

# Tetrandrine inhibits the proliferation and cytokine production induced by IL-22 in HaCaT cells

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## Abstract

**Objective:** To investigate the effect of tetrandrine (Tet) on HaCaT cell proliferation and cytokine expression induced by interleukin (IL)-22, and to investigate the underlying mechanism.

**Methods:** The half maximal inhibitory concentration (IC<sub>50</sub>) and antiproliferation effects of Tet on IL-22-treated HaCaT cells were analysed by MTT assay. Signal transducer and activator of transcription 3 (STAT3) expression was measured by reverse transcription plus real-time quantitative polymerase chain reaction (qPCR) and by Western blot. Phosphorylated (p)-STAT3 levels were also measured by Western blot. Cytokine production by HaCaT cells was analysed by enzyme-linked immunosorbent assay (ELISA) following administration of IL-22 and/or Tet.

**Results:** Tet displayed a dose-dependent inhibitory effect on HaCaT cell proliferation and reduced the phosphorylation level of STAT3 induced by IL-22, without affecting STAT3 mRNA and protein levels. Furthermore, co-incubation with Tet significantly down-regulated HaCaT cell production of tumour necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$ , IL-6, IL-20 and chemokine (C-C motif) ligand 20 (CCL20) induced by IL-22.

**Conclusions:** Tet inhibits proliferation and cytokine production in HaCaT cells, and the process may involve the inhibition of STAT3 phosphorylation.

## Keywords

Psoriasis, tetrandrine, IL-22, HaCaT cells, cytokines, STAT3

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## Introduction

Psoriasis is a chronic inflammatory skin disease mediated by immune cells. Worldwide prevalence of psoriasis is approximately 2–3%,<sup>1</sup> and prevalence in China varies between 0.13% and 1.23%.<sup>2</sup> The most common type of psoriasis, accounting for 90% of all cases, is psoriasis vulgaris,<sup>3</sup> in which red coloured papulosquamous plaques are well-delineated from the surrounding normal skin, and are covered by white or silvery scales. Psoriasis is recurrent, and associated with a variety of comorbidities including cardiovascular diseases, diabetes mellitus, obesity and even hyperlipidaemia, and seriously affects patients' quality of life.<sup>4,5</sup> However, the mechanism underlying the pathogenesis of psoriasis remains largely unknown.

Psoriasis development is accompanied by the enhanced expression of cytokines,<sup>6</sup> such as interferon (*IFN*)-gamma, interleukin (*IL*)-2, and *IL*-22.<sup>7,8</sup> *IL*-22 is mainly produced from activated T cells and natural killer (NK) cells, and sequentially induces the rapid proliferation of keratinocytes, which specifically express the *IL*-22 receptor, thus leading to the pathogenesis of psoriasis.<sup>9</sup> Additionally, high levels of *IL*-22 have been detected in the sera of patients with psoriasis, and have been positively correlated with disease severity.<sup>10</sup> Keratinocyte cell lines induced by *IL*-22, for example, HaCaT cells, are often used as models for the study of psoriasis.<sup>11</sup> Moreover, lines of evidence reveal that activation of the signal transducer and activator of transcription 3 (STAT3) has been involved in cytokine expression and the development of psoriasis.<sup>12–14</sup>

Tetrandrine (Tet) is a natural nonselective  $\text{Ca}^{2+}$  channel blocker with anti-inflammatory effects.<sup>15,16</sup> It is widely used in the treatment of rheumatism, joint pain, neuralgia and cancer,<sup>17–19</sup> however, the effect of Tet on psoriasis remains unclear.

The aim of the present study was to measure the *in vitro* antiproliferative effect of Tet on HaCaT cells, and to analyse the effect of Tet on *STAT3* expression and phosphorylated (P)-*STAT3* levels and cytokine production in HaCaT cells induced by *IL*-22 using reverse transcription and real-time quantitative polymerase chain reaction (qPCR), Western blot and enzyme-linked immunosorbent assay (ELISA).

## Materials and methods

### Cell culture and treatment

The HaCaT cells (Cell Bank, Chinese Academy of Sciences, Shanghai, China) were cultured in T-25 culture flasks at 37°C/5%  $\text{CO}_2$  in complete medium comprising Dulbecco's Modified Eagle Medium (DMEM; Cat No. SH30243.01B; HyClone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Cat No. SH30070.02; HyClone). HaCaT cells in logarithmic growth phase were harvested and seeded as described in subsequent sections for treatment under the following experimental conditions with or without recombinant human *IL*-22 (Cat No. ab49821; Abcam, Cambridge, MA, USA) and Tet (Purity >98%; Shanghai base Industrial Co., Ltd., Shanghai, China): initial test of antiproliferative effects of Tet at a range of doses from 40–240  $\mu\text{M}$ , incubated with HaCaT cells for 48 h; negative control group (without *IL*-22 or Tet), *IL*-22 group (treated with 100  $\mu\text{g}/\text{ml}$  *IL*-22 alone), Tet low dose group (treated with 100  $\mu\text{g}/\text{ml}$  *IL*-22 and 43  $\mu\text{M}$  Tet), and Tet high dose group (treated with 100  $\mu\text{g}/\text{ml}$  *IL*-22 and 86  $\mu\text{M}$  Tet), all incubated for 72 h at 37°C/5%  $\text{CO}_2$  under each treatment condition.

### MTT assay

The HaCaT cells in logarithmic growth phase were harvested and seeded into

96-well plates at  $2 \times 10^3$  cells per well in complete medium and incubated at  $37^\circ\text{C}/5\%$   $\text{CO}_2$  to allow cells to adhere. The medium was then replaced with complete medium containing Tet and/or IL-22, or complete medium only (control). Three replicates were set up for each group. Following incubation for 24–72 h, the medium was replaced with serum-free medium containing 5 mg/ml MTT (Cat No. C0009; Beyotime, Shanghai, China) and the cells were incubated for a further 4 h at  $37^\circ\text{C}/5\%$   $\text{CO}_2$ . Finally, the crystals were dissolved by the addition of dimethyl sulphoxide and the absorbance value at 490 nm was measured using a micro-plate reader. Inhibition of cell proliferation by Tet was calculated as  $\% \text{ inhibition} = (1 - A_{\text{Tet}} / A_{\text{control}}) \times 100$  (where  $A$  = absorbance value at 490 nm). The half maximal inhibitory concentration (IC50) was calculated using SPSS software, version 21.0 (SPSS Inc., Chicago, IL, USA).

### Western blot

Interleukin-22 is known to activate janus kinase (JAK)-STAT3, extracellular signal-regulated kinase (ERK)1/2/mitogen-activated protein kinase (MAPK), and p38/MAPK pathways to regulate the proliferation and differentiation of keratinocytes after binding to its receptor.<sup>20</sup> Therefore, to clarify the mechanism underlying the inhibition of HaCaT cell proliferation by Tet, protein levels of P-STAT3 and STAT3 were measured. The HaCaT cells in logarithmic growth phase were harvested and seeded into 6-well plates at a density of  $2 \times 10^6$  cells/well in complete medium and incubated at  $37^\circ\text{C}/5\%$   $\text{CO}_2$  to allow cells to adhere. The medium was then replaced with complete medium containing Tet and/or IL-22, or complete medium only (control). Experiments were performed in triplicate. Following incubation at  $37^\circ\text{C}/5\%$   $\text{CO}_2$  for 72 h, cells were lysed in radioimmunoprecipitation assay

(RIPA) buffer (Cat No. P0013B; Beyotime) and total protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Cat No. RTP7102; Real-Times Biotechnology Co., Ltd., Beijing, China). Proteins (20  $\mu\text{g}$ ) were separated by 10% sodium dodecyl sulphate (SDS)-polyacrylamide-gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane. After blocking with 5% bovine serum albumin BSA, the membrane was incubated with primary antibodies (anti-P-STAT3, 1:1500 [Cat No. ab30647; Abcam, Cambridge, UK], anti-STAT3, 1:1000 [Cat No. ab68153; Abcam] and anti- $\beta$ -actin, 1:5000 [Cat No. ab129348; Abcam]) diluted in primary antibody dilution solution (Cat No. P0023A; Beyotime) overnight at  $4^\circ\text{C}$ . After washing five  $\times$  5 min each with  $1 \times$  phosphate buffered saline-Tween, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:3000 [Cat No. ab6721; Abcam]) for 1 h at room temperature. Target protein levels were detected by the ChemiDoc<sup>TM</sup> Touch Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) and analysed using Image Lab software, version 3.0 (Bio-Rad Laboratories).  $\beta$ -actin served as an internal control to normalize target protein levels between the samples.

### RNA isolation, reverse transcription and real-time qPCR

Total RNA was extracted from HaCaT cells (in 6-well plates) that had been exposed to complete medium containing Tet and/or IL-22, or complete medium only (control), using TriZol reagent (Cat No. 10606ES60; YEASEN, Shanghai, China). Total RNA quality was assessed by agarose gel electrophoresis and the concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo scientific, Basingstoke, UK). The cDNA was synthesized from

1 µg RNA using TIANScript II cDNA kit (Cat No. KR107; TIANGEN, Beijing, China) according to the manufacturer's protocol. Gene expression levels were quantified by real-time qPCR using an iQ5 real-time qPCR instrument (Bio-Rad) and a 20-µl reaction mix comprising 7 µl ddH<sub>2</sub>O, 2 µl cDNA, 10 µl SuperReal PreMix (SYBR Green) (Cat No. FP204; TIANGEN) and 0.5 µl each of the following primer sequences: *STAT3*, forward 5'-GGAGGAGGCATTC GGAAAG -3' and reverse 5'-TCGTTGGT GTCACACAGAT-3'; *β-actin*, forward 5'-AACGGCTCCGGCATGTGCAA-3' and reverse 5'-CTTCTGACCCATGCCAC CA- 3'.

The qPCR reaction conditions were as follows: denaturation at 95°C for 2 min, 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min. The relative *STAT3* expression levels were calculated using the  $2^{-\Delta\Delta Ct}$  method, and *β-actin* served as the internal control.

## ELISA

In addition to keratinocyte proliferation, the inflammatory response is also shown to be amplified during the development of psoriasis.<sup>21</sup> As stated previously, the expression of pro-inflammatory factors, such as *TNF-α*, *IL-6*, *IL-8*, *IL-20*, and chemokine (C-C motif) ligand 20 (*CCL20*) was significantly up-regulated in the epidermis of psoriasis patients.<sup>9,22,23</sup> Thus, the production of these cytokines by HaCaT cells was investigated following treatment with IL-22 alone or with the addition of Tet.

Supernatant from HaCaT cells that had been incubated with Tet and/or IL-22 for 72 h at 37°C/5% CO<sub>2</sub> was harvested and cell debris was removed by centrifugation at 1000 × *g* for 10 min. Levels of *TNF-α*, *IL-6*, *IL-8*, *IL-20* and *CCL20* in the supernatants were measured using the following

ELISA kits (*IL-6* ELISA kit, Cat No. A90-2094; *TNF-α* ELISA kit, Cat No. A90-2128; *IL-8* ELISA kit, Cat No. A90-2097; and *IL-20* ELISA kit, Cat No. A90-2084; all from Abeomics [San Diego, CA, USA]; and *CCL20* kit, Cat No. KA0389; Abnova [Taipei City, Taiwan]) following the manufacturer's instructions. Briefly, 50 µl of standard, samples and blank were added to the well of a 96-well plate coated with the primary antibodies against the cytokines. The HRP-conjugated detection antibodies (100 µl) were then added and incubated for 1 h at 37°C. After washing five times, substrates A and B were added for a further 15-min incubation. Finally, the absorbance at 450 nm was read in a micro-plate reader immediately after adding the stop solution. A standard curve was produced to quantify the concentration of cytokines from the different samples.

## Statistical analyses

Data are presented as mean ± SD and were analysed using SPSS software, version 21.0 (SPSS Inc.). Between-group comparisons were performed with analysis of variance (ANOVA) after performing a test for normality. Fisher's least significant difference (LSD) and Student-Newman-Keuls (SNK) tests were used when there was homogeneity of variance, otherwise, Tamhane's T2 or Dunnett's T3 tests were used. A *P* value < 0.05 was considered to be statistically significant.

## Results

### *Tet inhibits the proliferation induced by IL-22 in HaCaT cells*

The effect of Tet on proliferation of HaCaT cells was initially investigated at Tet concentrations of 40, 60, 80, 120, 160, and 240 µM incubated for 48 h. HaCaT proliferation was found to be inhibited by Tet in a

dose-dependent manner, with increasing Tet concentrations resulting in increased inhibition of proliferation (Figure 1a). The IC<sub>50</sub> value was calculated to be  $86.02 \pm 6.45 \mu\text{M}$ .

The possible role of Tet in the inhibition of proliferation by HaCaT cells treated with IL-22 was explored using Tet at 86  $\mu\text{M}$  (the IC<sub>50</sub> value; high Tet), and at 43  $\mu\text{M}$  (half of the IC<sub>50</sub> value; low Tet). Following treatment with IL-22 only for 72 h, the 490 nm absorbance values increased in all groups over time (Figure 1b). In the groups treated with IL-22 and either high (86  $\mu\text{M}$ ) or low (43  $\mu\text{M}$ ) Tet concentrations, the proliferation of HaCaT cells was significantly decreased at 72 h (Figure 1b). Moreover, the inhibition of proliferation was higher in the high Tet group compared with low Tet group ( $P < 0.05$ ; Figure 1b), suggesting that proliferation of HaCaT cells may be inhibited by Tet in a dose-dependent manner.

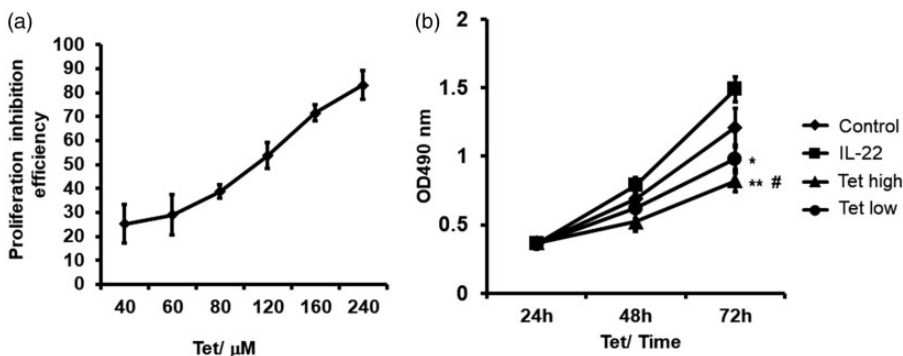
### Tet reduces IL-22 induced STAT3 phosphorylation in HaCaT cells

The level of P-STAT3 protein was significantly up-regulated after 72-h incubation

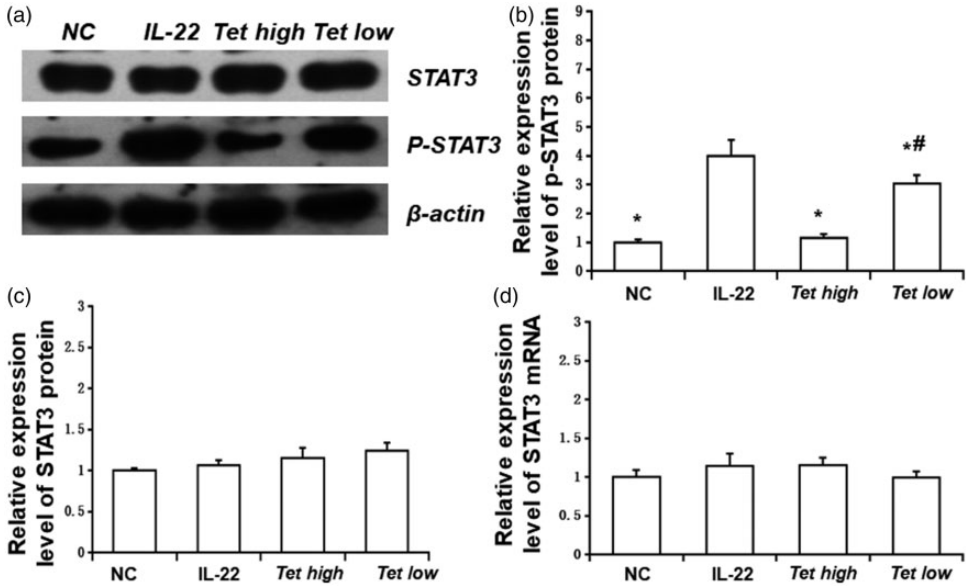
with IL-22 only, and this increased level of P-STAT3 was significantly reduced by co-incubation with Tet at both 43 and 86  $\mu\text{M}$  concentrations ( $P < 0.05$ ; Figure 2a and b). In addition, the reduced level of P-STAT3 was significantly higher with the higher Tet concentration ( $P < 0.05$  versus 43  $\mu\text{M}$  Tet; Figure 2a and b). STAT3 protein and mRNA levels were not significantly changed by IL-22 with or without Tet (Figure 2c and d), suggesting that Tet may inhibit the promotion of STAT3 phosphorylation by IL-22, which may contribute to the inhibition of HaCaT cell proliferation.

### Tet reduces IL-22 induced cytokine production in HaCaT cells

Levels of TNF- $\alpha$ , IL-6, IL-8, IL-20 and CCL20 proteins in the supernatant of HaCaT cells were all increased following 72-h incubation with IL-22 only (Figure 3a–e). When co-incubated with Tet, the enhanced production of cytokines by IL-22 was significantly reduced in a dose-dependent manner (Figure 3a–e), suggesting that Tet may inhibit the production



**Figure 1.** Inhibition of *in vitro* proliferation by tetrandrine (Tet) in interleukin (IL)-22 treated HaCaT cells: (A) the effect of Tet at a range of concentrations (40, 60, 80, 120, 160, or 240  $\mu\text{M}$ ) on inhibition of *in vitro* proliferation of HaCaT cells over 48 h ( $n = 3$ ); (B) the effect of Tet (43 or 86  $\mu\text{M}$ ) on *in vitro* proliferation of HaCaT cells induced by IL-22 over 72 h ( $n = 3$ ); Tet high, 86  $\mu\text{M}$  Tet plus 100  $\mu\text{g}/\text{ml}$  IL-22; Tet low, 43  $\mu\text{M}$  Tet plus 100  $\mu\text{g}/\text{ml}$  IL-22; Control, DMEM plus 10% fetal bovine serum; OD, optical density. Data presented as mean  $\pm$  SD; \* $P < 0.05$ , \*\* $P < 0.01$  versus IL-22 group; # $P < 0.05$ , Tet high versus other groups (all one-way analysis of variance)



**Figure 2.** Down-regulation of signal transducer and activator of transcription 3 (STAT3) phosphorylation by tetrandrine (Tet) in HaCaT cells treated with interleukin (IL)-22: (A) representative Western blot showing STAT3 and phosphorylated (P)-STAT3 protein levels following 72 h incubation with IL-22 (100  $\mu$ g/ml) alone, or IL-22 (100  $\mu$ g/ml) plus 43  $\mu$ M Tet (Tet low) or 86  $\mu$ M Tet (Tet high), with  $\beta$ -actin as the loading control; and (B) relative levels of P-STAT3 protein ( $n = 3$ ); (C) relative levels of STAT3 protein ( $n = 3$ ); and (D) relative expression of STAT3 mRNA ( $n = 3$ ), all following 72 h incubation with IL-22 (100  $\mu$ g/ml) alone, or IL-22 (100  $\mu$ g/ml) plus 43  $\mu$ M Tet (Tet low) or 86  $\mu$ M Tet (Tet high); NC, negative control (DMEM plus 10% fetal bovine serum). Data presented as mean  $\pm$  SD; \* $P < 0.05$ , versus IL-22 group; ## $P < 0.05$ , versus Tet high group (all one-way analysis of variance)

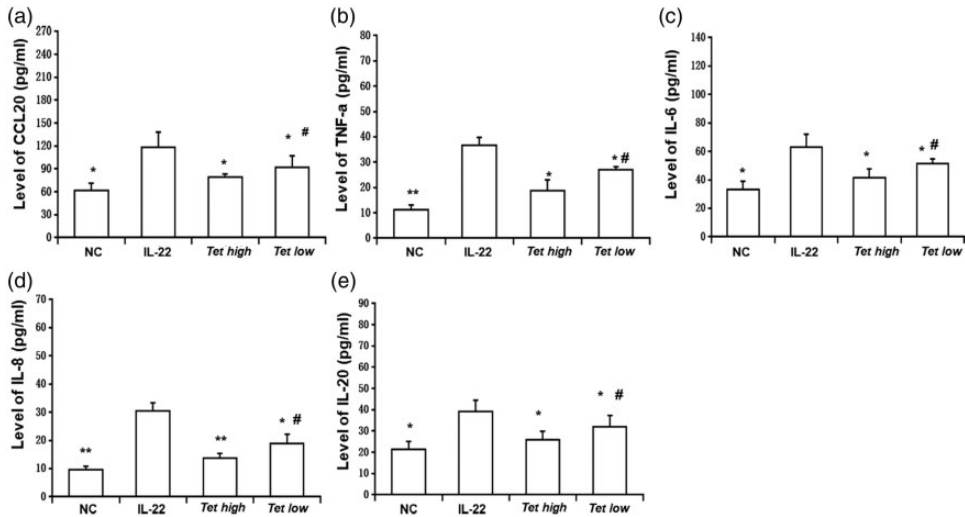
of TNF- $\alpha$ , IL-6, IL-8, IL-20 and CCL20 in HaCaT cells.

## Discussion

As a chronic inflammatory skin disease, psoriasis affects 2–3% of individuals worldwide, and seriously compromises the quality of life of patients.<sup>1,4,5</sup> IL-22 signalling is considered to play an important role in psoriasis, since IL-22 expression is highly positively correlated with disease severity<sup>10</sup> and IL-22 receptors are specifically expressed in keratinocytes.<sup>9</sup> Tet, as an anti-inflammation drug, is widely used in clinics in China,<sup>24</sup> however, the role of Tet in psoriasis remains to be demonstrated.

In the present study, Tet was demonstrated to have a dose-dependent inhibitory effect on the *in vitro* proliferation of HaCaT cells, and was shown to reduce STAT3 phosphorylation levels induced by IL-22 without affecting STAT3 mRNA and protein levels. Furthermore, co-incubation of Tet with IL-22 significantly down-regulated the IL-22 induced production of TNF- $\alpha$ , IL-6, IL-8, IL-20 and CCL20 by HaCaT cells.

Signal transducer and activator of transcription 3 (STAT3) has been reported to mediate the expression of a variety of genes in cellular processes, such as cell growth and apoptosis.<sup>25</sup> One important finding of the present study was that IL-22



**Figure 3.** Reduction of interleukin (IL)-22 induced cytokine production by HaCaT cells following incubation with tetrandrine (Tet): (A–E) the production of tumour necrosis factor (TNF)- $\alpha$ , IL-6, IL-8, IL-20 and chemokine (C-C motif) ligand 20 (CCL20) after 72-h incubation with IL-22 (100  $\mu$ g/ml) alone, or IL-22 (100  $\mu$ g/ml) plus 43  $\mu$ M Tet (Tet low) or 86  $\mu$ M Tet (Tet high) (all  $n = 3$ ); NC, negative control (DMEM plus 10% fetal bovine serum). Data presented as mean  $\pm$  SD; \* $P < 0.05$ , \*\* $P < 0.01$  versus IL-22 group; # $P < 0.05$ , versus Tet high group (all one-way analysis of variance).

induced P-STAT3 in HaCaT cells was notably reduced by coincubation with Tet. Combined with the result that Tet was shown to inhibit HaCaT cell proliferation, it would make sense for future studies to verify the potential role of P-STAT3 in Tet-mediated antiproliferation effects.

In conclusion, Tet was found to greatly reduce the *in vitro* production of IL-22-induced TNF- $\alpha$ , IL-6, IL-8, IL-20 and CCL20 by HaCaT cells. Combined with the results that Tet was shown to inhibit HaCaT cell proliferation, the present authors hypothesize that the anti-inflammatory role of Tet may be associated with its inhibitory effect on cell proliferation. Therefore, developing an effective drug such as Tet may be a good strategy to target the rapid proliferation of keratinocytes and the enhanced expression of inflammatory mediators in psoriasis.

Collectively, the present study demonstrates the role of Tet in the *in vitro* inhibition of proliferation and cytokine production by HaCaT cells, and STAT3 phosphorylation may be involved in this process. The present findings expand our understanding of the diverse functions of Tet and contribute to developing new strategies for psoriasis treatment.

#### Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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