

Teng-Long-Bu-Zhong-Tang induces p21-dependent cell senescence in colorectal carcinoma LS174T cells via histone acetylation

Bing Hu^{1,2}

Hong-Mei An³

Shuang-Shuang Wang^{1,2}

Jia-Lu Zheng^{1,2}

Xia Yan^{1,2}

Xiao-Wei Huang^{1,2}

Jian-Hui Tian^{1,2}

¹Department of Oncology, Longhua Hospital, Shanghai University of Traditional Chinese Medicine,

²Institute of Traditional Chinese Medicine in Oncology, Shanghai Academy of Traditional Chinese Medicine, ³Department of Science and Technology, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, People's Republic of China

Abstract: Teng-Long-Bu-Zhong-Tang (TLBZT) is a Chinese herbal formula for colorectal carcinoma treatment. TLBZT effectively induces cell senescence in colorectal carcinoma, accompanied by p21 upregulation. In this study, we further explored the role of p21 in TLBZT-induced cell senescence, as well as the mechanism by which TLBZT upregulates p21. Specific knockdown of p21 expression by small interfering RNA significantly attenuated TLBZT-induced cell senescence in human colorectal carcinoma LS174T cells. Silencing of p53 by small interfering RNA did not affect TLBZT-induced p21 upregulation. Meanwhile, TLBZT inhibited histone deacetylase activity. Furthermore, TLBZT increased acetylation levels of histone H3 and H4, enhancing their binding to the p21 promoter. These data suggested that TLBZT induces cell senescence in LS174T cells through a mechanism involving p21 upregulation via histone H3 and H4 acetylation. This study provides new insights into the application of TLBZT for colorectal carcinoma treatment.

Keywords: colorectal carcinoma, TLBZT, cell senescence, p21, histone acetylation

Introduction

Colorectal cancer is the third most frequently diagnosed cancer in males and the second in females worldwide.¹ The overall survival of patients with early-stage colorectal carcinoma has improved in the past decades. However, treatment outcomes of metastatic colorectal cancer are less than satisfactory, even though targeted therapeutic drugs are currently used.^{2,3} Ethnomedicine constitutes another resource for the treatment of colorectal carcinoma. Most ethnic drugs are nontoxic with low cost, and have been used for thousands of years. There are abundant clinical experiences which are a resource for the development of new drugs for colorectal carcinoma treatment.

In China, Traditional Chinese Medicine (TCM) plays an important role in colorectal cancer treatment. TCM effectively enhances the curative effects of chemotherapy while reducing its toxic side effects, palliates clinical syndromes, prevents recurrence and metastasis, improves the quality of life and immune function, and prolongs survival in colorectal cancer.^{4,5} Cancer patients are treated with multiple Chinese herbs based on the syndromes (Zheng) and diseases present in individual patients, indicating that TCM is a form of personalized treatment. Most Chinese herbs are medicated in a prescription and applied as decoction.

Based on the TCM principles and clinical practice, we have established a herbal formula for colorectal cancer treatment, namely, Teng-Long-Bu-Zhong-Tang (TLBZT). TLBZT effectively induces cell senescence, accompanied with p21 upregulation and reduced retinoblastoma protein (RB) phosphorylation in colorectal carcinoma.^{6,7}

Correspondence: Bing Hu
Department of Oncology, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, 725 South Wanping Road, Shanghai 200032, People's Republic of China
Tel +86 21 6438 5700
Email bearhu@hotmail.com

However, the relationship between TLBZT-induced cell senescence and p21 upregulation remains unclear. This study assessed whether TLBZT induction of cell senescence is dependent on p21 and explored the potential mechanism by which TLBZT upregulates p21.

Materials and methods

Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum were obtained from Hyclone (Logan, UT, USA). Antibodies against acetylated histone H3 (ACh3) and H4 (ACh4), histone H3 and H4, p21, p53, and β -actin were obtained from Cell Signaling Technology (Danvers, MA, USA). Cellular Senescence Detection Kit was purchased from Cell Biolabs (San Diego, CA, USA). Histone deacetylase (HDAC) activity assay kit and Trichostatin A (TSA) were from BioVision (San Francisco, CA, USA). Chromatin immunoprecipitation (ChIP) assay kit was obtained from Beyotime Institute of Biotechnology (Jiangshu, People's Republic of China). Small interfering RNAs (siRNAs) targeting p21 and p53, and control siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine™ 2000 was from Thermo Fisher Scientific (Waltham, MA, USA). Sodium valproate (SVP) was purchased from Sigma-Aldrich (St Louis, MO, USA).

TLBZT extraction

The main herbs in TLBZT formula (Chinese patent ZL200910197565.2) are roots of *Actinidia chinensis* (Teng-Li-Geng, 30 g), *Solanum nigrum* (Long-Kui, 15 g), *Duchesnea indica* (She-Mei, 15 g), *Atractylodes macrocephala* (Bai-Zhu, 9 g), Coix seed (Yi-Yi-Ren, 30 g), and *Viscum coloratum* (Hu-Ji-Sheng, 15 g). All these herbs were from Longhua Hospital according to the original proportions, and decocted twice with eight-fold volume of distilled water for 1 h. The resulting decoction was filtered and centrifuged twice at 12,000 rpm for 30 min to remove insoluble ingredients. The supernatants were mixed with an equal volume of ethanol and incubated at 4°C overnight, and centrifuged at 12,000 rpm for 30 min. The resulting supernatants were lyophilized, weighed, dissolved in DMEM, and adjusted to 400 mg/mL. Finally, the preparation was sequentially passed through 0.45 and 0.22 μ m filters, for sterilization. The gas chromatography–mass spectrometry profile of TLBZT has been described previously.⁷

Cell culture

Human colorectal carcinoma LS174T cells were obtained from Cell Bank of Type Culture Collection of the Chinese

Academy of Sciences. LS174T cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified environment containing 5% CO₂.

siRNA treatment

For siRNA transfection, LS174T cells were cultured in six-well plates to 60% confluency, and 80 pmol of specific or control siRNA was introduced into the cells using Lipofectamine™ 2000, according to the manufacturer's recommendations.⁸ After 24 h of transfection, the cells were treated with TLBZT and subjected to further assays.

Cell senescence detection

siRNA-transfected or nontransfected LS174T cells (1.5×10^3) were seeded in 96-well plates and treated with different doses of TLBZT and SVP or TSA for 5 days. Cell senescence was detected with a commercial kit. Briefly, the cells were lysed at 4°C for 5 minutes and centrifuged at 12,000 rpm for 10 minutes at 4°C. Cell lysates were collected and incubated with fluorometric SA- β -Gal substrate at 37°C, protected from light, for 2 h. Fifty microliters of the reaction mixture was transferred to a 96-well plate, stopped by adding 200 μ L of stop solution, and read with a fluorescence plate reader at 360 nm (excitation)/465 nm (emission). The results were expressed as fold of control.

Western blot

Western blot was performed as described previously.^{8,9} Briefly, the collected cells were lysed, and total protein was separated by 8%–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% non-fat milk, washed, and probed with the indicated antibodies. Blots were washed and incubated with IRDye 700- and IRDye 800-conjugated secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA, USA), and visualized on Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

HDAC activity detection

TLBZT-treated or untreated LS174T cells were collected and assessed for HDAC activity with a specific kit, according to the manufacturer's instructions. Briefly, the cells were lysed at 4°C for 5 minutes and centrifuged at 12,000 rpm for 10 minutes at 4°C. Cell lysates were collected and incubated with HDAC colorimetric substrate at 37°C for 1 h. The reaction mixture was read in a plate reader at 405 nm. The results were expressed as fold of control.

ChIP-quantitative polymerase chain reaction

ChIP was performed with a commercial assay kit using antibodies against AcH3 and AcH4, or control IgG, according to the manufacturer's manual. Purified DNA was used as a template for quantitative polymerase chain reaction (qPCR) amplification using p21 promoter-specific primers.¹⁰ The results were expressed as % of input: % input = $2^{-(Ct(\text{ChIP}) - Ct(\text{input}))} \times 100\%$.

Statistical analysis

Results are mean \pm standard deviation (SD) from at least two independent triplicate experiments. Differences between control and TLBZT treatments were analyzed by one-way analysis of variance. Differences were considered significant at $P < 0.05$.

Results

TLBZT-induced cell senescence is dependent on p21

We have previously demonstrated that TLBZT-induced cell senescence in LS174T cells is accompanied by p21 upregulation.^{6,7} In this study, we further assessed whether TLBZT-induced cell senescence is associated with p21. As shown in Figure 1, p21 knockdown by specific siRNA significantly abrogated TLBZT-induced cell senescence. These observations suggested TLBZT-induced cell senescence is dependent on p21.

TLBZT-induced p21 expression is independent of p53

In a previous study, we found that p53 expression did not change upon TLBZT treatment.⁶ Therefore, we also determined whether TLBZT-related p21 upregulation is associated with p53. As shown in Figure 2, p53 was expressed in LS174T cells at low levels. Consistent with previous findings, TLBZT did not alter p53 expression. Knockdown of p53 expression by specific siRNA did not affect p21 expression. These

findings demonstrated that TLBZT induced p21 expression in a p53-independent manner.

TLBZT inhibits HDAC activity

It has been reported that HDAC inhibition could upregulate p21 and induce cell senescence.^{11,12} We, therefore, assessed the effects of TLBZT on HDAC activity. Interestingly, TLBZT inhibited HDAC activity in a dose-dependent manner (Figure 3A). In addition, inhibition of HDAC activity by SVP and TSA enhanced the effects of TLBZT on LS174T cell senescence (Figure 3B and 3C). These findings suggested that HDAC inhibition contributed to TLBZT-induced cell senescence in LS174T cells.

TLBZT induces histone H3 and H4 acetylation

HDACs regulate gene transcription by deacetylating the α -acetyl lysine residue within the NH₂-terminal tail of core histones, including histone H3 and H4. The effects of TLBZT on histone H3 and H4 acetylation in LS174T cells were detected by Western blot. As shown in Figure 4, TLBZT increased the acetylation of histone H3 and H4 in a dose-dependent manner.

TLBZT upregulates histone H3 and H4 acetylation at the p21 promoter region

ChIP assays and qPCR were performed to detect AcH3 and H4 at the p21 promoter region by using specific antibodies and primers. As shown in Figure 5, qPCR revealed a significant increase of p21 promoter fragments in ChIP-precipitated DNA from TLBZT-treated LS174T cells. These observations suggested increased acetylation of histone H3 and H4 at the p21 promoter region upon TLBZT treatment.

Discussion

Cell senescence is a state of irreversible cell cycle arrest caused by a variety of stimuli. Senescent cells maintain some

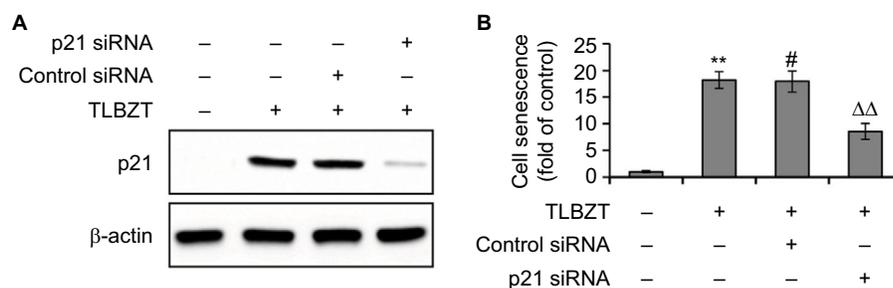


Figure 1 Role of p21 in TLBZT-induced cell senescence.

Notes: After 24 h of transfection with p21 siRNA or control siRNA, LS174T cells were treated with 200 $\mu\text{g}/\text{mL}$ of TLBZT for 5 days and subjected to Western blot analyses (A) and cell senescence detection (B). Data are representative of three independent experiments. ** $P < 0.01$ vs control, # $P > 0.05$ vs TLBZT, $\Delta\Delta P < 0.01$ vs control siRNA.

Abbreviations: siRNA, small interfering RNA; TLBZT, Teng-Long-Bu-Zhong-Tang.

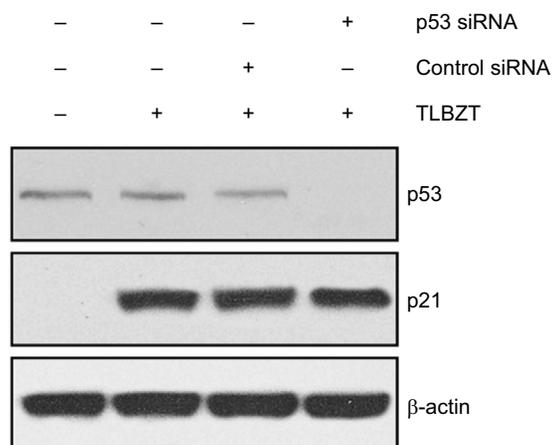


Figure 2 Role of p53 in TLBZT-induced p21 expression.
Notes: After 24 h of transfection with p53 siRNA or control siRNA, LS174T cells were treated with 200 µg/mL of TLBZT for 5 days and subjected to Western blot probing p53 and p21; β-actin was used as a loading control.
Abbreviations: siRNA, small interfering RNA; TLBZT, Teng-Long-Bu-Zhong-Tang.

metabolic activity, but can no longer proliferate, even when stimulated with mitogens. Cell senescence plays an important role in tumorigenesis suppression, and is considered one of the useful mechanisms for anticancer therapy.^{13–15} Chemotherapeutic agents, such as cisplatin, doxorubicin, and camptothecin, have been reported to inhibit growth of cancer cells by inducing cell senescence.^{16–18} Natural products such as curcumin, resveratrol, and β-asarone have demonstrated cell senescence-inducing effects in cancer cells.^{19–21}

Cell senescence is closely related to the activation of p21/pRB/E2F signaling.^{22,23} P21, also known as cyclin-dependent kinase inhibitor 1A, CDK-interacting protein 1, or wild-type p53-activated fragment 1, can inhibit a variety of cyclin/CDK complexes and induce the hypophosphorylation or dephosphorylation of protein RB.^{24–26} Hypophosphorylated pRB binds to E2F and prevents it from activating target

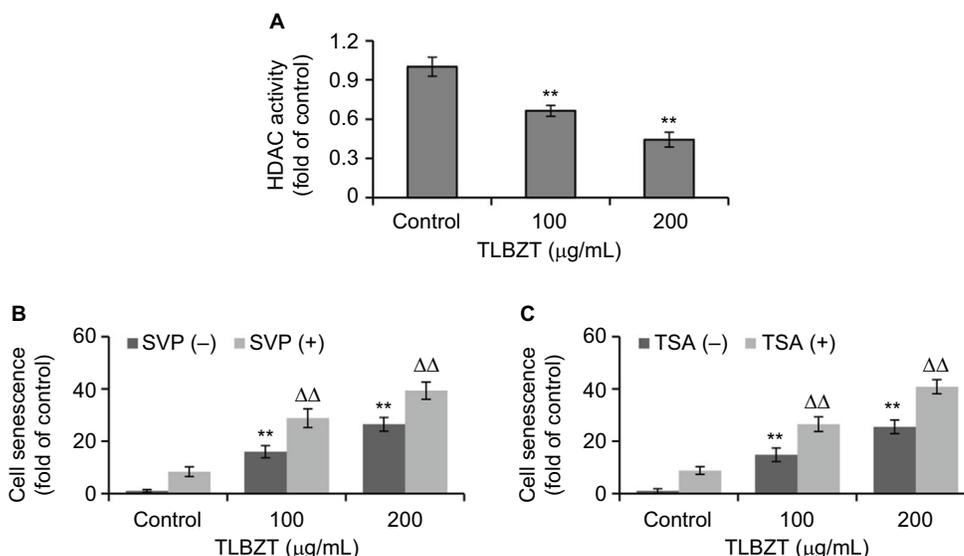


Figure 3 Effects of TLBZT on HDAC activity.
Notes: LS174T cells were collected after TLBZT treatment at 100 and 200 µg/mL for 5 days, and subjected to HDAC activity detection according to the manufacturer’s protocol (A). LS174T cells were treated with TLBZT and SVP (200 µM) (B) or TSA (10 nM) (C) for 5 days and subjected to cell senescence detection. Data are from three independent experiments. ***P*<0.01 vs control, ^{ΔΔ}*P*<0.01 vs SVP (-) or TSA (-).
Abbreviations: HDAC, histone deacetylase; SVP, sodium valproate; TLBZT, Teng-Long-Bu-Zhong-Tang; TSA, trichostatin A.

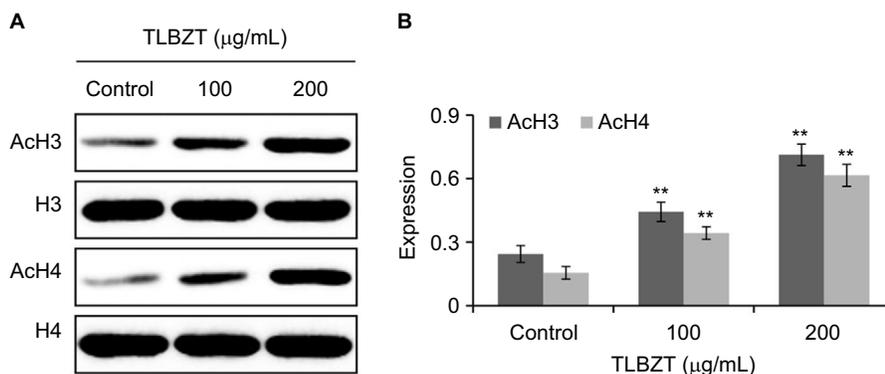


Figure 4 Effects of TLBZT on H3 and H4 acetylation.
Notes: LS174T cells were treated with 100 and 200 µg/mL of TLBZT for 5 days and subjected to Western blots using indicated antibodies (A). Histone acetylation was quantified by the Quantity One software and expressed as fold of histone (B). ***P*<0.01 vs control.
Abbreviations: AcH3, acetylated histone H3; AcH4, acetylated histone H4; H3, histone H3; H4, histone H4; TLBZT, Teng-Long-Bu-Zhong-Tang.

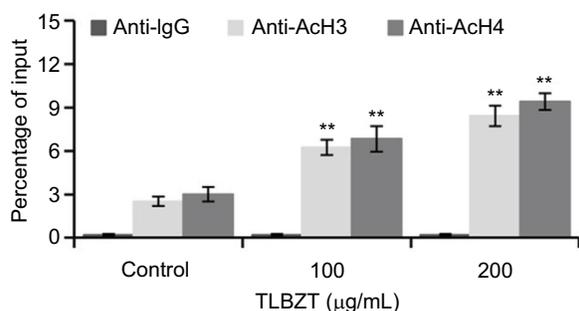


Figure 5 Effects of TLBZT on histone H3 and H4 acetylation at the p21 promoter region.

Notes: LS174T cells were treated with 100 and 200 µg/mL of TLBZT for 5 days and subjected to ChIP assay using AcH3 and AcH4 antibodies or control IgG. Precipitated genomic DNA was amplified by qPCR and expressed as a percentage of total input genomic DNA. Data are representative of three independent experiments. ** $P < 0.01$ vs control.

Abbreviations: AcH3, acetylated histone H3; AcH4, acetylated histone H4; ChIP, chromatin immunoprecipitation; qPCR, quantitative polymerase chain reaction; TLBZT, Teng-Long-Bu-Zhong-Tang.

genes essential in the cell cycle, which may lead to cell cycle arrest. In this study, p21 knockdown significantly abrogated TLBZT-induced cell senescence in LS174T cells, suggesting TLBZT-induced cell senescence is dependent on p21.

Transcription of p21 can be activated both in p53-dependent and -independent manners.^{26,27} The classical p53–p21 pathway is usually activated by DNA damage in response to genotoxic agents and other stimuli. Based on the nature of DNA damage, p53 may selectively discriminate between promoters in inducing target genes, thereby regulating their expression and promoting apoptosis, senescence, or autophagy.^{28,29} DNA-damaging drugs may induce p53–p21-dependent cell senescence in cancer cells.^{30–32} Unlike cytotoxic chemotherapeutic drugs, the herbs in TLBZT are nontoxic. As demonstrated previously, p53 expression does not change in LS174T cells upon TLBZT treatment.⁶ Meanwhile, siRNA knockdown of p53 did not affect TLBZT-induced p21 expression, suggesting that TLBZT upregulated p21 expression in a p53-independent manner.

In addition to p53 regulation, other mechanisms contribute to p21 upregulation, for example, BRCA1, E2F1, E2F3, and HDAC inhibitors.³³ HDAC inhibitors release HDAC1 from the Sp1 site and promote acetylation of histone H3 and H4 at the p21 promoter region, easing the access of transcription factors to induce p21 expression.²⁶ Sodium butyrate (NaBu), a well-known inhibitor of class I and II HDACs, upregulates p21 and induces cell senescence in various cancer cells.^{11,34,35} In this study, TLBZT inhibited HDAC activity; HDAC inhibitors, SVP and TSA, enhanced the effects of TLBZT on LS174T cell senescence. TLBZT also increased histone H3 and H4 acetylation at the p21 promoter

region. These findings suggested that TLBZT upregulates p21 expression through histone acetylation.

Conclusion

In summary, the current findings demonstrated that TLBZT induces cell senescence in LS174T cells in a mechanism dependent on p21 upregulation via histone H3 and H4 acetylation. This study provides new insights into the application of TLBZT for colorectal carcinoma treatment.

Disclosure

The authors report no conflicts of interest in this work.

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