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ORIGINAL ARTICLE

The Effects of Tofacitinib-Mediated Janus Kinase/Signal Transducers and Activators of the Transcription Signal Pathway Inhibition on Collagen Biosynthesis in Hepatic and Skin Fibroblast Cell Culture

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ABSTRACT

Objectives: This study aims to investigate the effects of Janus kinase/signal transducers and activators of the transcription (JAK/STAT) pathway inhibition on collagen biosynthesis in fibroblast cell culture by tofacitinib.

Materials and methods: BJ-CRL-1474® (skin) and BRL3A® (hepatic) fibroblast cell cultures were proliferated in a suitable medium. Tofacitinib was administered to fibroblast cells proliferating in 96-well flasks at concentrations of 25, 50, 100, 200, 400, and 800 nM. Tissue inhibitor of metalloproteinase-1 (TIMP-1), matrix metalloproteinase-3 (MMP-3), transforming growth factor beta 1 (TGF-β1), and hydroxyproline levels were measured using the enzyme-linked immunosorbent assay method.

Results: Tofacitinib showed cytotoxic effect on skin and liver cell culture. The cytotoxic effect of tofacitinib started at 100 nM ($p<0.05$). The highest effect was obtained at 800 nM. The time-dependent cytotoxic effect of tofacitinib was significantly higher at all concentrations after 72 hours than at 24 and 48 hours ($p<0.05$). The level of TGF-β1 was significantly lower even at a tofacitinib concentration of 25 nM ($p<0.05$). There were significant decreases in MMP-3, TIMP-1, and hydroxyproline levels after tofacitinib administration ($p<0.05$).

Conclusion: Tofacitinib inhibited fibroblast cell proliferation in a concentration-dependent manner in a fibroblast cell culture. However, further extensive animal and human studies are necessary to determine the clinical significance of this effect.

Keywords: Collagen, fibroblast cell culture, Janus kinase/signal transducers and activators of the transcription, tofacitinib.

The pathogenesis of fibrosis and the diseases that manifest with fibrosis (e.g., hepatic fibrosis, scleroderma, diabetic nephropathy, rheumatoid arthritis (RA), idiopathic pulmonary fibrosis, and arteriosclerosis) are characterized by excessive synthesis and deposition of the extracellular matrix and impairment of normal tissue function. Fibroblasts are the most important cells for the homeostasis of tissue repair, with many pro- and anti-fibrotic cytokines, proteins, and enzymes also contributing to maintaining this balance.[1] The most important mediator of fibrotic response is transforming growth factor beta (TGF-β), which stimulates collagen type-1 synthesis and extracellular matrix (ECM) production. The TGF-β receptor has intracellular tyrosine kinase activity,[1] and its activation is also regulated by intracellular Smad pathways. The TGF-β/Smad signaling pathway is controlled by the mitogen-activated protein kinase cascade.[2]

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The most important actors in inter- and intracellular communication are the cell surface receptors. When a signaling molecule binds to the receptor, this initiates several intracellular responses. It was reported that Janus kinase/signal transducers and activators of the transcription (JAK/STAT) pathway directly affects transcription factors, including tyrosine kinase proteins and transcription factors.[3] There are four different known types of JAK enzyme, which are located at the cell membrane: JAK1, JAK2, JAK3, and tyrosine kinase 2 (Tyk2).[4] On the other hand, seven different types have been defined for signal transducers and activators of transcription (STATs), which are located in the cytoplasm and activated by JAK: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6.[5] The abnormal activation of the JAK/STAT signaling pathway was associated with many hematologic malignancies (acute myeloid leukemia and myelodysplastic syndromes).[6] As a result of its interaction with class II cytokine receptor-related cytokines, such as interleukin (IL)-2 and IL-4, the JAK1 protein is responsible for metastasis, tumor progression, and drug resistance.[7] Some mutations (V617F) in the JAK2 protein are associated with hematologic proliferative disorders.[6] Tyk2 is associated with proinflammatory cytokines, such as IL-6, IL-10, and IL-12. The JAK3 protein is mostly found in hematopoietic cells and activated by cytokines, such as IL-2, IL-4, IL-9, and IL-17, which all contain the common gamma (δ) domain. JAK3 has significant effects on the development and migration of T lymphocytes. JAK3 has also been shown to be effective in colon cancer migration, renal fibrosis, and myocardial ischemia.[8]

The treatment options for diseases with increased collagen production, such as scleroderma and liver fibrosis, are limited. Studies on the drugs that inhibit excessive collagen synthesis, and therefore prevent fibrosis, are ongoing. Currently, some targeted therapies are available.[9]

Tofacitinib (CP 690.550) is a disease-modifying, targeted, and immunomodulatory drug that has been orally administered in the treatment of RA in recent years.[10] Tofacitinib acts by blocking JAK1 and JAK3. Consequently, signalization mediated by these cytokines, and particularly by IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, is inhibited. However, there is as yet insufficient data on the use of tofacitinib in inflammatory rheumatic disorders.[11] In this study, we aimed to investigate the effects of JAK/STAT pathway inhibition on collagen biosynthesis in fibroblast cell culture by tofacitinib.

MATERIALS AND METHODS

This study was performed in the cell culture laboratory of Sivas Cumhuriyet University Faculty of Medicine between September 2017 and March 2018. The study protocol was approved by the Sivas Cumhuriyet University Faculty of Medicine Ethics Committee (approval dated 25/11/2016 with number 2016-11/21). The study was conducted in accordance with the principles of the Declaration of Helsinki.

The study consisted of three main steps: cell proliferation, viability and proliferation assays, and enzyme-linked immunosorbent assay (ELISA) measurements. In the first stage, the cell line was grown under appropriate conditions and medium. The cells were plated in 96-well plates, each containing 100 µL of cells per well. These cell wells were grouped as control cells and cells exposed to various drug doses. Each group had its own control group.

BJ-CRL-1474 CRL-2522® (skin, human-derived cell line) and BRL3A® (hepatic, rat derived cell line) were purchased from American Type Culture Collection. These fibroblast cell cultures were thawed for passaging, then incubated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, L-glutamine-penicillin-streptomycin solution, phosphate-buffered saline, Roswell Park Memorial Institute 1,640 medium, and L-glutamine at 37°C in a carbon dioxide (CO_2) incubator until a single cell was formed. After dilution, inoculation was performed so that each well of the 96-well plates contained 100 µL of cells. These plates were then incubated in a CO_2 incubator at 37°C after dosing.

The XTT method was used for the cell viability and proliferation assay. This method is based on the principle of the cleavage of XTT, a tetrazolium salt, into orange formazan components by the metabolically active cells. The resulting dye is soluble in water, and the dye can be quantitated at the given wavelengths by a spectrophotometer. In our study, a 96-well plate was inoculated, and a sufficient number of cells was obtained after

incubation. Tofacitinib (Xeljanz®) was used after dissolving in sterile distilled water. Tofacitinib was administered to fibroblast cells proliferating in 96-well flasks at the concentrations of 25, 50, 100, 200, 400, and 800 nM, respectively. Cell viability and quantity were read by a "micro reader" spectrophotometer and the data were saved.

The other cytokine levels that we evaluated in our study, in addition to fibroblast viability, were tissue inhibitor of metalloproteinase-1 (TIMP-1), matrix metalloproteinase-3 (MMP-3), TGF-β1, and hydroxyproline levels. In the ELISA method, first, the samples (not the standards) were acidified and then neutralized to ensure they are in the immunoreactive form before measuring the above-mentioned substances. For cell culture supernatants, 20 µL of 1N hydrochloric acid was added for each 100 µL sample and incubated at room temperature for 10 minutes. Then, the mixture was neutralized with 20 µL of 1N sodium hydroxide. After neutralization and dilution in all wells, the plates were incubated at room temperature for a sufficient amount of time and readings were obtained at 450 nM wavelength. The data were saved.

Statistical analysis

The IBM SPSS version 25.0 statistical program (IBM Corp., Armonk, NY, USA) was used for the analysis of our data. The normality of the data was checked using the Kolmogorov-Smirnov statistical test. Data obtained as absorbance were presented as mean and standard deviation (mean±SD). ANOVA test was used to evaluate the significance of the difference between means. If one or all of the assumptions were not met, the Mann Whitney U test was used for two independent groups, the Wilcoxon test for two conjugate groups, and the Kruskal Wallis test for more than two independent groups. The p-value smaller than 0.05 was considered statistically significant.

RESULTS

Tofacitinib had concentration-dependent cytotoxic activity. This effect started at 100 nM ($p<0.05$), and the highest antiproliferative effect was observed at 800 nM (Figure 1). The activities at 100 and 200 nM were not significantly different ($p>0.05$); however, the activities at 400 and 800 nM were higher ($p<0.05$) compared to the activities at 100 and 200 nM (Table 1). This effect was similar in both the hepatic and skin fibroblast cells. The difference between the effects on hepatic and skin fibroblast cells was 5%, which was not statistically significant ($p>0.05$) (Figure 1).

The cytotoxic activity of tofacitinib increased with increasing exposure time. The cytotoxic effects of tofacitinib at 50 and 100 nM at 48 and 72 hours were statistically significantly higher than at 24 hours ($p<0.05$). The cytotoxic effects at 200, 400, and 800 nM were significantly higher at 72 hours than at 24 and 48 hours ($p<0.05$) (Table 1). The mentioned effect was similar in

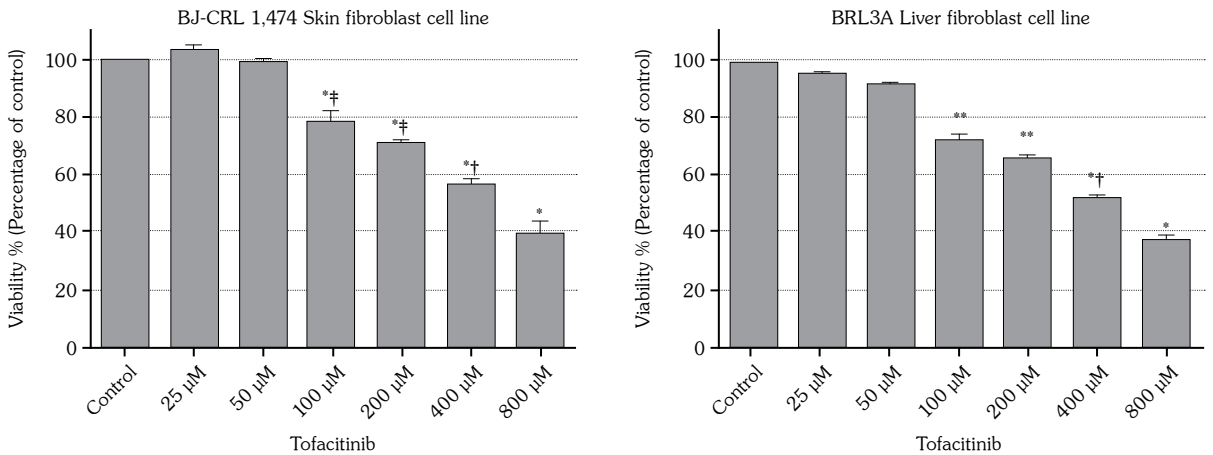

Figure 1. Evaluation of cytotoxic effect of tofacitinib on fibroblast cell lines by concentration.
* $p<0.05$; ‡ $p<0.05$; ** $p<0.05$.

Table 1. Evaluation of cytotoxic effect of tofacitinib on fibroblast cell lines by time and concentration

Tofacitinib	Viability (Percentage of the control)			*p*
	24 hours	48 hours	72 hours	
	Mean±SD	Mean±SD	Mean±SD	
Control	100	100	100	-
25 µM	103.0±7.6	96.0±4.5	105.0±3.2	-
50 µM	99.6±6.8*	89.7±6.1**	124.5±5.6**	<0.05
100 µM	78.9±8.2*	73.1±5.6**	98.6±7.6**	<0.05
200 µM	71.8±5.9*	47.4±48*	89.8±5.3**	<0.05
400 µM	56.8±6.4*	25.4±4.2*	71.0±4.1**	<0.05
800 µM	39.7±7.6*	14.8±4.1*	49.6±48**	<0.05

SD: Standard deviation; * p<0.05; ** p<0.05.

both the hepatic and skin fibroblast cells. The difference between the effects on hepatic and skin fibroblast cells was 5%, which was not significant (p>0.05) (Figure 2).

Tofacitinib resulted in a statistically significant decrease in TGF-β1 level starting from concentrations as low as 25 nM (p<0.05). The decreases at 100 and 200 nM were higher than at 25 and 50 nM (p<0.05). The decreases in TGF-β1 level at tofacitinib concentrations of 400 and 800 nM were more than at 100 and 200 nM (p<0.05). The TGF-β1 levels did not differ when comparing the 400 and 800 nM concentrations (p>0.05). The effect of tofacitinib on MMP-3 level started at a concentration of 100 nM (p<0.05). The differences between the 100, 200, and 400 nM concentrations in terms of the decrease in MMP-3 level were not significant (p>0.05). The decrease in MMP-3 level at a tofacitinib concentration of 800 nM was significantly more than at 100 and 200 nM (p<0.05) (Table 2). Tofacitinib resulted in a statistically significant decrease in TIMP-1 level starting at the 100 nM concentration (p<0.05). The differences between the 200, 400, and 800 nM concentrations in terms of the decrease in TIMP-1 level were not statistically significant (p>0.05). However, the decrease in TIMP-1 level was higher at these

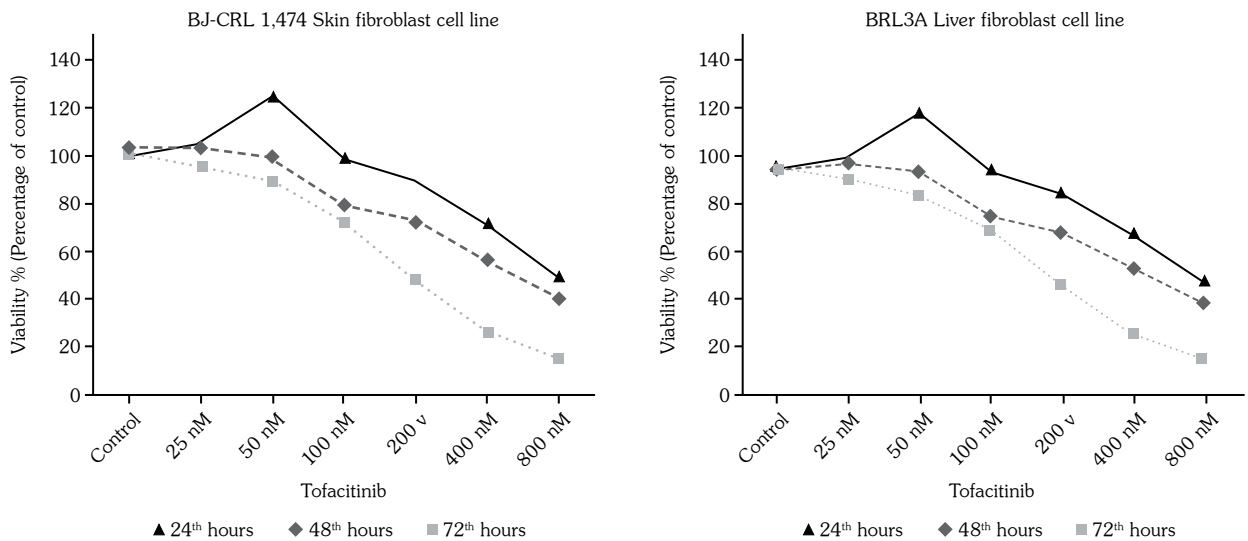

Figure 2. Evaluation of cytotoxic effect of tofacitinib on fibroblast cell lines by time.

Table 2. Effect of tofacitinib on transforming growth factor beta 1, matrix metalloproteinase-3, tissue inhibitor of metalloproteinase-1 and hydroxyproline levels

	TGF-1β (pg/mg)	MMP (units/mL)	TIMP-1 (pg/mL)	OH-Proline (Multiple of the control)
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Control	23.4±2.6	12.9±2.6	860.5±23.3	0.91±0.13
25 µM	18.6±3.5*	11.3±2.8	840.2±21.9	0.91±0.13
50 µM	17.2±1.9*	10.8±1.9	800.0±20.3	0.85±0.08
100 µM	11.3±2.1**	4.2±1.1*	621.3±25.6*	0.61±0.07
200 µM	10.6±1.8**	3.8±0.9*	324.3±17.5**	0.52±0.05
400 µM	3.6±0.9†	3.6±0.6*	321.9±16.2**	0.48±0.03
800 µM	2.1±0.7†	1.2±0.4**	310.2±18.8**	0.12±0.02

TGF-β: Transforming growth factor beta; MMP: Matrix metalloproteinase; TIMP-1: Tissue inhibitor of metalloproteinase-1; OH: Hydroxy; SD: Standard deviation; * $p<0.05$; ** $p<0.05$; † $p<0.05$.

three concentrations than at 100 nM ($p<0.05$). Tofacitinib resulted in a statistically significant decrease in hydroxyproline level starting from the 100 nM concentration ($p<0.05$). The MMP-3 levels did not differ between the 100, 200, and 400 nM concentrations ($p>0.05$). The decrease in hydroxyproline level at a tofacitinib concentration of 800 nM was statistically significant compared to the decrease at all the other tested tofacitinib concentrations ($p<0.05$) (Table 2).

DISCUSSION

In this study, we have shown that tofacitinib inhibits cell proliferation and collagen synthesis in skin and liver fibroblast cell culture. This effect was both dose- and concentration-dependent. Tofacitinib, a selective JAK 1/3 inhibitor, is currently used in the treatment of RA. The effect of JAK/STAT pathway inhibition on collagen synthesis after fibroblast activation is unclear. Since the principal causes of mortality in these diseases are pulmonary, liver, pancreatic, and renal fibrosis, a majority of the studies in the literature have focused on these organs and their cells. The number of studies showing that the JAK/STAT pathway has a role in pulmonary fibrosis is limited. Pedroza et al.[12] have demonstrated that STAT3 contributed to the development of pulmonary fibrosis via epithelial damage and fibroblast-myofibroblast differentiation. In another study concerning IL-27, which inhibits JAK/STAT and TGF-β1/Smad, Dong et al.[13] have shown that IL-27 caused decreased fibroblast activation and, therefore, decreased collagen synthesis in pulmonary fibrosis. Masamune et al.[14] have stimulated pancreatic satellite cells collected from mouse pancreatic cells with platelet-derived growth factor and shown that the JAK/STAT pathway was also activated in addition to the Raf/MEK/ERK pathway and that the former played a role in the development of pancreatic fibrosis.

We have revealed that tofacitinib reduced cell proliferation and viability depending on the concentration in a fibroblast cell culture (Figure 1). This result supports the notion that fibroblast viability decreases with the inhibition of the JAK/STAT pathway, which is in line with other studies. Similarly, Gu et al.[15] have reported that SHR0302, a JAK inhibitor, induces a decrease in hepatic fibrosis in hepatic satellite cells. In another study investigating the effect of tofacitinib on renal cells with renal fibrosis, Yan et al.[16] have found that tofacitinib caused a decrease in myofibroblast transformation, synthesis of extracellular matrix components, and fibroblast development.

One of the important points here is the fact that the effect is dose-dependent (i.e., increases with increasing dose). In our study, the cytotoxic effect has increased with increasing exposure time (Figure 2). The highest cytotoxic effect was seen at 72 hours. Similarly, other studies have reported that the effect of JAK inhibitors increases over time.[17] Based on our data, the effect of tofacitinib increases with increasing dose and exposure time.

Transforming growth factor beta-1 is the main stimulant of fibroblasts and is also secreted from fibroblasts. This effect of TGF-β1 has been shown in numerous studies.[18] In a study conducted on both mouse and human hepatic cell cultures, Dropmann et al.[19] have found abnormally higher levels of TGF-β1 and TGF-β2 in liver cells in both liver fibrosis and liver cancer. These authors have even shown that TGF-β2 stimulation led to an increase in TGF-β1 receptors.

In another study, anti-inflammatory and antimitotic drugs were administered to mouse cochlear cells both *in vitro* and *in vivo*, preventing fibrosis that develops after cochlear implantation.[20] These authors have found that the TGF-β1 and IL-1β levels were high before treatment, but decreased after the administration of the drugs.[20] In another study concerning bleomycin-induced pulmonary fibrosis, fluorofenidone was administered to investigate its anti-fibrotic effect. Using an animal model, Wu et al.[21] have shown that collagen production and TGF-β1 levels were decreased after administering fluorofenidone. As indicated in many studies, TGF-β1 has an important role in the fibrotic response. However, as mentioned above, TGF-β1 uses intracellular Smad pathways. Similar to the other studies, in our study, we have demonstrated the effect that leads to decreased fibroblast activity and, therefore, a drop in TGF-β1 levels. However, it is difficult to claim that this effect (inhibition of TGF-β1) is a direct effect of JAK-kinase inhibition by tofacitinib. This is because Yan et al.[16] have shown that the administration of tofacitinib decreased fibrosis and collagen synthesis without affecting TGF-β1 levels in renal cell culture in renal fibrosis. TGF-β1 is both the cause and result of collagen synthesis. In our study, we have revealed that tofacitinib decreased TGF-β1 levels and we believe that tofacitinib decreased the TGF-β1 level by reducing fibroblast proliferation.

Matrix metalloproteinases are a crowded family of enzymes that are responsible for collagen catabolism.[22] Some MMPs can stimulate cell proliferation. There are many studies concerning MMPs in the literature. Gao et al.[23] have administered tofacitinib to synovial cells obtained from patients with psoriatic arthritis and found that tofacitinib statistically significant inhibited STAT3 and STAT1, in addition to stimulating suppressor of cytokine signaling 3 (SOCS3) and protein inhibitor of activated STAT3 (PIAS3). In the same study, the authors have also observed decreased IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), MMP-9/MMP-2, and MMP-3 levels, and MMP-3/TIMP-3 ratio.[23] Glazewska et al.[24] have measured serum MMP-2, MMP-3, MMP-9, MMP-12, TIMP-2, and TIMP-3 levels in 49 patients with psoriasis using ELISA and conducted a receiver operating characteristic analysis. These authors have detected higher levels of MMP-2, MMP-3, MMP-9, TIMP-3, and lower levels of MMP-12.24 Flisiak et al.[25] have conducted a study on psoriasis patients and found higher serum MMP-1 and TIMP-1 levels in comparison to the control group. Our study has also shown that MMP-3 and TIMP-1 levels were lower than the control group after tofacitinib administration, which is in line with the literature. However, some studies have reported low levels of MMP-3. In a study by Zhou et al.,[26] IWR-1, a WNT/β-catenin pathway inhibitor, was administered to the fibroblasts obtained from normal and keloid tissue. These authors have reported increased levels of MMP-1, MMP-3, and MMP-13; the enzymes responsible for collagen catabolism increased after administering the drug.[26] As mentioned above, the MMP family consists of many enzymes. Studies have shown that each enzyme in this family could have different properties. This is why some MMP types increase, whereas others decrease in the same disease. Because of a lack of consensus in the literature, further extensive studies are required on this subject.

Furthermore, hydroxyproline is a molecule that has a role in collagen synthesis and is included in the collagen structure.[27] The level of hydroxyproline increases in parallel with the increase in collagen synthesis. In a study by Akimoto et al.,[28] it was shown that the administration of heparin caused a decrease in hydroxyproline level in hepatic cell culture. Another study was conducted on corneal keratinocytes. In this study, Stachon et al.[29] have collected cells from the corneas of patients with keratoconus and normal subjects, wherein they measured various parameters and hydroxyproline levels in cell culture. Compared to the control group, these authors have found that hydroxyproline levels were lower in the cells with keratoconus (i.e., cells with impaired

collagen synthesis).[29] In our study, we have also found that hydroxyproline levels were lower than the control group. Obtaining a lower level of hydroxyproline after administering tofacitinib supports the notion that it has a role in decreasing collagen synthesis.

One of the limitations of our study is that it was performed in healthy cell cultures. The effects on diseased cell cultures remain unknown. Another limitation is that the study was entirely *in vitro*. Although our findings are valuable and remarkable, these trends might not be reproducible in diseased patients, such as those with scleroderma.

In conclusion, we have shown that tofacitinib decreases fibroblast proliferation and viability in a fibroblast cell culture (both skin and hepatic) and that this effect increases with increasing dose and exposure time. Similarly, the levels of TGF-β1, MMP-3, TIMP-1, and hydroxyproline (markers of fibroblast activation) were lower after administering tofacitinib relative to the control condition. Based on these promising findings, more comprehensive and larger studies that include animal models and clinical studies are necessary to reveal the clinical significance of tofacitinib in disease treatment.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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