Inhibition of the SphK1/S1P signaling pathway by melatonin in mice with liver fibrosis and human hepatic stellate cells

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Inhibition of the SphK1/S1P signaling pathway by melatonin in mice with liver fibrosis and human hepatic stellate cells

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Running head: Melatonin, Sphingolipids and Fibrosis
Abstract

The sphingosine kinase 1/sphingosine 1-phosphate (SphK1/S1P) system is involved in different pathological processes, including fibrogenesis. Melatonin abrogates activation of hepatic stellate cells (HSCs) and attenuates different profibrogenic pathways in animal models of fibrosis, but it is unknown if protection associates with its inhibitory effect on the SphK1/S1P axis. Mice in treatment groups received carbon tetrachloride (CCl₄) 5 µL/g body wt i.p. twice a week for 4 or 6 weeks. Melatonin was given at 5 or 10 mg/kg/day i.p., beginning two weeks after the start of CCl₄ administration. At both 4 and 6 weeks following CCl₄ treatment, liver mRNA levels, protein concentration and immunohistochemical labelling for SphK1 increased significantly. S1P production, and expression of S1P receptor (S1PR)1, S1PR3 and acid sphingomyelinase (ASMase) were significantly elevated. However, there was a decreased expression of S1PR2 and S1P lyase (S1PL). Melatonin attenuated liver fibrosis, as shown by a significant inhibition of the expression of α-smooth muscle actin (α-SMA), transforming growth factor (TGF)-β and collagen (Col) I. Furthermore, melatonin inhibited S1P production, lowered expression of SphK1, S1PR1, S1PR3 and ASMase, and increased expression of S1PL. Melatonin induced a reversal of activated human HSCs cell line LX2, as evidenced by a reduction in α-SMA, TGF-β, and Col I expression. Melatonin-treated cells also exhibited an inhibition of the SphK1/S1P axis. Antifibrogenic effect of SphK1 inhibition was confirmed by treatment of LX2 cells with PF543. Abrogation of the lipid signaling pathway by the indole reveals novel molecular pathways that may account for the protective effect of melatonin in liver fibrogenesis.

Keywords: hepatic stellate cells; liver fibrosis; melatonin; sphingolipids

1. Introduction
Sphingosine 1-phosphate (S1P), a pleiotropic bioactive lipid mediator, is formed by the phosphorylation of sphingosine by sphingosine kinases (SphKs) and regulates an array of biological activities in various cell types [1]. SphK1 is the predominant isoform of the enzyme in many cells, and catalyzes the formation of S1P, which after binding to S1P receptors (S1PRs) activates different downstream signaling pathways and induces diverse cellular responses [2]. S1P does not accumulate in cells/tissues under normal condition; intracellularly generated S1P by SphKs is either secreted out via S1P transporter or degraded by S1P lyase (S1PL), a key enzyme involved in the terminal breakdown of S1P into hexadecenial and ethanolamine phosphate in mammalian tissues [3].

Liver fibrosis is the excessive accumulation of extracellular matrix proteins that occurs in various forms of chronic liver diseases. Advanced liver fibrosis results in hepatic dysfunction and irreversible cirrhosis. During fibrogenesis hepatic stellate cells (HSCs) undergo activation to a α-smooth muscle actin (α-SMA)-positive myofibroblastic phenotype and synthesize excess extracellular matrix components, particularly collagen [4]. Different studies have shown that the SphK1/S1P system is crucial in fibrosis in numerous organs and its effects mainly relate to transforming growth factor-β (TGF-β), a key mediator in the pathogenesis of fibrosis [2]. Thus, it has been found that SphK1 and S1PRs plays a role in differentiation of mouse and human lung fibroblasts mediated by TGF-β [5], collagen production by cardiac fibroblasts [6], or transdifferentiation of myoblasts in myofibroblast [7]. It is also known that overexpression of S1PL attenuates TGF-β-induced and S1P-induced differentiation of human lung fibroblasts, and knockdown of S1PL in mice augments bleomycin-induced pulmonary fibrosis [8,9]. Concerning liver fibrosis, it has been found that TGF-β induces activation of mouse bone marrow-derived mesenchymal stem cells (BMSCs) via SphK1-up-regulation [10], and it has been recently shown that production of collagen a1 is increased by SphK1 in
human BMSCs and hepatogenic fibrotic myofibroblasts after TGF-α treatment [2]. It is also known that SphK1 is upregulated in exosomes derived from CCl₄-treated mice [11].

Melatonin is a secretory product of the pineal gland that, in addition to regulating circadian rhythms, modulates several molecular pathways of inflammation, oxidative stress, and cellular injury [12,13]. Moreover, different studies have shown a protective role of melatonin against fibrosis in different organs, including the liver. It is known that the indole abrogates activation of HSCs induced in vitro [14], and different authors have demonstrated that melatonin attenuates multiple profibrogenic pathways in rats or mice with fibrosis induced by CCl₄, bile duct ligation, dymethylnitrosamine or thioacetamide [15]. Although in most studies only preventive effects before the onset of liver toxicity have been shown, we have recently demonstrated that delayed melatonin treatment allowing initial activation of HSCs ameliorates fibrosis in CCl₄-treated mice, with a downregulated expression of profibrogenic factors which associates to inhibition of endoplasmic reticulum stress and autophagy [16,17].

However, almost no information exist on the relationship between melatonin and sphingolipid metabolism, and only one research indicating an association of melatonin with SphK1 inhibition in prostate cancer cells has been published until now [18]. This study was aimed to identify if antifibrotic effects of melatonin associate with inhibition of SphK1/S1P axis. Herein we demonstrated that melatonin treatment, in addition to the impairment in HSCs activation and expression of profibrogenic factors, impairs the lipid signaling pathway in CCl₄-treated mice. These effects were also validated in vitro in human HSCs cell line LX2, suggesting a potential relevance in human liver fibrosis.

2. Experimental Procedures

2.1. Animal Experiments and Drug Treatment
Male C57BL/6J mice (Harlan Laboratories; BCN, Spain) weighing 20-25 g were used in this study. The animals were acclimated to the temperature (22±2°C) and humidity (55±5%) of controlled rooms with a 12:12 h light-dark cycle for at least week prior to experiments. They were allowed access to mice chow and water *ad libitum*. Mice in treatment groups received CCl$_4$ at a dose of 5 µL/g body weight (10% CCl$_4$ in corn oil) via intraperitoneal injection twice a week for 4 weeks or 6 weeks. Melatonin (Sigma; St Louis, MO) was administered via intraperitoneal injection (5 or 10 mg/kg/day), beginning two weeks after the start of CCl$_4$ administration. Melatonin was dissolved into absolute ethanol and further dilutions were made in saline; the final concentration of ethanol was 5%. Mice that received corn oil injection or melatonin injection only served as sham controls. Each group consisted of eight mice. The study protocol was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and was specifically approved by the Ethics Committee of the University of León. At the end of the experiment, mice were anesthetized with ketamine/xylazine cocktail and sacrificed. Serum samples were collected from each mouse and stored at -80°C to determine the serum biochemical parameters. Livers were harvested 24 h after the last injection of CCl$_4$.

### 2.2. LX2 culture and Treatments

Human HSCs cell line LX2 was kindly provided by Dr. Prieto, CIMA, Navarra, Spain. Stock cells routinely were grown at monolayers in a 5% CO$_2$ humidified incubator at 37°C. The cultured medium used was Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were maintained in T-75 culture flasks. After 24 h, the medium was replaced with fresh medium containing 2% FBS. Control cells were incubated with 0.05% DMSO, Control+Mel cells were incubated with melatonin (500 µM) dissolved in 0.05% DMSO and TGF-β cells were
incubated with 0.05% DMSO and 2 ng/mL TGF-β (R&D System, Minneapolis, USA).

Treated-TGF-β cells were incubated with 2 ng/mL TGF-β and melatonin (100 and 500 µM) dissolved in 0.05% DMSO. Cells were incubated for 24 h. For inhibition studies, the specific SphK1 inhibitor PF543 (Merck Millipore, Germany) [19] was added 1 h before TGF-β stimulation. PF543 (10 µM) was dissolved in 0.01% DMSO.

2.3. Immunohistochemical Staining

Liver tissue samples were recovered, fixed in 10% buffered formalin and embedded in paraffin. Sections (4 µM) were dewaxed and hydrated through graded ethanol, cooked in 25 mM citrate buffer, pH 6.0, in a pressure cooker for 10 min, transferred into boiling deionized water and let to cool for 20 min. Tissue sections were then treated with 3% hydrogen peroxide to inactivate endogenous peroxidase activity. The slides were incubated with mouse anti SphK1, S1PR1 and S1PR3 (Abcam, Cambridge, UK) antibodies overnight at 4°C. Subsequently, the sections were incubated for 30 min using the EnVision+ system and developed with a solution of 3-3-diaminobenzidine (DAB) (Vector Lab; Burlingame, CA, USA). The slides were stained with hematoxylin for 10 s and mounted. The specificity of the technique was evaluated by negative controls (omitting the incubation with the primary antibody and incubating it with non-immune sera). Positive areas were quantified using the software Image J (NIH, Bethesda, MD, USA).

2.4. Real-Time RT-PCR

Total RNA was obtained from frozen mouse liver and LX2 cells using a Trizol reagent (Life Technologies, Madrid, Spain) and quantified using a Nano Drop1000 spectrophotometer (Thermo Scientific; Wilmington, DE, USA). Residual genomic DNA was removed by incubating RNA with RQ1 RNase-free DNase (Promega; Madison, WI, USA). RNA integrity
was confirmed by formaldehyde gel electrophoresis. Total RNA (1 µg) was reverse transcribed as described [20] and mRNA was determined by real-time PCR analysis using SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and the appropriate primers for human and mouse (Table 1). Relative changes in gene expression levels were determined using the \(2^{-\Delta\Delta CT}\) method [21,22]. The cycle number at which the transcripts were detectable (Ct) was normalized to the cycle number of GADPH gene detection, referred to as \(\Delta CT\).

2.5. Western Blot Analysis

Western blot analyses were performed on liver tissue and LX2 cells. Extracts were homogenized in 1 mL RIPA buffer containing protease and phosphatase inhibitor cocktails (Roche Diagnostics GmbH), maintaining temperature at 4°C throughout all procedures. Then the homogenate was incubated on ice for 30 min and finally the samples were centrifuged at 13,000g for 30 min at 4°C. The supernatant fraction was stored at -80°C in aliquots until use. Protein concentration was measured by Bradford assay. Equal amounts of protein extracts (20-50 µg) were separated by 7-12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred electrically to polyvinylidene difluoride membranes (Merck Millipore) [23]. The membranes were then blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 30 min at 37°C [24] and probed overnight at 4°C with polyclonal anti-TGF-β, Col I (Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-SMA, SphK1, S1PL (Abcam) antibodies at 1:200-1:1,000 dilution with PBST containing 2.5% non fat dry milk. Equal loading of protein was demonstrated by probing the membranes with a rabbit anti-β-Actin polyclonal antibody (1:2,000; Sigma). After washing with TBST, the membranes were incubated for 1 h at room temperature with secondary HRP conjugated antibody (1:5,000; Dako, Glostrup, Denmark). After washing with TBST, the membranes
were incubated for 1 h at room temperature with secondary HRP conjugated antibody (Dako, 1:5,000), and visualized using ECL detection kit (Amersham Pharmacia; Uppsala, Sweden) [25]. Band intensities were quantified using the software Image J (NIH, Bethesda, MD, USA).

2.6. Assay for S1P Levels
S1P was tested by ELISA using a commercial kit (Echelon Biosciences, UT, USA). Quantitative S1P assays were done in duplicate on all liver homogenate samples according to manufacturer’s instructions. The levels were expressed as pmol/µg of protein.

2.7. Statistical Analysis
Results are expressed as mean values ± standard error of the mean (SEM). Data were compared by analysis of variance (ANOVA); when the analysis indicated the presence of a significant difference, the means were compared with the Newman-Keul’s test. Significance was accepted when \( P \) was less than 0.05. Values were analyzed using the statistical package SPSS 19.0 (IBM Corporation, Armonk, NY, USA).

3. Results
3.1. MelatoninInduces an Antifibrogenic Effect in CCl\(_4\)-Treated Mice
To confirm the antifibrogenic effect of melatonin, the expression of genes related to fibrogenesis using quantitative real-time PCR and Western blot was analyzed. We found augmented expression of \( \alpha \)-SMA, TGF-\( \beta \) and Col I in CCl\(_4\)-treated mice, and this increase was significantly prevented by melatonin administration at both 4 and 6 weeks (Table 2, Fig. 1).

3.2. Melatonin Inhibits the SphK1/S1P Pathway
It has been demonstrated that sphingosines play an important role in the development of fibrosis. We first investigated whether chronic CCl₄ injury is associated with up-regulation of the hepatic sphingosine pathway, by examining the expression of SphK1 and S1P levels in CCl₄-administered mice. Our data indicated that SphK1 mRNA expression, protein concentration and liver immunostaining increased significantly (Table 2, Fig. 1 and 2). S1P production, measured by ELISA, was significantly higher in mice receiving CCl₄ chronically (Fig. 2). On the contrary, S1PL mRNA expression and protein concentration were markedly diminished (Table 2, Fig. 1). All these effects were significantly abrogated by melatonin at 4 and 6 weeks (Table 2, Fig. 1 and 2). Considering the relationship between S1P and ceramide pathways we also analysed ASMase expression, observing a significant upregulation by CCl₄ treatment that was prevented by melatonin administration (Table 2).

### 3.3. Melatonin Downregulates S1PR1 and S1PR3 Expression

To study the effects of melatonin on S1PRs, we first measured mRNA levels of S1PR1, S1PR2 and S1PR3. S1PR1 and S1PR3 were significantly upregulated in mice treated with the toxic, whereas S1PR2 levels decreased markedly. These changes were abrogated by melatonin at both time points (Table 2). To further confirm involvement of S1PR1 and S1PR3 in the action of melatonin an immunohistochemical analysis was performed, confirming that the indole caused a significant decrease in immunostaining at both 4 and 6 weeks (Fig. 3).

Effects induced by melatonin on the levels and expression of the different factors involved in the fibrogenic process were dose-dependent, reaching values that were generally lower in mice treated with 5 mg/kg body wt melatonin when compared to animals receiving the high dose (10 mg/kg body wt) of the indole (Table 2, Fig. 1 and 2).
3.4. Melatonin Inhibits Expression of Profibrogenic Factors and the SphK1/S1P axis in TGF-β-Treated LX2 Cells

The protective action of melatonin was also validated in vitro in the LX2 cell line. When cells were incubated with TGF-β, exhibited a marked increase in the mRNA levels of α-SMA, TGF-β, Col I, SphK1, S1PR1, S1PR3 and ASMase, whereas expression of S1PL and S1PR2 decreased. Those effects were prevented by melatonin in a concentration-dependent manner when administered at two different doses (100 or 500 µM) (Table 3). Treatment with the inhibitor PF543 resulted in a 77% decrease of SphK1 protein concentration and also induced a significant impairment of α-SMA and Col I expression. Additional administration of melatonin at both 100 and 500mM did not cause any additional effect in the expression of the fibrogenic markers (Fig. 4).

4. Discussion

S1P is now recognized as a pivotal regulator of fibrosis diseases and the relevance of SphK1/S1P signaling has been shown in different animal models of liver fibrosis, human fibrotic samples and HSCs [26,27]. Previous studies have also found that melatonin may play a regulatory role in fibrotic pathogenesis and have protective effects against fibrosis in various organs and tissues, including the liver [15]. The results here obtained in a murine model of liver fibrogenesis and in a line of human HSCs suggest that inhibition of the SphK1/S1P axis may be involved in the antifibrotic effect of melatonin.

As previously reported, when melatonin was given to mice two weeks after the start of CCl₄ administration to allow initial activation of HSCs, the expression of profibrogenic genes was inhibited [16]. The antifibrogenic effects of melatonin were also tested in the present study in TGF-β-activated LX2 cells, a line derived from normal HSCs that are immortalized. Although earlier studies suggested that these cells are fully transdifferentiated myofibroblasts
with a low sensitivity towards TGF-β [28], we found an appropriate response to TGF-β that confirms other recent research with an adequate experimental setting involving early passages of cells [29]. Melatonin treatment-dependent reversal of activated LX2 cells was evidenced by a reduction in α-SMA, TGF-β and Col I expression, similarly to results by other authors when LX2 cells are treated with ursodeoxycholyl lysophosphatidylethanolamide [29] or the energy blocker 3-bromopyruvate [30]. Antifibrotic effects of melatonin associated both in vivo and in vitro to dose-dependent inhibition of the SphK1/S1P axis, with lowered S1P levels and a downregulation of SphK1 expression. Our data are consistent with previous reports showing that, after administration of SphK1 inhibitors, the intensity of α-SMA immunostaining and the expression of profibrogenic cytokines are markedly suppressed in bile duct-ligated mice and CCl₄-treated mice [31]. To better support the conclusion that down-regulation of the SphK1/S1P system is not simply associated with the drug's antifibrogenic action, but an actual cause-effect relationship exists between the two events, effects of the SphK1 inhibitor PF543 [32] were tested in LX2 cells in the presence and absence of melatonin treatment. Data obtained support the interest of targeting SphK1 to facilitate development of therapies for liver fibrosis.

S1P exerts its functions by binding to a family of five G protein-coupled receptors (S1PR types 1-5). S1PR1, S1PR2, and S1PR3 are expressed by a wide variety of tissues, while S1PR4 expression is confined to lymphoid and haematopoietic tissue and S1PR5 expression to the central nervous system [33]. By binding to its receptors, S1P modulate many physiological or pathological processes, and there is increasing evidence that supports a critical role of S1P receptors in mediating its profibrotic action in various cellular contexts [34]. Although S1P is crucial in liver fibrosis, different reports suggest that its mode of action may show differences concerning the role of S1PRs in various species. Thus, in human fibrotic samples S1PR1 and S1PR3 are strongly expressed, whereas expression of S1PR2 is
massively decreased [35]. However, in mice liver it was reported years ago that after 6 weeks of CCl₄ treatment S1P3, but not S1P1 and S1P2, was markedly upregulated [36]. There was also a report that CCl₄- and dimethylnitrosamine-induced liver fibrosis was reduced in S1PR2 knockout mice [37]. Nevertheless, our research indicates that, both in liver from fibrotic mice and human TGF-β-treated LX2 cells, S1PR1 and S1PR3 expression increases and there is a lowered expression of S1PR2. In addition, changes in the different S1PRs were abolished by melatonin treatment. These data support previous findings that blockade of S1PR1/3 with VCPC23019 administration attenuates the extension of liver fibrosis and angiogenesis in murine models of hepatic fibrosis [31]. Moreover, it is known that, contrary to the stimulatory effects of S1PR1 and S1PR3, S1PR2 inhibits cell migration in most cell types [38]. In fact, it has been reported that S1P-stimulated fibrogenic activity of LX2 cells is mimicked by the S1PR1 agonist SEW2871 or silencing of S1PR3 by siRNA, and abrogated by the antagonist W146; however, pretreatment with the S1PR2 inhibitor JTE-013 or silencing of S1PR2 indicates a negative role of the receptor [35]. In any case, although it is possible that distinct cell types respond to S1P in a different fashion, and the exact contribution of S1P receptors to liver fibrosis should be further elucidated, our current evidence suggests that in CCl₄-treated mice S1PR1 and S1PR3, but not S1PR2 are required for S1P-induced profibrogenic activity.

S1PL expression also appears to play a role in the development of fibrogenesis and it is known that in lung fibroblasts TGF-β increases S1PL expression and this effect is abolished by treatment of cells with anti-TGF-β antibody [26]. It has been recently shown that while SphK1 deficiency protects mice against bleomycin-induced pulmonary fibrosis, S1PL deficiency exacerbates fibrogenesis, suggesting that S1PL is a novel endogenous suppressor of pulmonary fibrosis [8,9]. However, enhanced levels of S1P in human liver biopsies and serum of fibrotic patients, have been previously proposed to result from increased SphK activity because expression of S1PL mRNA remained unchanged [35]. Our data indicate that,
both in CCl₄-treated mice and LX2 cells, S1PL expression were markedly reduced, and that this effect was significantly abrogated by melatonin in a dose-dependent manner. Findings support the possibility that regulation of S1P by the balance between SphK1 and S1PL may be a pathway that promotes development of fibrogenesis.

Different studies show that SphK1 is a downstream mediator of TGF-β signaling. Thus, it is known that decrease of SphK1 expression by siRNA blocks TGF-β-mediated upregulation of TIMP-1, a protein which plays an important role in matrix modelling and degradation [39]. We have recently reported that in melatonin-treated mice there is a decreased TIMP-1 expression [16], which could thus be a consequence of the inhibition in the SphK1/S1P axis and explain, at least in part, the abrogation of TGF-β mitogenic effects. However, it is also possible that S1P acts via indirect mechanisms through a crosstalk with the inflammatory and oxidative pathways. In high fructose-fed rats and Buffalo rat liver (BRL3A) cells stimulated with fructose, it appears that SphK1/S1P axis plays an hepatoprotective role through it activation by NF-kappaB by inflammatory cytokines [40]. However, although the antioxidant curcumin ameliorates fibrosis associated to nephropathy by inhibiting the SphK1/S1P pathway, in SphK<sup>WT</sup>-transfected glomerular mesangial cells overexpression of SphK1 is prevented by curcumin in a concentration-dependent manner, suggesting an effect independent from its antioxidant capacity [41].

Ceramide has also been investigated as a potential regulator of liver fibrosis and it could be proposed that S1P acts via indirect mechanisms in an antifibrotic manner by perturbing the ceramide/S1P rheostat [34]. Ceramides can be generated through sphingomyelin hydrolysis by acid sphingomyelinase (ASMase) [42]. Inhibition of ASMase has been shown to reduce liver ceramide levels and attenuate liver fibrosis in a rat model of Wilson’s disease [43], and ASMase haploinsufficient mice are protected from the development of liver fibrosis in both cholestasis- and CCl₄-induced models of this disease [44]. Data here obtained indicate that in
mice with chronic administration of CCl₄ and in LX2 cells ASMase expression is significantly reduced by melatonin, which gives an additional insight into the potential role of changes in sphingolipid pathways in the antifibrotic effect of the indole.

5. Conclusion

Our study demonstrates that the antifibrotic effect of melatonin, administered two weeks after CCl₄ treatment to allow the initial activation of HSCs, associate in mice to an inhibition of the SphK1/S1P axis. Beneficial effects involve impairment in S1P production, lowered expression of SphK1, S1PR1 and S1PR3 and increased expression of S1PL. Moreover, melatonin treatment induced a reversal of activated human hepatic stellate LX2 cells, which also exhibited an inhibition of the lipid signaling pathway. Although the precise mechanisms of melatonin in liver fibrosis remain to be elucidated and additional studies are required, our findings reveal novel molecular pathways that may account for the protective effect of melatonin in liver fibrogenesis. Considering that melatonin has very high safety profile and no deaths or serious toxicity associated to its usage has been reported, data obtained set the stage for further evaluation of its therapeutic potential.

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The authors have no conflicts of interest to declare.

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FIGURE LEGENDS

FIG 1 Effect of CCl₄ and melatonin on liver expression of TGF-β, Col I, SphK1, S1PL and ASMase in mice. Mice in treatment groups received CCl₄ at a dose of 5 µL/g body weight twice a week for 4 weeks or 6 weeks (w). Melatonin 5 mg/kg (5Mel) or 10 mg/kg (10Mel) was given to mice receiving CCl₄ or vehicle (Control+Mel). Protein from cell extracts was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by β-Actin bands. Band intensities were quantified using NIH Image J software. (A): Representative Western-blot photographs. (B): Histograms representing the result of densitometric quantification. Values are expressed as means S.E.M (n=8). aP < 0.05, compared with Control. bP < 0.05, compared with CCl₄ same period. cP < 0.05, compared with 5Mel same period.

FIG 2 Liver SphK1 immunohistochemistry and S1P levels in CCl₄-administered and melatonin-treated mice. Mice in treatment groups received CCl₄ at a dose of 5 µL/g body weight twice a week for 4 weeks or 6 weeks (w). Melatonin 5 mg/kg (5Mel) or 10 mg/kg (10Mel) was given to mice receiving CCl₄ or vehicle (Control+Mel). (A): Photomicrographs of sections of liver samples taken from (a) Control; (b) Control+Mel; (c) CCl₄ 4w; (d) CCl₄ 6w; (e) CCl₄+5Mel 4w; (f) CCl₄+5Mel 6w; (g) CCl₄+10Mel 4w; (h) CCl₄+10Mel 6w. Paraffin-embedded sections were stained with SphK1 antibody. Original magnification: 200x. (B): Image analysis of the area of SphK1 staining. Positive areas were quantified using the NIH Image J software. (C): Level of S1P in liver homogenates analyzed by ELISA using a commercial kit. Values are expressed as means S.E.M (n=8). aP < 0.05, compared with Control. bP < 0.05, compared with CCl₄ same period. cP < 0.05, compared with 5Mel same period.
FIG 3 Liver S1PR1 and S1PR3 immunohistochemistry in CCl₄-administered and melatonin-treated mice. Mice in treatment groups received CCl₄ at a dose of 5 µL/g body weight twice a week for 4 weeks or 6 weeks (w). Melatonin 5 mg/kg (5Mel) or 10 mg/kg (10Mel) was given to mice receiving CCl₄ or vehicle (Control+Mel). (A and C): Photomicrographs of sections of liver samples taken from (a) Control; (b) Control+Mel; (c) CCl₄ 4w; (d) CCl₄ 6w; (e) CCl₄+5Mel 4w; (f) CCl₄+5Mel 6w; (g) CCl₄+10Mel 4w; (h) CCl₄+10Mel 6w. Paraffin-embedded sections were stained with S1PR1 or S1PR3 antibodies. Original magnification: 200x. (B and D): Image analysis of the area of S1PR1 and S1PR3 staining. Positive areas were quantified using the NIH Image J software. Values are expressed as means S.E.M (n=8).

aP < 0.05, compared with Control.
bP < 0.05, compared with CCl₄ same period.
cP < 0.05, compared with 5Mel same period.

dFIG 4 Effects of PF543 and melatonin on expression of SphK1, α-SMA and Col I in LX2 cells. Activated TGF-β cells were incubated for 24 h with 2 ng/mL TGF-β and melatonin at a dose 100 mM (100Mel) or 500 mM (500Mel). The specific SphK1 inhibitor PF543 (10 µM) was added 1 h before TGF-β stimulation. Protein from cell extracts was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by β-Actin bands. Band intensities were quantified using the NIH Image J software. (A): Representative Western-blot photographs. (B): Histograms representing the result of densitometric quantification. Values are expressed as means S.E.M (n=6).

aP < 0.05, compared with Control.
bP < 0.05, compared with TGF-β.
cP < 0.05, compared with 100Mel.
dP < 0.05, compared with 500Mel.
### TABLE 1 Primers used in this study

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<td>ACTCTCCACCCGCAATTACGAT</td>
<td>CAAGACAGTGAAGCTACAGGTG</td>
</tr>
<tr>
<td>S1PL</td>
<td>ACCAGACCCTTTCACACATTT</td>
<td>ACTGCCACATGTGCAAGGAT</td>
</tr>
<tr>
<td>ASMase</td>
<td>CAAGCTTGAAGAGCGCTCTCATC</td>
<td>AGGCAGGACATCGCATCTG</td>
</tr>
<tr>
<td>β-Actin</td>
<td>AATCGTGCCTGACATCAAGAGA</td>
<td>GCCATCTCCTGCTCGAAGTCT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human gene</th>
<th>Sense primer (5’-3’)</th>
<th>Antisense primer (3’-5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SMA</td>
<td>GACAGCTACGTTGGGTACCGAA</td>
<td>CGGGTACTTCCAGGTCAGGAT</td>
</tr>
<tr>
<td>TGF-β</td>
<td>TCGTGGAACATGACAAGGAATTC</td>
<td>AAGATCAAAACCGGAAACTCTCGAT</td>
</tr>
<tr>
<td>Col I</td>
<td>GAGACTGGTTCTTCTTCTGCTGTAAGTGTGTGTAACTG</td>
<td>CCCCGTGACACACATCAAGAC</td>
</tr>
<tr>
<td>SphK1</td>
<td>TGGACGCTTCCCTTGACACAT</td>
<td>TGGTACAGGAGGTCTATTG</td>
</tr>
<tr>
<td>S1PR1</td>
<td>ACTCCAGCCACCAGGTGT</td>
<td>AGGTGACAGGCGCAACAA</td>
</tr>
<tr>
<td>S1PR2</td>
<td>CCATCTTCTCCATCATCTCTGTTG</td>
<td>GAGCCGACCACGCGATAGA</td>
</tr>
<tr>
<td>S1PR3</td>
<td>ACAACTCCAGCGGCTCATCAT</td>
<td>TGAACACGCTACCAAAATCA</td>
</tr>
<tr>
<td>S1PL</td>
<td>GCTCTGGGATCCCGTGTAT</td>
<td>TTCAGTCTTCACCCTAGCA</td>
</tr>
<tr>
<td>ASMase</td>
<td>CTGACTCTCCGGTTCTCTCTTG</td>
<td>AGGTGATGGCGGTAATAG</td>
</tr>
<tr>
<td>β-Actin</td>
<td>TTCCCGACAGAGAGTAGAAGA</td>
<td>GCCGATCCACACGGAGTACT</td>
</tr>
</tbody>
</table>

*Abbreviations: α-SMA, α-smooth muscle actin; ASMase, acidic sphingomyelinase; Col I, collagen I; Mel, melatonin; S1PL, sphingosine 1-phosphate lyase; S1PRs, sphingosine 1-phosphate receptors; SphK1, sphingosine kinase 1; TGF-β, transforming growth factor-β.*
TABLE 2 Effect of treatment with melatonin on messenger RNA levels of genes related to fibrosis and sphingolipid metabolism in CCl$_4$-treated mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control+Mel</th>
<th>CCl$_4$ 4w</th>
<th>CCl$_4$+5Mel 4w</th>
<th>CCl$_4$+10Mel 4w</th>
<th>CCl$_4$ 6w</th>
<th>CCl$_4$+5Mel 6w</th>
<th>CCl$_4$+10Mel 6w</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SMA</td>
<td>100 ± 6</td>
<td>97 ± 5</td>
<td>596 ± 24$^a$</td>
<td>491 ± 9$^{ab}$</td>
<td>356 ± 13$^{abc}$</td>
<td>657 ± 24$^a$</td>
<td>564 ± 19$^{ab}$</td>
<td>391 ± 20$^{abc}$</td>
</tr>
<tr>
<td>TGF-β</td>
<td>100 ± 3</td>
<td>99 ± 5</td>
<td>252 ± 32$^a$</td>
<td>206 ± 4$^b$</td>
<td>108 ± 3$^b$</td>
<td>367 ± 1$^a$</td>
<td>253 ± 32$^b$</td>
<td>174 ± 5$^{bc}$</td>
</tr>
<tr>
<td>Col I</td>
<td>100 ± 5</td>
<td>90 ± 6</td>
<td>986 ± 83$^a$</td>
<td>871 ± 57$^{ab}$</td>
<td>532 ± 44$^{abc}$</td>
<td>1217 ± 5$^a$</td>
<td>1023 ± 54$^b$</td>
<td>970 ± 33$^{bc}$</td>
</tr>
<tr>
<td>SphK1</td>
<td>100 ± 6</td>
<td>131 ± 11</td>
<td>2728 ± 556$^a$</td>
<td>1499 ± 54$^{ab}$</td>
<td>1051 ± 24$^{abc}$</td>
<td>2000 ± 192$^a$</td>
<td>1526 ± 24$^{ab}$</td>
<td>1147 ± 31$^{abc}$</td>
</tr>
<tr>
<td>S1PR1</td>
<td>100 ± 12</td>
<td>114 ± 9</td>
<td>386 ± 38$^{ab}$</td>
<td>164 ± 40$^{b}$</td>
<td>96 ± 14$^{bc}$</td>
<td>283 ± 15$^a$</td>
<td>206 ± 58$^{ab}$</td>
<td>125 ± 20$^{bc}$</td>
</tr>
<tr>
<td>S1PR2</td>
<td>100 ± 9</td>
<td>82 ± 10</td>
<td>13 ± 5$^a$</td>
<td>44 ± 1$^{abc}$</td>
<td>92 ± 6$^{abc}$</td>
<td>6 ± 2$^a$</td>
<td>51 ± 3$^{abc}$</td>
<td>66 ± 8$^{abc}$</td>
</tr>
<tr>
<td>S1PR3</td>
<td>100 ± 11</td>
<td>118 ± 7</td>
<td>352 ± 22$^{ab}$</td>
<td>155 ± 4$^{ab}$</td>
<td>145 ± 7$^{ab}$</td>
<td>219 ± 31$^a$</td>
<td>115 ± 2$^b$</td>
<td>139 ± 7$^{ab}$</td>
</tr>
<tr>
<td>S1PL</td>
<td>100 ± 6</td>
<td>92 ± 13</td>
<td>30 ± 2$^a$</td>
<td>63 ± 3$^{ab}$</td>
<td>83 ± 5$^{bc}$</td>
<td>23 ± 3$^a$</td>
<td>48 ± 3$^{ab}$</td>
<td>63 ± 3$^{ab}$</td>
</tr>
<tr>
<td>ASMase</td>
<td>100 ± 6</td>
<td>123 ± 3</td>
<td>225 ± 14$^{a}$</td>
<td>122 ± 12$^b$</td>
<td>77 ± 15$^{bc}$</td>
<td>133 ± 23</td>
<td>112 ± 8</td>
<td>80 ± 4</td>
</tr>
</tbody>
</table>

Mice in treatment groups received CCl$_4$ at a dose of 5 µL/g body weight twice a week for 4 weeks or 6 weeks (w). Melatonin 5 mg/kg (5Mel) or 10 mg/kg (10Mel) was given to mice receiving CCl$_4$ or vehicle (Control+Mel). mRNA was determined by real-time PCR analysis and the appropriate primers. Relative changes in gene expression levels were determined using the $2^{ΔΔCt}$ method. Abbreviations: α-SMA, α-smooth muscle actin; ASMase, acid sphingomyelinase; Col I, collagen I; Mel, melatonin; S1PL, sphingosine 1-phosphate lyase; S1PRs, sphingosine 1-phosphate receptors; SphK1, sphingosine kinase 1; TGF-β, transforming growth factor-β. Values are expressed as means ± standard error of the mean. $^a$P < 0.05, compared with control. $^b$P < 0.05, compared with CCl$_4$ same period. $^c$P < 0.05, compared with 5Mel same period.
TABLE 3 Effect of treatment with melatonin on messenger RNA levels of genes related to fibrosis and sphingolipid metabolism in LX2 cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>Control+Mel</th>
<th>TGF-β</th>
<th>TGF-β+100Mel</th>
<th>TGF-β+500Mel</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SMA</td>
<td>100 ± 5</td>
<td>84 ± 8</td>
<td>285 ± 21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>175 ± 4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>130 ± 6&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGF-β</td>
<td>100 ± 7</td>
<td>113 ± 8</td>
<td>168 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>110 ± 5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>67 ± 7&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Col I</td>
<td>100 ± 6</td>
<td>82 ± 5</td>
<td>395 ± 17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>271 ± 11&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>235 ± 12&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>SphK1</td>
<td>100 ± 7</td>
<td>126 ± 8</td>
<td>505 ± 35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>400 ± 24&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>305 ± 22&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>S1PR1</td>
<td>100 ± 6</td>
<td>84 ±10</td>
<td>224 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>152 ± 6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>130 ± 4&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>S1PR2</td>
<td>100 ± 4</td>
<td>90 ± 6</td>
<td>25 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46 ± 5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>86 ± 5&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>S1PR3</td>
<td>100 ± 6</td>
<td>91 ± 6</td>
<td>257 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>183 ± 9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>143 ± 3&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>S1PL</td>
<td>100 ± 8</td>
<td>84 ± 7</td>
<td>30 ± 10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53 ± 6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>78 ± 9&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>ASMase</td>
<td>100 ±6</td>
<td>89 ± 5</td>
<td>210 ± 13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>162 ± 14&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>83 ± 2&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Activated TGF-β cells were incubated for 24 h with 2 ng/mL TGF-β and melatonin at a dose 100 mM (100Mel) or 500 mM (500Mel). mRNA was determined by real-time PCR analysis and the appropriate primers. Relative changes in gene expression levels were determined using the 2<sup>-ΔΔct</sup> method. Abbreviations: α-SMA, α-smooth muscle actin; ASMase, acidic sphingomyelinase; Col I, collagen I; Mel, melatonin; S1PL, sphingosine 1-phosphate lyase; S1PRs, sphingosine 1-phosphate receptors; SphK1, sphingosine kinase 1; TGF-β, transforming growth factor-β. Values are expressed as means ± standard error of the mean. <sup>a</sup>P < 0.05, compared with control. <sup>b</sup>P < 0.05, compared with TGF-β. <sup>c</sup>P < 0.05, compared with 100Mel.
Fig 1
164x189mm (150 x 150 DPI)
Fig 2
190x150mm (100 x 100 DPI)
Fig 3
284x180mm (150 x 150 DPI)
Fig 4
176x143mm (150 x 150 DPI)