20 h. Apoptosis was determined by Annexin V-FITC/PI double staining. *P<0.05 and **P<0.001 indicate a statistically significant difference versus vehicle-stimulated cells. Wildtype (3c) and $S1P_3^{(-/-)}$-fibroblasts (3d) were stimulated with the indicated concentrations of FTY720-P for 1 h followed by the addition of 100 ng/ml Actinomycin and 20 ng/ml TNFα for 20h. Apoptosis was determined by Annexin V-FITC/PI double staining. *P<0.05 and **P<0.001 indicate a statistically significant difference versus vehicle-stimulated cells.

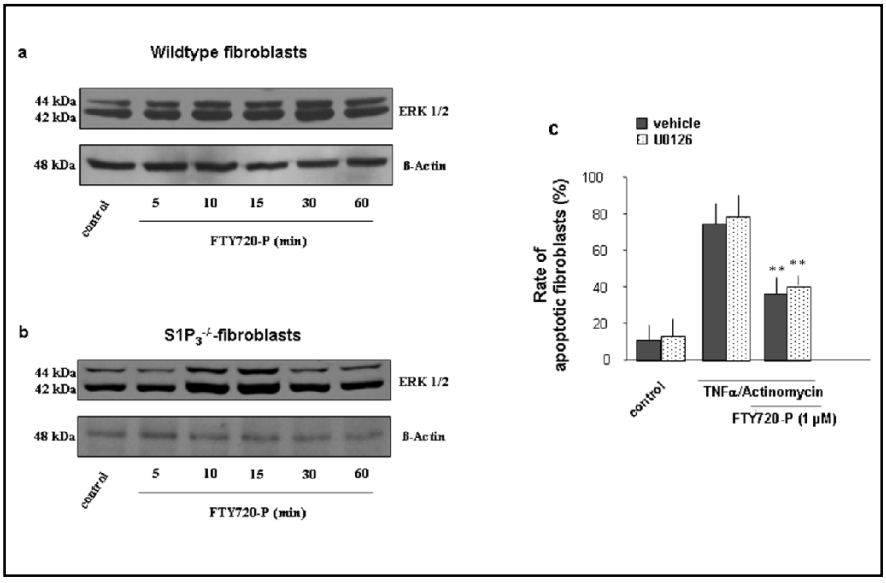

Fig. 4. MAP kinase independent anti-apoptotic effects of FTY720-P. ERK-phosphorylation in wildtype- and $S1P_3^{(-/-)}$ fibroblasts in response to FTY720-P (1 μM) for the indicated time periods was measured by Western blot analysis. For control experiments β-actin protein levels were measured (4a/b).To test an involvement of the MAP kinase pathway in apoptosis, fibroblasts were pretreated with 10 μM of U0126 for 45 min (4b) before applying 1 μM of FTY720-P for 1h followed by the addition of Actinomycin (100 ng/ml) and TNFα (20 ng/ml) for 20 h. Apoptosis was determined by Annexin V-FITC/PI double staining. *P<0.05 and **P<0.001 indicate a statistically significant difference versus vehicle-stimulated cells (4c).

we tested the ability of FTY720-P to prevent TNF-α/actinomycin-induced apoptosis in the presence of the non-selective $S1P_3$-antagonist suramin and the $S1P_1/S1P_3$ antagonist VPC23019. Indeed, when fibroblasts were preincubated with either suramin or VPC23019, the capability of FTY720-P to inhibit apoptosis was significantly reduced suggesting an involvement of $S1P_3$ (Fig. 3a/b). To further verify the importance of this receptor subtype, fibroblasts were isolated from wildtype and $S1P_3$-deficient mice. In analogy to human fibroblasts, FTY720-P protects wildtype fibroblasts from TNF-α/actinomycin-induced cell death (Fig. 3c). However, when fibroblasts, obtained from $S1P_3$-knock-out mice, were treated with TNF-α/actinomycin, FTY720-P did not exert a significant antiapoptotic effect (Fig. 3d).

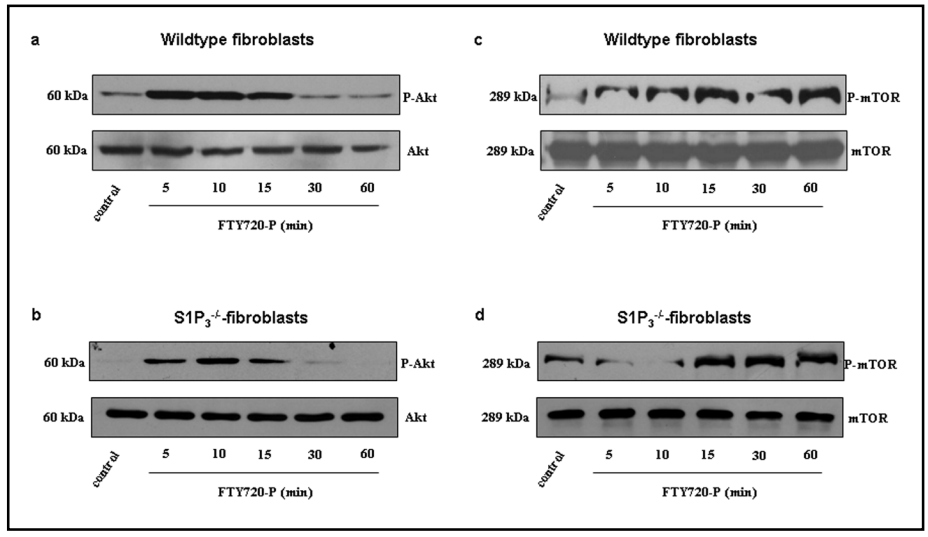

Fig. 5. Akt and mTOR are not involved in prosurvival properties of FTY720-P. Phosphorylation of Akt was determined in wildtype- and $S1P_3^{(-/-)}$ fibroblasts after stimulation with FTY720-P (1 μM) for the indicated time periods (5a/b). For control experiments unphosphorylated Akt was determined. Phosphorylation of mTOR was measured by Western blot of cell lysates of wildtype and $S1P_3^{(-/-)}$ fibroblasts stimulated with FTY720-P (1 μM) for the indicated time periods (6c/d). For control experiments unphosphorylated protein levels of mTOR were measured.

Classical antiapoptotic pathways like ERK, Akt and mTOR production are not involved in the cytoprotective property of FTY720-P

As it is well understood that S1P mediates most of its effects via GPCRs, it was of interest if PTX is able to block the antiapoptotic properties of FTY720-P. When fibroblasts were preincubated with PTX, the inhibitor of the $G_{\alpha i/o}$-subunit totally abolished the cytoprotective action of FTY720-P (data not shown). Then further experiments were performed to clarify the downstream pathways of $G_{i/o}$-proteins. Several pathways like MAPK1/2, PI3K/Akt, or mTOR have been associated with the cytoprotective behaviour of S1P depending on the cell type. To investigate whether the MAPK-pathway plays a role in the antiapoptotic action, the ability of FTY720-P to phosphorylate ERK1/2 of human and murine fibroblasts was tested. Indeed, FTY720-P was able to induce a transient phosphorylation of ERK1/2 suggesting that this pathway may be involved in the cytoprotective action (Fig. 4a). To test this, fibroblasts were preincubated with U0126, which prevents the activation of ERK1/2. As shown in Fig. 4b, FTY720-P did not lose its cytoprotective effect despite inhibition of the ERK1/2 pathway. Similar results were obtained with another MAPK inhibitor, namely PD98059 (data not shown). Finally, FTY720-P was also able to stimulate ERK1/2 in $S1P_3$-knockout fibroblasts although this receptor is essential for its protective property (Fig. 4c).

The PI3K/Akt signaling pathway has been shown to be of great importance in regulation of survival. Therefore, Western blot studies were carried out to examine the influence of FTY720-P on the phosphorylation of Akt. Although FTY720-P induced a strong phosphorylation of Akt in both human and wildtype fibroblasts already visible after 5 min with a peak at 10 min, it also provoked a similar phosphorylation in $S1P_3$ knockout fibroblasts (Fig. 5a/b). These data indicate that activation of Akt is not essential for the antiapoptotic effect of FTY720-P.

It has been reported that activation of mTOR triggers cytoprotective effects in various cell types. Even though mTOR is a downstream target of PI3K/Akt other studies have been proposed a PI3K/Akt independent activation. Measurement of mTOR-phosphorylation indicated that FTY720-P is capable to stimulate this pathway in human and murine fibroblasts. However, mTOR was also activated in $S1P_3$-deficient fibroblasts suggesting that this pathway is not involved in FTY720-P-mediated cytoprotection (Fig. 5c/d). Congruently, the mTOR inhibitor rapamycin was used to investigate if this compound leads to an attenuation of the antiapoptotic behaviour of FTY720-P. Indeed, FTY720-P decreased TNF-α/actinomycin-induced apoptosis even in the presence of rapamycin indicating that this pathway is not required for FTY720-P-mediated survival (data not shown).

Involvement of the intrinsic pathway in the cytoprotective property of FTY720-P

As classical extrinsic pathways are not involved in FTY720-P-mediated survival, modulation of the intrinsic apoptotic pathway was examined more closely. It is not clear if both pathways can be activated separately or if they are always connected with each other.

Hence, we utilised JC-1, a specific cationic dye for displaying the membrane potential of mitochondria in cells. Therefore, cells were exposed to TNF-α/actinomycin after preincubation with FTY720-P and then subjected

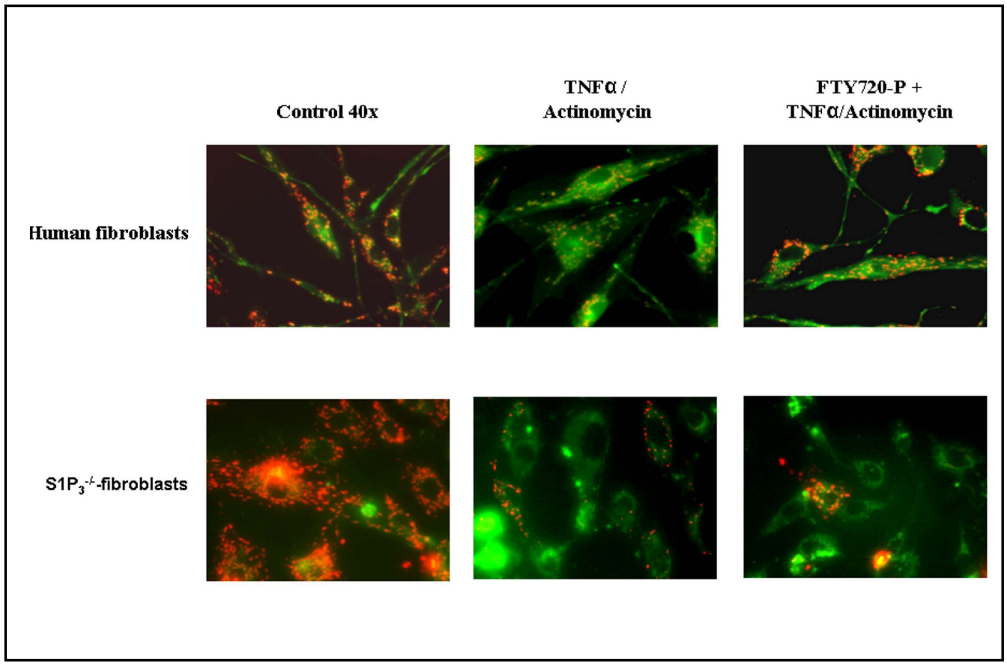

Fig. 6. FTY720-P inhibits TNF-α/Act induced alteration of mitochondrial potential in human fibroblasts. Fibroblasts were pretreated with 1 µM of FTY720-P for 1 h followed by the addition of 20 ng/ml TNF-α and 100 ng/ml Actinomycin for 20 h. $S1P_3^{(-/-)}$ fibroblasts were pretreated with 1 µM of FTY720-P for 1 h followed by the addition of 20 ng/ml TNF-α and 100 ng/ml Actinomycin for 20 h. Then cells were incubated with the fluorescent dye JC1 (5 µg/ml) for 30 min. A mainly red coloured cell encodes for a lively, non-apoptotic fibroblast whereas a prominent diffuse green displays apoptotic cells.

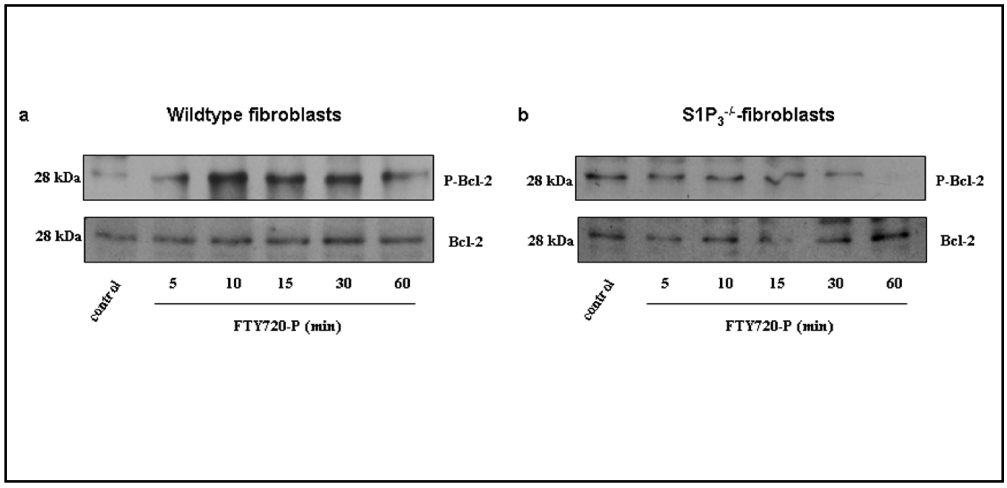

Fig. 7. FTY720-P induces Bcl-2 phosphorylation via the $S1P_3$ receptor subtype. For the examination of Bcl-2-protein phosphorylation in response to FTY720-P, Western blot analysis of P-Bcl-2 was determined. Wildtype and $S1P_3^{(-/-)}$ fibroblasts were stimulated with FTY720-P (1 µM) for the indicated time periods and cell lysates were used for Western Blot analysis. For control experiments Bcl-2 protein levels were measured.

to JC-1 staining to examine the changes in the mitochondrial membrane potential. Pretreatment with FTY720-P stabilized the mitochondria of human and murine fibroblasts compared with the apoptotic control. Most interestingly, when $S1P_3$ knockout cells were tested with FTY720-P no increase in membrane potential compared to apoptotic control could be detected (Fig. 6). Thus, in contrast to all other examined pathways the mitochondrial membrane potential shows a clear linkage to the $S1P_3$ receptor subtype. It is well established that the mitochondrial membrane potential is stabilized by the Bcl-2 protein subfamily. When the Bcl-2 protein itself is phosphorylated it exerts its antiapoptotic property. Indeed, FTY720-P induced a strong phosphorylation of Bcl-2 in human and murine fibroblasts. In congruence with measurement of the mitochondrial membrane potential, Bcl-2 phosphorylation in response to FTY720-P did not occur in $S1P_3$ deficient fibroblasts (Fig. 7). This proves that this receptor subtype is crucial for the inhibition of the intrinsic pathway to prevent cells from undergoing apoptosis after stimulation with FTY720-P.

Discussion

The novel immunomodulator FTY720 has been the topic of a huge number of studies over the last years. The unique mode of action as an immunosuppressant and the possible usability as a drug for the treatment of relapsing multiple sclerosis account for that. In addition, FTY720 has been shown to have properties beyond its effect on immunomodulation.

Depending on the cell type FTY720 has been reported to induce apoptosis via different mechanisms. FTY720 induces cell death in several human haematopoietic cells like human lymphocytes [41], HL-60 cells [42], Jurkat T-cells [22, 23] and multiple myeloma cells [21]. Further cytotoxic effects of FTY720 have been indicated in glioma cells [43], prostate [24-26] and liver cells [20, 33, 44]. But it should be considered that concentrations of FTY720 to induce apoptosis are in the micromolar range, which is 100 fold higher than plasma levels in response to therapeutic concentrations of FTY720 [45, 46]. At these high concentrations S1P receptor independent actions seem likely as induction of apoptosis in CD^{4+} cells was shown to be PTX independent [30]. As it is widely accepted that FTY720 is phosphorylated by sphingosine kinases and thus showing similarity to S1P, antiapoptotic properties seem possible, too. Confirming this assumption, it was demonstrated that FTY720 protects oligodendrocytes from apoptosis contributing to its use in the possible treatment for multiple sclerosis [36]. An antiapoptotic effect of FTY720-P has also been detected in endothelial cells [47]. In our study we not only show that FTY720-P protects primary human fibroblasts from TNFα/actinomycin-induced apoptosis already at physiological concentrations. In contrast, we even demonstrate that the nonphosphorylated form can still act apoptotic and moreover increases TNFα/actinomycin-induced cell death. Taking the results of the silencing experiments for SphK2, we suggest that FTY720 is phosphorylated *in vitro* but the remaining parent substance counteracts the antiapoptotic action of FTY720-P. This goes along with a previous report where phosphorylation of FTY720 was not necessary for its cytotoxicity [32].

Different S1P receptor subtypes have been identified for mediating cytoprotective effects of S1P depending of the cell type. In adult human mature oligodendrocytes the $S1P_5$ receptor subtype has been indicated to mediate the effects of FTY720 [19]. In isolated cardiomyocytes the $S1P_1$ receptor selective agonist SEW2871 was able to protect these cells from cell death [18], whereas a previous study identified the $S1P_2$ and $S1P_3$ receptor subtypes to be involved [48]. The vascular endothelial growth factor (VEGF) protects human endothelial cells from apoptosis via upregulation of Akt mediated by the $S1P_3$ receptor [49]. Most recently, $S1P_3$ has been reported to be responsible in fibroblasts which were rescued from cell death by treatment with the glucocorticoid dexamethasone. More precisely, treatment with dexamethasone leads to an upregulation of SphK1 and the increased amount of intracellular S1P is transported to the surface of the cell to act in an autocrine fashion [17]. Congruently we present in this study that in $S1P_3$ deficient cells no antiapoptotic action of FTY720-P was detectable.

The process of apoptosis plays an important role in maintaining cell homeostasis. Many diseases involve too much apoptosis, e.g. neurodegenerative diseases, or too little apoptosis as in cancer. The molecular mechanism of apoptosis involves diverse signalling pathways [10]. The MAP kinase pathway has been accepted as one major pathway to prevent cells from apoptosis beside its numerous effects in cell proliferation, migration, adhesion and many others [50]. Accordingly it was no big surprise to find this pathway downregulated by FTY720 in cancer cell lines. For example, this was documented in breast cancer cells [32] and leukaemia cell lines [31]. Furthermore an involvement of MAP kinases in mediating FTY720 induced apoptosis has been indicated in human Jurkat T lymphocytes [22]. Interestingly, a downregulation of ERK has also been found in human smooth muscle cells without affecting apoptosis or necrosis [51]. On the other hand, several studies exist showing that FTY720 is able to activate ERK depending on the cell type. Thus, FTY720 provokes a phosphorylation of ERK in cultured brain cells [52] and endothelial cells [47]. The activation of ERK by FTY720 has also been linked to its cytoprotective effects in oligodendrocytes [36]. Most interestingly, although FTY720-P is able to induce ERK activation, our studies ruled out this pathway as being important for the protection of human fibroblasts as ERK signalling could also be detected in $S1P_3$ knockout cells.

One of the most prominent anti-apoptotic signalling pathways in mammalian cells implicates the activation of PI3K. Moreover, PI3K has been identified to be one of the most important activators of PKB/Akt [53]. This kinase and its corresponding signalling pathway control cell death or survival [54]. Comparable to the impairment of ERK activity by FTY720, it has been published that Akt is downregulated by FTY720 in leukaemia, myeloma, hepatoma and pancreatic cancer cells [21, 23, 28, 33]. In contrast, in cells where FTY720 shows prosurvival behaviour a phosphorylation of Akt could be detected at nanomolar concentrations [47, 52]. In addition, in human umbilical vein endothelial cells (HUVECs) Akt phosphorylation by FTY720-P occurred via activation of the $S1P_3$ receptor subtype. Surprisingly, we discovered that FTY720-P and its parent molecule induce a transient activation of Akt and this activation could also be provoked in $S1P_3$ receptor deficient fibroblasts. This is in contrast to a previous finding where stimulation of Akt by FTY720-

P was mediated by this receptor subtype in murine embryonic fibroblasts [55].

The mTOR pathway plays a role in the process of autophagy and proliferation of cells. Over the last years this regulating pathway gained importance because it displays a new possible target for cancer therapy. mTOR can be activated by different stimuli, whereas Akt is the predominant activator but Akt independent ways are established, too [56]. No direct interaction of FTY720 and mTOR has been reported so far. But the sphingolipid S1P is able to activate this pathway in human NIH 3T3 fibroblasts [57, 58] and vascular smooth muscle cells [59] to mediate mitogenic actions. Most recently it has been demonstrated that activation of mTOR by S1P can be independent of Akt [14]. We hereby report that both forms of FTY720 evoke a phosphorylation of mTOR. But again a similar result was obtained from experiments with $S1P_3$ knockout cells.

The complex mechanism of apoptosis warrants investigations in other signalling pathways, including mitochondrial membrane potential and the Bcl-2 protein family, as none of the classical extracellular antiapoptotic pathways could be linked to the protective actions of FTY720-P in our studies. There exist a number of studies that link Bcl-2 and alteration in mitochondrial membrane potential to cytotoxic properties of FTY720. For example, FTY720 induced apoptosis in pancreatic cancer cells is connected to downregulation of Bcl-2 amongst other prosurvival pathways [28]. In addition, in human renal cancer cells an involvement of Bcl-2 in FTY720 triggered apoptosis has also been documented [27]. A decreased expression of Bcl-2 was also found in a murine prostate cancer model [25]. Older studies already showed that overexpression of Bcl-2 stabilises mitochondrial membrane potential abolishing the apoptotic action of FTY720 in Jurkat T cells [41, 60, 61]. Furthermore, FTY720 is able to cleave other Bcl-2 family members like Bax and Bid, which leads to apoptosis Jurkat and multiple myeloma cells [21, 62]. In contrast, after being released from apoptotic Jurkat cells, S1P is able to upregulate Bcl-2 in human primary macrophages promoting cell survival [63]. In line with these findings, it was reported that protection of human fibroblasts from apoptosis by calcitriol is mediated by S1P involving an upregulation of Bcl-2/Bax rheostat [15]. Most recently it has been shown, that dexamethasone protects fibroblast from apoptosis via the formation of S1P and an activation of the Bcl-2 family protein Bcl-xL [64]. In human keratinocytes S1P has also been indicated to stimulate the expression of the Bcl-2 protein [65]. In our study we clearly indicate that FTY720-P stabilises the mitochondrial membrane potential. The result that preincubation with FTY720 did not have an effect substantiates the double edged behaviour of this molecule depending on its status of phosphorylation. Furthermore, we could show that stabilisation of the mitochondrial membrane potential and phosphorylation of Bcl-2 did not occur in $S1P_3$ knockout fibroblasts. These findings contribute to the clarification of the molecular mechanisms of treatment with the immunomodulator FTY720.

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