Journal of Traditional and Complementary Medicine 13 (2023) 561–567

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Baicalin inhibits the replication of the hepatitis B virus by targeting TRIM25

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ARTICLE INFO

Article history:
Received 20 December 2022
Received in revised form
20 March 2023
Accepted 30 May 2023
Available online 31 May 2023

Keywords:
HBV
Baicalin
TRIIM25
IFIT3

ABSTRACT

Objective: Baicalin, which is a key bioactive constituent obtained from *Scutellaria baicalensis*, has been utilized in traditional Chinese medicine for many centuries. Although it has been reported that Baicalin (BA) can inhibit the replication of the Hepatitis B virus (HBV), the exact mechanism behind this process remains unclear. Interferon-stimulated genes (ISGs) are crucial in the process of antiviral defense. We aim to investigate whether BA can regulate the expression of ISGs, and thereby potentially modulate the replication of HBV.

Methods: The study involved the use of CRISPR/Cas9 technology to perform knockout experiments on TRIM25 and IFIT3 genes. The expression of these genes was confirmed through techniques such as immunoblotting or Q-PCR. The levels of HBsAg and HBeAg were measured using ELISA, and the expression of interferon-stimulated genes was detected using a luciferase assay.

Results: It is interesting to note that several ISGs belonging to the TRIM family, including TRIM5, TRIM25, and TRIM14, were induced after BA treatment. On the other hand, members of the IFIT family were reduced by BA stimulation. Additionally, BA-mediated HBV inhibition was found to be significantly restored in HepG2 cells where TRIM25 was knocked out. Additional research into the mechanism of action of BA found that prolonged treatment with BA activated the JAK/STAT signaling pathway while simultaneously inhibiting the NF-kB pathway.

Conclusion: The findings of our study indicate that TRIM25 has a significant impact on the regulation of HBV replication following BA treatment, providing additional insight into the mechanisms by which BA exerts its antiviral effects.

1. Introduction

Hepatitis B Virus (HBV) is a 3.2 kb DNA virus, which specifically targets hepatocytes by binding to the NTCP receptor. Hepatocytes are the cells that are infected by HBV and provide the site for the initiation of its life cycle. During the replication of HBV, seven proteins are expressed, including Small S, Middle S, Large S, polymerase, preCore, Core, and HBx.[1] The World Health Organization (WHO) reports that HBV infects almost 2 billion people globally, with over 240 million developing chronic infections. These chronic infections can lead to an increased risk of developing liver cirrhosis and eventually hepatocellular carcinoma (HCC). Despite the availability of a highly effective genetically engineered vaccine that has been used for nearly 30 years to prevent HBV infection, the virus continues to pose a significant public health threat.[2–4] HBV

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.
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https://doi.org/10.1016/j.jtcme.2023.05.009

infection is linked to rising death rates across the globe, and there are currently no curative therapies available for patients infected with HBV. This results in almost one million people dying each year due to severe chronic hepatitis, fibrosis, cirrhosis, and primary liver cancer.[5,6]

Our immune system responds to microbial infections by inducing three types of interferons: including type I, II, and III interferons. Interferon, especially type I interferon, production is among the initial defense mechanisms of the immune response.[7] The TRIM family members are essential in the induction of hundreds of interferon-stimulated genes (ISGs), which contribute significantly to the broad antiviral activity of type I interferons.[8] TRIM5α is known to block HIV-1 infection by targeting the HIV-1 capsid,[9] while TRIM5γ inhibits HBV replication by promoting the degradation of HBx in association with TRIM31.[10] Moreover, TRIM14 reduces HBV replication by binding to the C-terminal of the HBx protein and inhibiting the formation of the HBx-DDB1-Smc complex through its SPRY domain11. The IFIT gene family is a well-known group of interferon-stimulated genes that play a critical role in immune defense.[12] IFIT1 binds to the cap 0 (m (7)GppN) viral RNA and inhibits RNA translation,[13] and its C-terminal domain interacts with human IFIT3, which can modulate the protein stability of IFIT1 and also alter its RNA binding specificity, thereby blocking viral RNA translation.[14] Interestingly, IFIT3 was found to promote HBV replication in our previous study.[15]

Traditional Chinese Medicine (TCM) has been effectively used for the prevention and treatment of SARS-Cov2-infected patients since its outbreak in 2019,[16] as well as for SARS-Cov.[17] When compared to western medicine, herbal medicine has the advantage of being relatively inexpensive, readily available, and having fewer toxicities or side effects in clinical application.[18] As a flavonoid derived from *Scutellaria baicalensis*, Baicalin (BA) has a range of pharmacological functions, including antioxidant, anti-cancer,[19] and anti-inflammatory activities.[20] Moreover, research has reported that BA can inhibit HBV replication by downregulating the expression of HNF1α and HNF4α.[21] In this study, we found that BA has a dual role in regulating the expression of ISGs. It upregulates TRIM families including TRIM25, TRIM15, and TRIIM5γ, while downregulating IFITs, such as IFIT3, upon BA stimulation. It was suggested that the regulation of ISGs by BA may be attributed to STAT1 and NF-κB. Additionally, the inhibition of HBV replication by BA was found to be dependent on TRIM25 proteins, which were stabilized by BA. Our study provides additional information on how BA exhibits its antiviral activity and may encourage the use of TCM in the treatment of HBV-infected patients.

1.1. BA inhibits HBV replication

To study the impact of BA on HBV replication, we conducted a cell cytotoxicity assay to determine the appropriate dose range of BA (0–800 μM). HepG2 cells were treated with BA, and their viability was assessed using a CCK8 Kit after 24, 48, or 72 h of treatment. The results revealed that concentrations of the drug below 50 μM did not cause any significant damage to the cells (Fig. 1A–C). Thus, HepG2 cells transfected with pHBV1.2 plasmids were treated with BA at a concentration of either 25 or 50 μM. After 48 h of treatment, the levels of HBsAg in the supernatant were significantly inhibited by BA, and the levels of HBV DNA and pgRNA also showed similar inhibition (Fig. 1D–F). These data confirmed that BA acts as an inhibitor in the regulation of HBV replication.

1.2. BA regulates the expression of ISGs

The most crucial innate immune response to viral infection is the induction of interferons. Interferons induce the expression of hundreds of ISGs, which can obstruct different stages of the viral life cycle.[22] To investigate whether BA can regulate the expression of ISGs, HepG2 cells were treated with different doses of BA and for various time points. Q-PCR was performed to analyze the mRNA level of various members of TRIMs (TRIM14, TRIM25, and TRIM5) and IFITs (IFIT1, IFIT2, IFIT3, and IFIT5), which are known as classical ISGs. It was observed that the mRNA levels of TRIM25 was significantly induced but not of TRIM5, and TRIM14 was induced slightly (Fig. 2A), Additionally, the mRNA levels of IFITs were significantly inhibited by BA in a dose-dependent manner (Fig. 2B). We further verified the results by Western blot analysis and confirmed that the mRNA level of TRIM proteins was indeed induced (Fig. 2C), and conversely, that of IFIT was decreased (Fig. 2D). Considering all the results, it can be suggested that BA may regulate the expression of ISGs through different mechanisms.

1.3. TRIM25 is important in BA-mediated HBV inhibition

In our previous reports, we showed that TRIM25 and IFIT3 have opposite roles in regulating HBV replication.[10,15] Since we have observed that BA can regulate the expression of these two genes, we aim to further investigate if TRIM25 and IFIT3 have any role in the BA-mediated inhibition of HBV. Our data indicated that there was no significant difference between IFIT3 WT and KO HepG2 cells (Fig S1). However, as expected, BA inhibited HBV replication in HepG2 WT cells, while in the TRIM25 knockout cells, the levels of HBV DNA, pgRNA, and HBsAg were significantly increased compared to WT cells (Fig. 3A–D). Notably, the BA-mediated inhibition of HBV replication was rescued after TRIIM25 KO, indicating that TRIM25 played an important role in this process.

1.4. BA promotes TRIM25 expression and stabilizes the TRIM25 protein level

To investigate the mechanism by which BA regulates TRIM25 expression, HepG2 cells were treated with BA at different doses and cells were collected at different time points. The results indicated that TRIM25 was induced at later time points and high doses (Fig. 4A), which was consistent with the ISRE Luciferase results (Fig. 4B). Additionally, STAT1 was also activated after long-term BA treatment but not after short-term treatment (Fig. 4C–D). Moreover, by blocking protein synthesis with CHX, it was found that the TRIM25 protein in the HepG2 cells was significantly more stable after BA stimulation (Fig. 4E). Interestingly, we also found that NF-kB was inhibited by BA treatment in HepG2 cells (Fig S2), which might be the reason why IFIT3 expression was reduced after BA stimulation. Taken together, our data suggest that BA plays a dual role in the regulation of antivirus gene expression, and our proposed working model suggests that TRIM25 plays a crucial role in the inhibition of HBV replication mediated by BA (Fig. 5).

2. Discussion

HBV-related diseases continue to pose a serious threat to human health.[23,24] TCM has been used in clinical practice to treat a wide range of diseases,[25,26] including HBV infection. In this study, we found that BA, a monomer derived from Chinese medicine, can inhibit HBV replication by targeting TRIM25. TRIM proteins, including TRIM25, TRIM5γ, and TRIM14, were induced in HepG2 cells by BA stimulation. BA also stabilized the TRIM25 protein, leading to more effective inhibition of HBV replication. However, when TRIM25 was knocked out in HepG2 cells, the antiviral effects of BA against HBV were rescued. Interestingly, we also found that IFITs were downregulated by BA stimulation. Furthermore, prolonged BA stimulation activated STAT1, while NF-κB basal

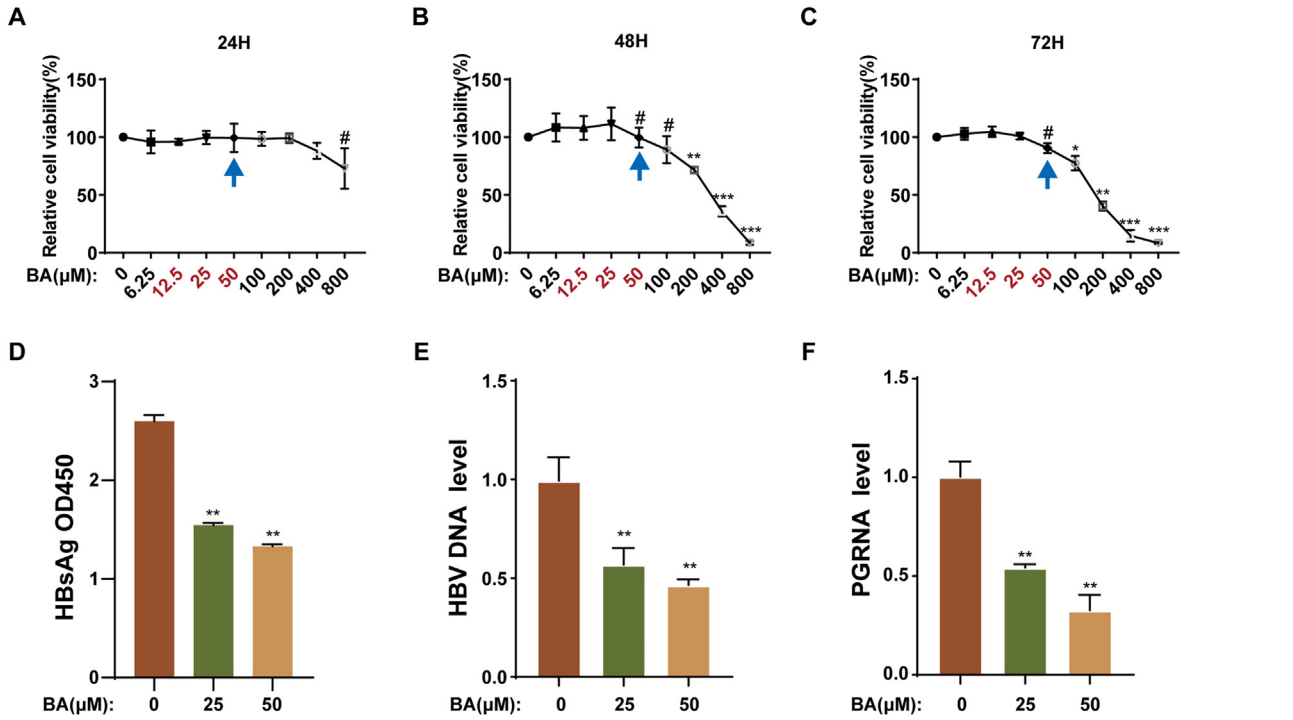

Fig. 1. Baicalin inhibits HBV replication (A–C) HepG2 cells were treated with different concentrations of baicalin. After 24, 48, or 72 h, CCK8 was added to detect cell viability. As shown in the figure, cell viability was expressed as the proportion of the control group. (D) Cellular models of HBV were constructed by transfecting HepG2 cells with pHBV1.3. The cells were treated with baicalin for 48 h and the supernatant was collected to perform ELISA aimed at detecting the expression of HBsAg. (E, F) HepG2 cells were transfected with pHBV1.3, and the cells were treated with baicalin for 48 h. Cell samples were collected for q-RT PCR to detect HBV DNA and pgRNA. Data were obtained from the mean ± SD of three independent trials. The results were analyzed by Student's t-test. *p < 0.05; **p < 0.01; ***p < 0.001.

activation was inhibited. These findings shed more light on the crosstalk of TCM with HBV.

IFN-α is commonly used as the first-line drug in the clinical treatment of HBV-infected patients.[27] By inducing the expression of hundreds of ISGs,[28] IFN inhibits the infection and replication of a variety of viruses, including HBV, SARS, EBV, etc.[10,11,29,30] STAT1 is a key transcription factor that plays a critical role in the induction of ISGs. In HepG2 cells stimulated with BA, we observed STAT1 activation after 24 h of treatment, which differs from the activation pattern observed in cells stimulated with IFN. This observation is in line with the kinetics of TRIM25 expression, as we found that TRIM25 mRNA was only induced after 24 h of stimulation. Interestingly, IFIT family members were reduced by BA stimulation. As is already known, IFITs are also ISGs that are significantly upregulated by IFNs; however, we previously found that IFITs were induced by HBx, which is an activator of NF-kB. Since BA has been shown to inhibit NF-kB activation,[31] it may play a dual role in regulating the induction of ISGs. On the one hand, it stimulates STAT1 and induces TRIM expression. On the other hand, it reduces IFITs by inhibiting NF-kB activation.

TRIM25 functions as an E3 ligase and plays a broad and essential role in the antiviral process.[32] As an ISG, TRIM25 has been shown to amplify the production of interferons.[33] We have reported the function of TRIM25 in the inhibition of HBV replication. TRIM25 was induced by IFN treatment in a STAT1-and STAT3-dependent manner. STAT3 also plays an indispensable role in IFN-mediated HBV inhibition.[34] In this study, we find Chinese herb BA can inhibit HBV replication through TRIM25. More research is required to investigate the precise mechanism by which BA or TRIM25 inhibits HBV replication. This could reveal additional targets for the development of therapeutic drugs to treat hepatitis B.

2.1. Cell culture, plasmids, and reagents

Human Embryonic Kidney 293 T (HEK293T) cells and HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum that had been inactivated, as well as penicillin (100 IU/mL) and streptomycin (100 mg/mL), and were incubated at 37 °C in a 5% CO_2 atmosphere.[35] The pHBV1.3 plasmid was provided by Dr. Lishan Su from the University of North Carolina and the pHBV1.2 plasmid was provided by Prof. Qiang Deng from Fudan University. The ISRE-Luc reporter plasmid was from Promega. Baicalin (HPLC≥98%) was purchased from Shanghai Yuanye Biotechnology Co. IFIT1, IFIT2, IFIT3, and IFIT5 antibodies were bought from SAB, TRIM25, p-STAT1, IκBα, *p*-IκBα, and TRIM5 antibodies were purchased from Cell Signaling Technology, and CHX (Cycloheximide) was from Sigma (C7698, Sigma).

2.2. Cell counting Kit-8 (CCK8)

The cells were seeded into 96-well plates and treated with various concentrations of baicalin in a complete medium containing 10% FBS and Penicillin-Streptomycin Solution, with a volume of 200 μl per well. The cells were treated with cck8 reagent at 24, 48, and 72 h of incubation and then incubated in a cell incubator at 37 °C for 1 h.[36] The absorbance at 450 nm was measured, and cell viability was calculated as a percentage of the control.

2.3. RNA extraction and quantitative real-time PCR (Q-PCR)

Total cellular RNA was extracted using Trizol according to the manufacturer's instructions. cDNA was synthesized using the TransScript First Strand cDNA Synthesis SuperMix kit (AT341-02, Transgen, China).[11] GAPDH was used as an internal reference

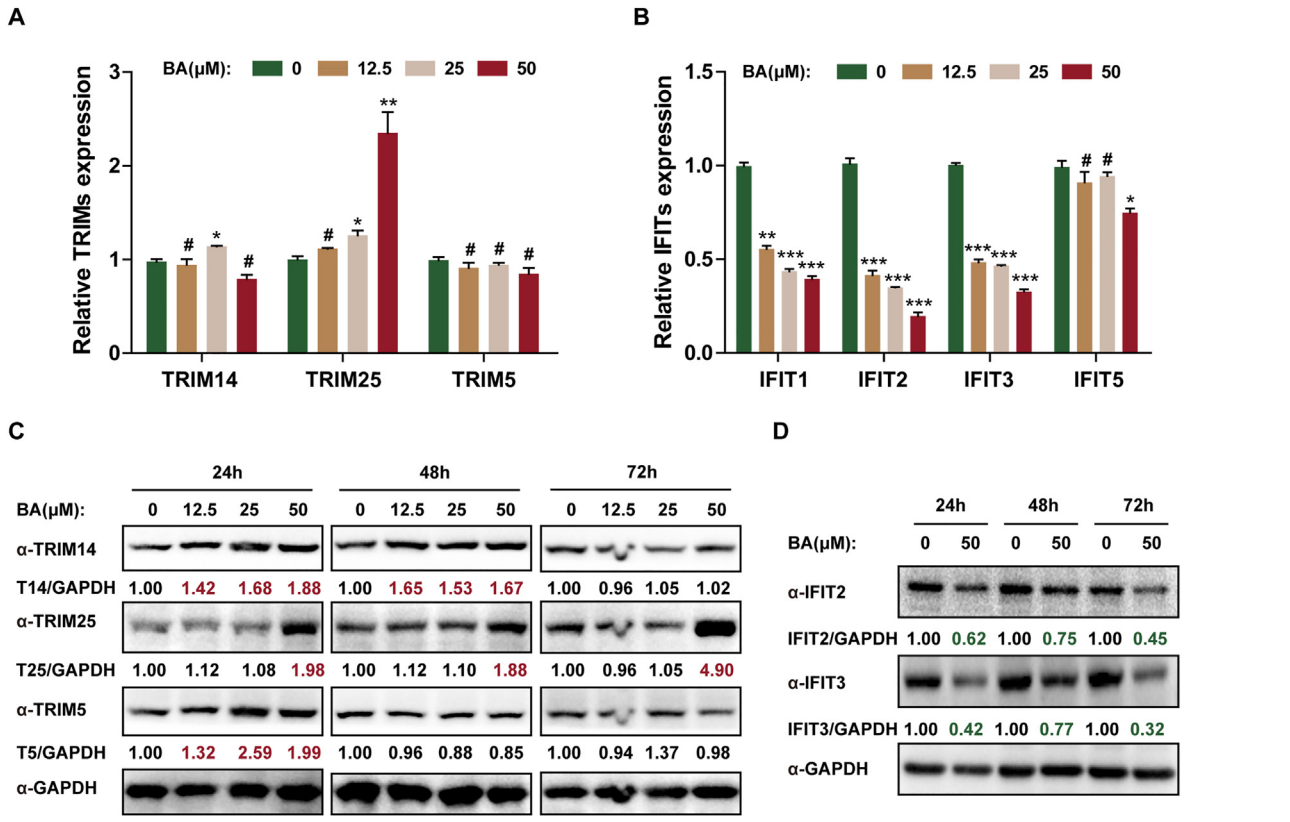

Fig. 2. Baicalin regulates the expression of Interferon-Stimulated Genes (A, B) HepG2 cells were transfected with PHBV1.3 plasmids, and the cells were treated with baicalin for 48 h. After the cells were harvested, total RNA was extracted and q-RT PCR was performed. (C) HepG2 cells were treated with different concentrations of baicalin. The effects of baicalin on the expression of TRIM14, TRIM25, and TRIM5 were evaluated after 24 h, 48 h, and 72 h, respectively. (D) HepG2 cells were treated with baicalin (50 μM) and the effect of baicalin on IFIT2 and IFIT3 proteins was evaluated at different treatment times. Data were obtained from the mean ± SD of three independent trials. The results were analyzed by Student's t-test. *p < 0.05; **p < 0.01; ***p < 0.001.

control, and real-time fluorescence quantitative PCR was performed in an ABI StepOne Plus Real-Time Quantitative Fluorescence PCR System (Applied Biosystems). The primer sequences used are listed in Supplementary Table 1.

2.4. CRISPR/Cas9 knockout

HepG2 cells were plated in 24-well plates for 16 h. Subsequently, they were transfected with plasmids encoding a puromycin-resistance gene, as well as Cas9 and TRIM25-or IFIT3-targeting sgRNA, using the Viafect transfection reagent (E4982, Promega). The selection of cells that were resistant to puromycin (2 μg/mL) was initiated 36 h after transfection. Thereafter, the cells were analyzed using specific antibodies through the process of Western blotting.[36] After a 2-day selection period, single-cell cloning was performed via serial dilution, with clonal cells plated and cultured in 96-well plates. After clonal lines had proliferated sufficiently, gene knockout was again confirmed via Western blotting, and this result was further verified through DNA sequencing of selected clones. The sgRNA sequences used are provided in Supplementary Table 1.

2.5. Western blotting

The cells were collected and experiments were conducted while keeping them on ice. Lysis buffer (20 mM HEPES, 20% glycerol, 1% NP-40, 1 mM MgCl2, 350 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, and 0.5 mM DTT) was added to the cells, and the samples were tapped every 10 min. After 30 min, the protein concentration of the samples was determined using Coomassie Plus Protein Assay Reagent (Thermo Scientific).[11] The protein samples were separated using SDS-PAGE and then transferred onto PVDF membranes. The blots were blocked with 5% skim milk prepared with BSA, and the membranes were then incubated with different antibodies.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Cells were transfected with plasmid pHBV1.2 or pHBV1.3 and treated with different concentrations of baicalin. Supernatants were collected after 48 h. The kit instructions were followed to detect the levels of HBsAg and HBeAg.[37]

2.7. Dual-Luciferase Reporter Assay

pGL4.45 ISRE-Luc plasmids were transfected into HepG2 cells along with the pGL4.74 Tk-Rluc reporter. After 24 h, the cells were treated with BA. Post treatment, the cells were lysed using a passive lysis buffer, and a Dual-Luciferase Reporter Assay System (E1910, Promega) was used to measure firefly and Renilla luciferase activity in these samples.[37]

Quantification and statistical analyses

GraphPad Prism 8 software (GraphPad Software, San Diego, CA) was used for data analyses; a two-tail unpaired *t*-test was used to assess between-group differences. $p < 0.05$ was considered to indicate statistical significance.[38]

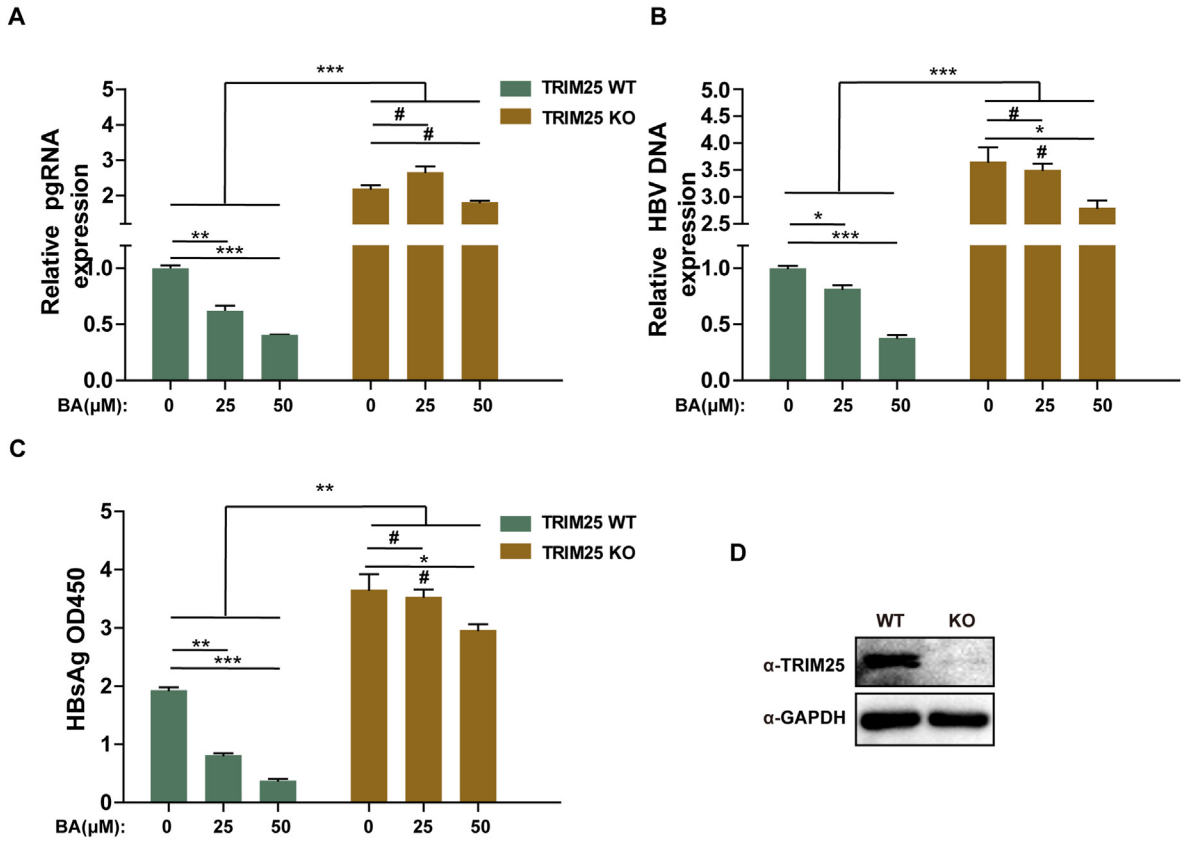

Fig. 3. TRIM25 plays an important role in the Baicalin-mediated HBV inhibition (A–C) HepG2 WT or TRIM25 KO cells were transfected with PHBV1.2 plasmids and treated with baicalin for 48 h. Total RNA was extracted for q-RT PCR, and the effect of baicalin on HBV is shown in the figure. The supernatant was collected for ELISA, which was then used to measure the level of HBsAg. (D) Difference in TRIM25 protein expression between TRIM25 KO HepG2 cells and wild-type cells is shown as indicated. Data were obtained from the mean ± SD of three independent trials. The results were analyzed by Student's t-test. *p < 0.05; **p < 0.01; p < 0.001.

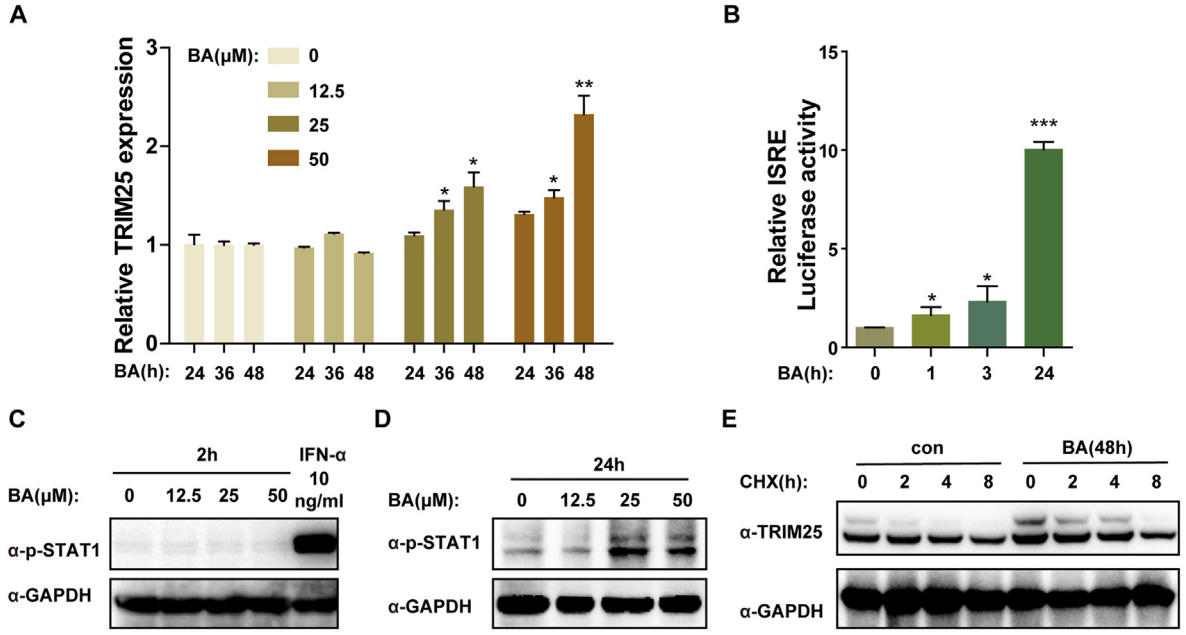

Fig. 4. Baicalin stabilizes TRIM25 expression and promotes TRIM25 expression through prolonged stimulation (A) HepG2 cells were treated with different concentrations of baicalin for 24 h, 36 h, and 48 h to assess the effect of baicalin on TRIM25 expression. (B) Co-transfection of the pGL4.7 TK-Luc reporter gene with ISRE-Luc or NF-κB luciferase reporter genes into HepG2 cells. The cells were collected 48 h after transfection and treated with baicalin (50 μM) for 1 h, 3 h, and 24 h before the collection of cell samples. The luciferase activity was then measured. (C, D) HepG cells were treated for 2 h or 24 h with baicalin, and the effect of baicalin on p-STAT1 was assessed using Western blot. IFNα (10 ng/mL) was used as a positive control. (E) HepG2 cells were transfected with Flag-TRIM25, and the cells were treated with baicalin for 48 h after transfection. CHX (10 μM) was added at 2 h, 4 h, and 8 h before the harvesting of cells. (F) HepG2 cells were treated for 2 h with baicalin, and the effect of baicalin on the NF-κB signaling pathway was assessed using Western blot. Data were obtained from the mean ± SD of three independent trials. The results were analyzed by Student's t-test. *p < 0.05; **p < 0.01; ***p < 0.001.

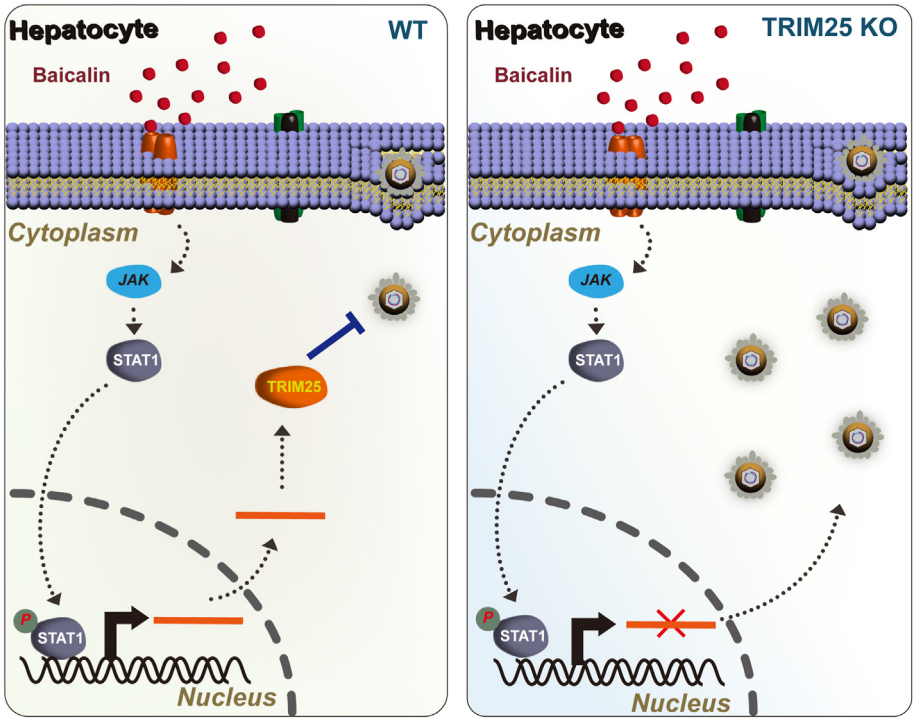

Fig. 5. Model that suggests baicalin can inhibit HBV replication by targeting TRIM25.

Data and code availability

The published article includes all datasets generated or analyzed during this study.

Author contributions

X.F. performed the experiments and drafted part of the paper; H.S. set up experiments for the Dual-Luciferase Reporter Assay; F.X. performed the CRISPR/Cas9 Knockout experiments; Q.W. and B.L. provided technical assistance and facility; and G.T. conceived the research project and wrote the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the 68th batch of first-class funding from the China Postdoctoral Science Foundation, Jilin Provincial Joint Fund Bethune Medical Project and an International cooperation project of Jilin Provincial Science and Technology Department (Grant Nos. 2020M680044, 20200201340JC and 20220402085GH to GT), the National Natural Science Foundation, China (Grant No. 81901592 to HS), Jilin University Excellent Young Teacher Training Program to GT.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2023.05.009.

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