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Dimethyl fumarate protection against collagen II degradation

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ABSTRACT

Degradation of collagen type II caused by pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) is one of the major pathological characteristics of osteoarthritis (OA). Dimethyl fumarate (DMF) is a medication approved by the US Food and Drug Administration (FDA) as an oral multiple sclerosis (MS) therapy. In this study, we found that DMF ameliorated collagen type II degradation by inhibiting the expression of MMP-1, MMP-3, and MMP-13 caused by TNF-α. Mechanistically, DMF attenuated MMPs expression by suppressing JAK/STAT3 pathway. These findings imply that DMF treatment might be a potential therapeutic strategy for chondroprotective therapy.

1. Introduction

Osteoarthritis (OA), a major cause of disability, is a degenerative joint disease [1]. Progressive loss of articular cartilage is one of the major pathological characteristics of OA. Pro-inflammatory cytokines including interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) disturb the metabolism balance of extracellular matrix (ECM) in chondrocytes, mainly composed by collagen type II. The activation of degradative enzymes such as the matrix metalloproteinases (MMPs) plays a pivotal role in the loss and degradation of proteoglycans and collagen the destruction of articular cartilage in patients who are suffering from osteoarthritis [2]. It has been reported that MMP-3 degrades proteoglycans and activates procollagenase in articular cartilage [3]. MMP-13 mainly targets type II collagen in cartilage for degradation [4]. Tumor necrosis factor alpha (TNF-α) was also involved in the progression of OA via up-regulating MMPs expression or triggering inflammatory process [5]. Currently, no treatment or drug is available to retard its progression. Thus, it is believed that disturbing the effects of MMPs or pro-inflammatory cytokine activity may reduce the cartilage degradation in OA.

Dimethyl fumarate (DMF) is a methyl ester of fumaric acid approved by the FDA as an oral multiple sclerosis (MS) therapy [6]. Multiple lines of evidence have demonstrated the detoxification and anti-inflammatory capabilities of DMF, which reduces with the production and release of inflammatory molecules, such as cytokines (TNF-α, IL-1β, and IL-6) and NO, and elevates the production of detoxification enzymes such as reduced-form nicotinamide adenine dinucleotide phosphate quinone reductase 1 and/or glutathione [7,8]. DMF also inhibits the expression of VCAM-1 in endothelial cells [9] and activates nuclear factor erythroid 2-related factor (Nrf2), a transcription factor which reduces the free radicals, prevents synthesis of reactive nitrogen species (RNS), and thus protects the CNS from degeneration and axonal loss [10]. However, little information regarding to the effects of DMF on MMPs and subsequent collagen type II has been reported before.

2. Materials and methods

2.1. Primary cell culture

The study was approved by the local ethics committee and guided by the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. All of participants have signed the written informed consent. Normal specimens of knee joint cartilage were obtained from normal femoral heads of patients undergoing prosthetic replacement due to fracture of the femoral neck or distal femoral tumor. Briefly, cartilage tissue was digested in 0.25% trypsin (Sigma–Aldrich, USA) at 37 °C for 30 min followed by digestion in 3 mg/ml collagenase type I (Sigma–Aldrich, USA) in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) (Gibco, UK) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco, UK), 2 mM glutamine (Gibco, UK), 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco, UK), 2.5 mg/ml amphotericin B (Sigma–Aldrich, USA), and 50 mg/ml ascorbic acid (Sigma–Aldrich, USA) (complete media) at 37 °C for 16 h. Isolated chondrocytes were cultured in monolayer in

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complete media in a humidified atmosphere of 5% CO_2 at 37 °C and harvested at passage 2.

2.2. RNA isolation and quantitative real time PCR

Chondrocytes were induced with TNF-α (10 ng/mL) with or without DMF (0, 1, 10, 50 μM) for 24 h. Chondrocytes were collected and total RNA was extracted by using TRIzol reagent (Invitrogen, USA). The concentration and purity of extracted total RNA were determined by using the absorbance at 260 and 280 nm. cDNA was synthesized by reverse transcription of RNA(2 μg) using Superscript First-Strand Synthesis System (Invitrogen, USA). Synthesized cDNA was used for PCR amplification using specific primers, a core kit for SYBR Green (Applied Biosystems, USA) in a Step One Real-Time PCR System (Applied Biosystems, USA). The following primers were used in this study: for

MMP-1: forward, 5′-GGGAATAAGTACTGGGCTGTTCAG-3′; reverse, 5′-CCTCAGAAAGAGCAGCATCGATATG; for MMP-3, forward, 5′- CCTCTATGGACCTCCCACAGAATC-3′; reverse, 5′-GGTGCT GACTGCATCGAAGGACAAA-3′; for

MMP-13: forward, 5′-CTGGCCTGCTGGCTCATGCTT-3′; reverse, 5′-CCTCAGAAAGAGCAGCATCGATATG-3′; for actin: forward, 5′-CACCTTCTACAATGAGCTGCGTGT-3′; reverse, 5′-CACAGCCTGGATA GCAACGTACA-3′.

2.3. MMP-1, 3 and -13 enzyme linked immunosorbent assays (ELISAs)

After indicated treatment, the culture medium was assessed by the enzyme-linked immunosorbent assay (ELISA). Commercial ELISA kits (R&D Systems, Abingdon, UK) were used according to the manufacturer's instructions to measure MMP-1, MMP-3 protein expression (ng/ml) and MMP-13 protein expression (pg/ml).

2.4. Western blot analysis

After indicated treatments, chondrocytes were collected and washed with ice-cold phosphate-buffered saline (PBS). The protein was extracted and protein concentration was evaluated by the Bicinchoninic Acid (BCA) Protein Assay kit. 20 μg of total extracted proteins were subjected to 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels, followed by transference onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocked with 5% dry fat milk in Tris-buffered saline plus 0.05% Tween-20 (TBST) for 1 h at room temperature (RT), the PVDF membranes were sequentially probed with primary antibodies overnight at 4 °C and the secondary antibody linked with horse radish peroxidase (HRP) (Pierce Biotechnology, USA) at room temperature (RT) for 1 h. Blots were developed with enhanced chemiluminescence (ECL) kit (Pierce Biotechnology).

The membranes were detected using Enhanced Chemiluminescence (ECL) reagents (GE Healthcare, USA) and exposed to X-ray films.

2.5. Statistical analysis

All data were expressed as arithmetic mean ± standard deviation (SD). The data were analyzed by one-way ANOVA. Differences were considered significant when P was less than 0.05.

3. Results

3.1. Effects of DMF on MMP-1, MMP-3, and MMP-13 gene expression

We analyzed the effects of DMF on MMP-1, MMP-3 and MMP-13 gene expression by quantitative real-time PCR. Chondrocytes un-stimulated with TNF-α showed low gene expression of MMP-1, MMP-3 and MMP-13, TNF-α significantly induced the gene expression of MMP-1, MMP-3 and MMP-13 and this induction was inhibited by DMF in a dose dependent manner (1, 10, 50 μM) (Fig. 1).

3.2. Effects of DMF on MMP-1, MMP-3 and MMP-13 protein levels

We next investigated whether DMF affected the TNF-α induced-protein levels of MMP-1, MMP-3 and MMP-13 in chondrocytes. As shown in Fig. 2, DMF significantly down-regulated the augmented protein levels of MMP-1, MMP-3 and MMP-13 which were induced by TNF-α in a dose dependent manner (1, 10, 50 μM).

3.3. Effects of DMF on collagen II degradation

The degradation of collagen II by MMPs has been a critical step leading to damage of the joints in OA patients. By Western blotting, the results showed that TNF-α decreased collagen II levels, which was ameliorated by treatment with DMF in a dose dependent manner (see Fig. 3).

3.4. DMF inhibits the activation of STAT3

JAK2/STATs pathways have been reported to be involved in activation of MMPs.

Among the seven STAT members downstream of JAK signaling pathways, STAT3 appears to be one of the most important regulators. A recent study has shown that the JAK2/STAT3 pathway is involved in the collagen II reduction in chondrocytes [11]. The

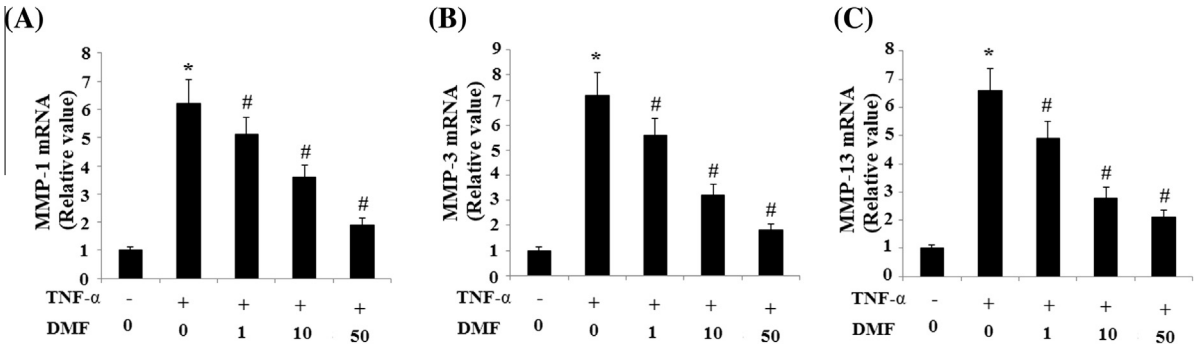

Fig. 1. Effects of dimethyl fumarate (DMF) on MMP-1, MMP-3, and MMP-13 gene expression. Human chondrocytes were treated with 10 μg/ml TNF-α in the presence or absence of DMF (0, 1, 10, 50 μM) for 24 h, and total RNA was assessed for MMPs mRNA expression by real time PCR. (A) MMP-1; (B) MMP-3; (C) MMP-13 (*P < 0.01 vs. nontreated control, n = 4; #P < 0.01 vs. TNF-α treated group).

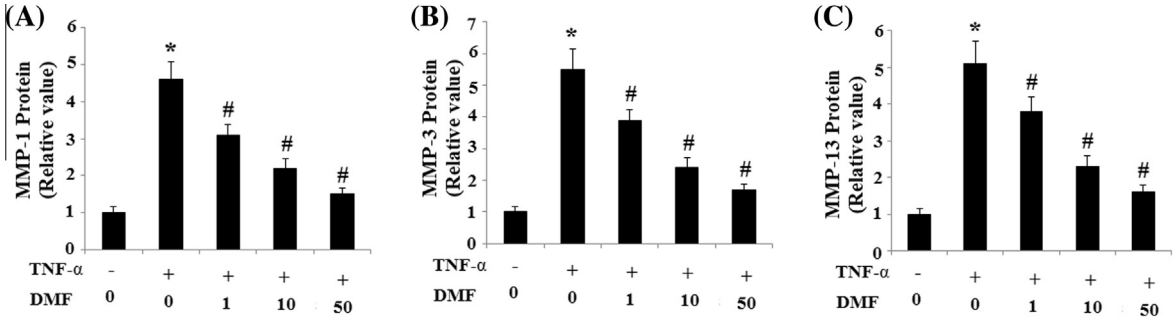

Fig. 2. Effects of Dimethyl fumarate (DMF) on MMP-1, MMP-3, and MMP-13 protein expression. Human chondrocytes were treated with 10 μg/ml TNF-α in the presence or absence of DMF (0, 1, 10, 50 μM) for 24 h, and MMPs expression was determined by the Elisa assay. (A) MMP-1; (B) MMP-3; (C) MMP-13 ($^{*}P < 0.01$ vs. non-treated control, $n = 4$; $^{\#}P < 0.01$ vs. TNF-α treated group).

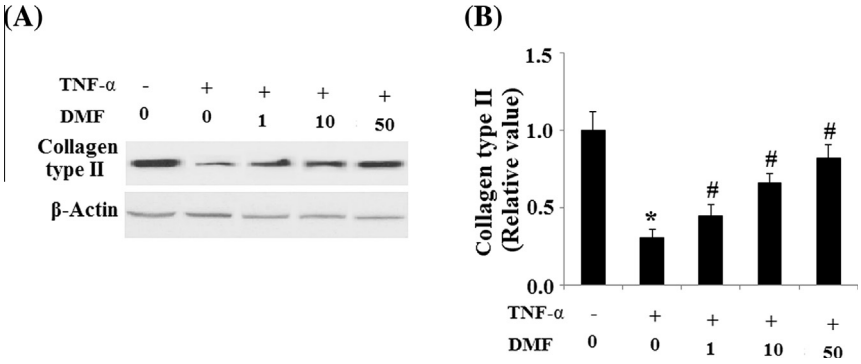

Fig. 3. Normal human chondrocytes were treated with TNF-α with or without various doses of DMF or the solvent, DMSO, for 24 h. The levels of collagen type II in total cell lysates were measured by Western blot analysis ($P < 0.01$ vs. control group; $^{\#}P < 0.01$ vs. TNF-α treated group).

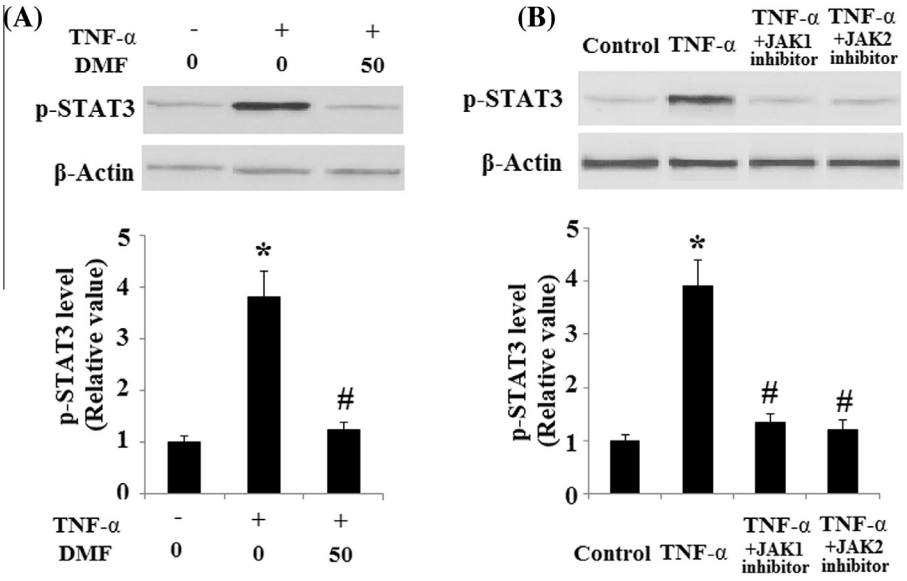

Fig. 4. DMF prevents the activation of JAK2/STAT3. Human chondrocytes were treated with TNF-α with or without various doses of DMF or the solvent, DMSO, for 24 h. (A) The expression level of phosphorylated STAT-3; (B) pre-treatment with either JAK1 or JAK2 inhibitor efficiently reduced AGE-induced STAT3 tyrosine phosphorylation ($P < 0.01$ vs. control group; $^{\#}P < 0.01$ vs. TNF-α treated group).

results indicated that TNF-α significantly induced expression of the tyrosine phosphorylated form of STAT3 24 h after stimulation. Notably, the activation was attenuated by treatment with DMF (Fig. 4A). Meanwhile, both JAK1 and JAK2 inhibitors successfully blocked TNF-α-induced STAT3 phosphorylation and DNA-binding activity (Fig. 4B).

4. Discussion

TNF-α is a pivotal cytokine in many inflammatory disorders including osteoarthritis. In particular, TNF-α induces MMPs, TNF-α, IL-8, and complement factor involved in the cartilage degradation *in vitro* [12]. It suppressed proteoglycan production and

stimulated collagen type II degradation. In this study, we found that DMF ameliorated collagen type II degradation by inhibiting the expression of MMP-1, MMP-3, and MMP-13. TNF-α has been reported to induce catabolic genes, such as matrix metalloproteinase-1 (MMP-1), MMP-3, MMP-9, and MMP-13 [13]. MMP-3 protein was reported to be expressed in the synovium and the surface of cartilage in the knee joints and in pannus-like tissue of patients with OA [14]. In addition to collagen type II, MMP-13 also degrades proteoglycan, types IV and type IX collagen, osteonectin and perlecan in cartilage. It has been suggested that inhibition of MMPs expression, especially MMP-1, MMP-3, MMP-13, may show some beneficial effects of chondroprotection on the pathological conditions such as OA [15].

Activation of JAK/STAT pathway or STAT signaling has been involved in chondrocytes of OA patients. The JAK/STAT3 pathway has been reported to regulate the induction of MMP-1 expression [16]. In addition, Legendre et al. (2005) reported that IL-6/sIL-6R treatment provoked MMP-1, -3 and -13 expressions via STAT1/3 activation in primary bovine chondrocytes [17]. In addition, it has been reported that IL-1β treatment induces MMP-13 and activates JAK/STAT signaling in SW1353 cells, particularly, via JAK2/STAT1/2 [18]. Our study demonstrated that DMF inhibits the activation of the JAK/STAT3 pathway, suggesting a novel inhibitory effect of DMF in inflammation. Consistent with our findings, it has been suggested that immunomodulatory mechanisms of action of DMF or its metabolite monomethyl fumarate (MMF) include inhibition of cytokine-induced nuclear translocation of the nuclear factor kappa B (NF-κB) [19], apoptosis of stimulated T cells [20], and increased production of the TH2 cytokines IL-4 and IL-5 in stimulated T cells, whereas generation of the TH1 cytokine interferon gamma (IFN-γ) [21]. The long-term effect of DMF in neuronal cells is most probably mediated via Nrf2 as other reported mechanisms such as the inhibition of the nuclear translocation of NF-κB [22].

In spite of its proven clinical efficacy, the mode of metabolism and the pharmacodynamics of DMF are still not completely understood. It has been shown that orally applied DMF for a considerable part is rapidly metabolized to methylhydrogen fumarate (MHF) at basic pH conditions as present in the upper intestine. Due to its high lipophilicity, DMF has been shown rapidly to penetrate into cells and may thus at least in part be absorbed after po application without being hydrolyzed. As a matter of fact, no detectable amounts of DMF were hitherto found in plasma samples after po administration [23]. However, in order to explore its potential therapeutic effects, DMF has been used to study various *in vitro* disease models. And the concentration of DMF used in these studies is mainly ranged from 1 μM to 75 μM. For example, a recent study indicated that treatment with DMF at the concentrations of 5, 20, and 50 μM in rat vascular smooth muscle cells (VSMCs) resulted in a dose-dependent suppression of calcification in rat VSMCs [24]. In addition, DMF was seen to inhibit TGF-β-stimulated type I collagen expression in a dose-dependent manner from 20–40 μM in mesangial cells (RMCs) [25]. Another study showed that 20 μM DMF caused an increase in relative HDAC1, HDAC2, and HDAC4 mRNA levels in astrocytes [26]. Kang and his colleagues showed that DMF treatment reduced the mRNA levels of adipogenic genes C/EBPb, C/EBPa, PPARc, SREBP-1c, FAS, and aP2 in a dose dependent manner at the concentrations of 25, 50, and 75 μM [27]. These findings support the concentrations of DMF we used in this study.

In conclusion, for the first time, our data demonstrated that DMF suppressed TNF-α- induced degradation of collagen type II in human chondrocytes. Mechanistically, DMF attenuated MMPs expression by suppressing JAK/STAT3 pathway. These findings imply that DMF treatment might be a potential therapeutic strategy for chondroprotective therapy.

References

[1] M. Okubo, Y. Okada, Destruction of the articular cartilage in osteoarthritis, Clin. Calcium 23 (2013) 1705–1713.
[2] W. Kullich, N. Fagerer, H. Schwann, Effect of the NSAID nimesulide on the radical scavenger glutathione S-transferase in patients with osteoarthritis of the knee, Curr. Med. Res. Opin. 23 (2007) 1981–1986.
[3] P.M. Lin, C.T. Chen, P.A. Torzilli, Increased stromelysin-1 (MMP-3), proteoglycan degradation (3B3- and 7D4) and collagen damage in cyclically load-injured articular cartilage, Osteoarthritis Cartilage 12 (2004) 485–496.
[4] T. Shiomi, V. Lemaître, J. D'Armiento, Y. Okada, Matrix metalloproteinases, a disintegrin and metalloproteinases, and a disintegrin and metalloproteinases with thrombospondin motifs in non-neoplastic diseases, Pathol. Int. 60 (2010) 477–496.
[5] K.S. Santangelo, G.J. Nuovo, A.L. Bertone, In vivo reduction or blockade of interleukin-1beta in primary osteoarthritis influences expression of mediators implicated in pathogenesis, Osteoarthritis Cartilage 20 (2012) 1610–1618.
[6] M. Marziniak, Multiple sclerosis: new treatment options, MMW Fortschr. Med. (2014) 69–73 (156 Spec 1, quiz 74).
[7] A. Wierinckx, J. Brevé, D. Mercier, et al., Detoxication enzyme inducers modify cytokine production in rat mixed glial cells, J. Neuroimmunol. 166 (2005) 132–143.
[8] P. Albrecht, I. Bouchachia, N. Goebels, et al., Effects of dimethyl fumarate on neuroprotection and immunomodulation, J. Neuroinflammation 9 (2012) 163.
[9] M. Vandermeeren, S. Janssens, M. Borgers, J. Geysen, Dimethylfumarate is an inhibitor of cytokine-induced E-selectin, VCAM-1, and ICAM-1 expression in human endothelial cells, Biochem. Biophys. Res. Commun. 234 (1997) 19–23.
[10] R.A. Linker, D.H. Lee, S. Ryan, et al., Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway, Brain 134 (2011) 678–692.
[11] C.Y. Huang, K.Y. Lai, L.F. Hung, W.L. Wu, F.C. Liu, L.J. Ho, Advanced glycation end products cause collagen II reduction by activating janus kinase/signal transducer and activator of transcription 3 pathway in porcine chondrocytes, Rheumatology (Oxford) 50 (2011) 1379–1389.
[12] W. Hui, G.J. Litherland, M. Jefferson, et al., Lithium protects cartilage from cytokine-mediated degradation by reducing collagen-degrading MMP production via inhibition of the P38 mitogen-activated protein kinase pathway, Rheumatology (Oxford) 49 (2010) 2043–2053.
[13] J.H. Yik, Z. Hu, R. Kumari, et al., Cyclin-dependent kinase 9 inhibition protects cartilage from the catabolic effects of proinflammatory cytokines, Arthritis Rheumatol. 66 (2014) 1537–1546.
[14] A. Shibakawa, H. Aoki, K. Masuko-Hongo, et al., Presence of pannus-like tissue on osteoarthritic cartilage and its histological character, Osteoarthritis Cartilage 11 (2003) 133–140.
[15] A. Vidal, M. Sabatini, G. Rolland-Valognes, P. Renard, J.C. Madelmont, E. Mounetou, Synthesis and in vitro evaluation of targeted tetracycline derivatives: effects on inhibition of matrix metalloproteinases, Bioorg. Med. Chem. 15 (2007) 2368–2374.
[16] A.K. Behera, C.M. Thorpe, J.M. Kidder, et al., Borrelia burgdorferi-induced expression of matrix metalloproteinases from human chondrocytes requires mitogen-activated protein kinase and janus kinase/signal transducer and activator of transcription signaling pathways, Infect. Immun. 72 (2004) 2864–2871.
[17] F. Legendre, P. Bogdanowicz, K. Boumediene, J.-P. Pujol, Role of interleukin 6 (IL-6)/IL-6R-induced signal transducers and activators of transcription and mitogen activated protein kinase/extracellular signal-related kinase in upregulation of matrix metalloproteinase and ADAMTS gene expression in articular chondrocytes, J. Rheumatol. 32 (2005) 1307–1316.
[18] H. Lim, H.P. Kim, Matrix metalloproteinase-13 expression in IL-1β-treated chondrocytes by activation of the p38 MAPK/c-Fos/AP-1 and JAK/STAT pathways, Arch. Pharm. Res. 34 (2011) 109–117.
[19] M. Vandermeeren, S. Janssens, H. Wouters, et al., Dimethylfumarate is an inhibitor of cytokine-induced nuclear translocation of NF-kappa B1, but not RelA in normal human dermal fibroblast cells, J. Invest. Dermatol. 116 (2001) 124–130.
[20] F. Treumer, K. Zhu, R. Gläser, U. Mrowietz, Dimethylfumarate is a potent inducer of apoptosis in human T cells, J. Invest. Dermatol. 121 (2003) 1383–1388.
[21] R. de Jong, A.C. Bezemer, T.P. Zomerdijk, T. van de Pouw-Kraan, T.H. Ottenhoff, P.H. Nibbering, Selective stimulation of T helper 2 cytokine responses by the anti-psoriasis agent monomethylfumarate, Eur. J. Immunol. 26 (1996) 2067–2074.
[22] K. Ghoreschi, J. Bruck, C. Kellerer, et al., Fumarates improve psoriasis and multiple sclerosis by inducing type II dendritic cells, J. Exp. Med. 208 (2011) 2291–2303.
[23] T.J. Schmidt, M. Ak, U. Mrowietz, Reactivity of dimethyl fumarate and methylhydrogen fumarate towards glutathione and N-acetyl-L-cysteine – preparation of S-substituted thiosuccinic acid esters, Bioorg. Med. Chem. 15 (2007) 333–342.

[24] C.M. Ha, S. Park, Y.K. Choi, et al., Activation of Nrf2 by dimethyl fumarate improves vascular calcification, Vascul. Pharmacol. S1537–1891 (2014) 00125–126.
[25] C.J. Oh, J.Y. Kim, Y.K. Choi, et al., Dimethylfumarate attenuates renal fibrosis via NF-E2-related factor 2-mediated inhibition of transforming growth factor-β/Smad signaling, PLoS One 7 (2012) e45870.
[26] S. Kalinin, P.E. Polak, S.X. Lin, et al., Dimethyl fumarate regulates histone deacetylase expression in astrocytes, J. Neuroimmunol. 263 (2013) 13–19.
[27] H.J. Kang, H.A. Seo, Y. Go, et al., Dimethylfumarate suppresses adipogenic differentiation in 3T3-L1 preadipocytes through inhibition of STAT3 activity, PLoS One 8 (2013) e61411.