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Original Article

Triptolide Inhibits HIV Transcription Elongation through Ubiquitin-Dependent Proteasomal Degradation of Viral Tat Protein

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Abstract:
Tat protein, one of human immunodeficiency virus (HIV-1) specifically encoded gene products, plays a crucial role in HIV-1 transcription, which functions as "on and off" switch in HIV-1 latency and activation. Previous study has shown that triptolide (TPL) inhibits HIV-1 replication by promoting proteasomal degradation of Tat protein, but the mechanism of TPL-induced Tat degradation still remains elusive. Here, we compared with other active ingredients extracted from *Tripterygium wilfordii*, and found that TPL has a better effect on inhibition in HIV-1 transcription in a time and dose dependent manner. Our study indicates that TPL induced Tat degradation in an ubiquitin proteasome pathway, which is neither through blocking CycT1 protection for Tat, nor affecting transcription or translation of Tat. CUL4B is involved in Tat degradation under natural condition while not TPL treatment. Although mass spectrometry shows that TPL induces Tat K19 and K71 ubiquitination, single site mutation of either K19 or K71 as well as other lysine residues does not protect Tat from TPL, except that K41R shows partial resistance to TPL-induced Tat degradation. SILAC (stable isotopic labeling by amino acids in cell culture) analysis indicates that 17 E3 ubiquitin ligases maybe involved in TPL-induced Tat degradation. These findings provide novel insight to block HIV replication by promoting Tat degradation.

Keywords: Tat, TPL, Ubiquitin proteasome degradation, Ubiquitination, E3 ligase

1. Introduction

Human Immunodeficiency Virus (HIV) is the cause of the spectrum of disease known as Acquired Immunodeficiency Syndrome (AIDS), which has claimed 40.1 million lives worldwide so far according to WHO. HIV is currently treated with antiretroviral therapy (ART) consisting of one or more medicines. ART does not cure AIDS but reduces the viral load to an undetectable level[1]. However, discontinuation or interruption of ART may result in viral rebound. Furthermore, antiretroviral drugs are at risk of becoming partially or fully inactive due to the emergence of drug-resistant virus[2]. As AIDS continues to be a major global public health issue, novel therapies to effectively restrict HIV are urgently needed.

The promoter-proximal pausing of RNA polymerase II (Pol II) on the integrated HIV proviral DNA has long been considered as a major rate-limiting step in viral transcription[3]. The HIV-encoded Tat protein recruits the host Super

Elongation Complex (SEC) to the paused Pol II by binding to the 5' end of the nascent viral RNA[4]. The formation of the Tat-SEC on the viral TAR RNA structure leads to activation of HIV transcriptional elongation[5]. In the absence of Tat, transcription initiates normally at the 5' long terminal repeat (LTR) but results in short, abortive viral transcripts[6]. In other words, Tat is essential for the expression of HIV genes in host cells.

There have been studies that focus on Tat degradation, which could potentially be utilized as HIV therapies. For instance, natural product triptolide caused Tat degradation and inhibited HIV replication[7]. Triptolide is extracted from plant *Tripterygium wilfordii*, which is a vine used in traditional Chinese medicine[8]. Besides triptolide (TPL), other bioactive compounds derived from *Tripterygium wilfordii* include Triponide, Triptophenolide, Celastrol, Wilforlide A, and Demethylzeylasteral, etc. Although TPL has been reported to be able to inhibit HIV replication through inducing proteasomal degradation of Tat, several questions remain unanswered: is the degradation is ubiquitin dependent, what are the E3 ubiquitin ligases involved in this process, which site is ubiquitinated?

In this study, we first identify triptolide as the most effective compound among *Tripterygium wilfordii* extracts. We show that TPL inhibits HIV transcription at the elongation stage. Our data demonstrate that triptolide inhibits HIV replication by promoting Tat in ubiquitin-dependent proteasomal degradation, while not affecting other P-TEFb partners degradation or CycT1 stabilization to Tat. Through mutagenesis, we identify K41 on Tat contributes partly to TPL-induced degradation. We also provide a list of candidates of E3 ligases that might be involved in Tat degradation.

1. Materials and Meathods

1.1. Western Blot Analysis

Briefly, cell lysates were harvested and boiled in loading buffer (0.08 M Tris, pH 6.8, with 2.0% SDS, 10% glycerol, 0.1 M dithiothreitol [DTT], and 0.2% bromophenol blue) and SDS-PAGE sample buffer followed by separation on a 10 to 12% polyacrylamide gel. Proteins were transferred onto nitrocellulose (NC) or PVDF membranes for Western blot analysis. After 48h, cells were lysed and immunoblotted with indicated antibodies. The primary antibodies used and the final dilutions were as follows: ELL2 (Bethyl Laboratories, A302-505A, 1: 1000), CycT1 (Santa Cruz Biotechnology, sc-10750, 1:1000), Anti-BRD4, -LARP7, -HEXIM1 and -CDK9 were generated in our own laboratory and have been described previously [27].

1.2. Luciferase Assay

For the luciferase, it is performed in the Hela-based NH1 and NH2 cell lines containing an integrated HIV-1 LTR-lucifersase reporter gene without or with Tat expression. Luciferase activities were measured using kit E1501 from Promega. Lysates were prepared from approximately equal amount of cells and normalized based on α–tubulin [28].

1.3. SiRNA Transfection

For efficient RNAi knockdown of human CUL4B, CUL4B siRNA1 (5'-GCC ACG UAC CGA UAC AGA ATT-3'), siRNA2 (5'-GGA GUU AUU UAG GGC UCA UTT-3'), siRNA3 (5'-GCA GAC AGA CUU AUU ACU UTT-3') and a scrambled siRNA control were synthesized and transfected into HEK293 cells using Lipofectamine 3000 transfection reagent (Life technologies).

1.4. Co-immunoprecipitation

Briefly, for anti-Flag IP, nuclear extracts (NEs) prepared from HeLa cells transfected with the indicated expressing constructs were incubated with anti-Flag agarose beads (Sigma) for 2 hr prior to washing and elution. For precipitations of endogenous proteins, NEs were incubated overnight with the specific antibodies or total rabbit IgG and then with protein A beads (Invitrogen) for 1 hr. After incubation, the immunoprecipitates were washed extensively with Buffer D (20 mM HEPES-KOH [pH7.9], 15% glycerol, 0.2 mM EDTA, 0.2% NP-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluride, and 0.3 M KCl) before elution with 0.1M glycine (pH 2.0). The eluted materials were analyzed by Western blotting with the indicated antibodies [29].

1.5. PCR

The primers used to generate site mutagenesis for Tat are designed by central overlapping primers

(COP) method (Wang et al., 2011)[30]. The primers are listed as flows, K12R-F, 5'-GAT CCT AGA CTA GAG CCC TGG AGG CAT CCA GGA AGT CAG CCT AAA AC-3', K12R-R, 5'-CTT CCT GGA TGC CTC CAG GGC TC-3'; K19R-F, 5'-GAA GCA TCC AGG AAG TCA GCC TAG AAC TGC TTG TAC CAA TTG CTA TTG-3', K19R-R, 5'-GTA CAA GCA GTT CTA GGC TGA CTT C-3'; K28/29R-F, 5'-CTG CTT GTA CCA ATT GCT ATT GTA GAA GGT GTT GCT TTC ATT GCC AAG TTT G-3', K28/29R-R, 5'-TGA AAG CAA CAC CTT CTA CAA TAG CAA TTG-3'; K41R-F, 5'-CAT TGC CAA GTT TGT TTC ATA ACA AGA GCC TTA GGC ATC TCC TAT GGC-3', K41R-R, 5'-TGC CTA AGG CTC TTG TTA TGA AAC A-3'; K50R-F, 5'-CTT AGG CAT CTC CTA TGG CAG GAG GAA GCG GAG ACA GCG ACG AAG-3', K50R-R, 5'-GTC TCC GCT TCC TCC TGC CAT AG-3'; K51R-F, 5'-GGC ATC TCC TAT GGC AGG AAG AGG CGG AGA CAG CGA CGA AGA CC-3', K51R-R, 5'-CTG TCT CCG CCT CTT CCT GCC A-3'; K71R-F, 5'-CAG ACT CAT CAA GTT TCT CTA TCA AGG CAA CCC ACC TCC CAA TCC C-3', K71R-R, 5'-GGT GGG TTG CCT TGA TAG AGA AAC-3'; K85R-F, 5'-GGG GAC CCG ACA GGC CCG AGG GAA GTC GAC ATG GAC TAC AAG-3'; K85R-R, 5'-TGT CGA CTT CCC TCG GGC CTG T-3'.

1.6. Quantitative PCR

The reactions were performed with Applied Biosystem 7300 Real-Time PCR System and Finnyzme F-410L SYBR Green RT-PCR reagents according to the manufacturers' instructions. Total mRNA was extracted and reverse transcription was performed. Then, the cDNA was subjected to Tat gene amplification using the primers used for qRT-PCR are as follows, Tat 5'-ATG GAG CCA GTA GAT CCT AGA C-3' and 5'-ACT TGG CAA TGA AAG CAA CAC-3'; Nascent region 5'-GTT AGA CCA GAT CTG AGC CT-3' and 5'-GTG GGT TCC C TA GTT AGC CA-3'; Promoter proximal region 5'-GTT AGA CCA GAT CTG AGC CT-3' and 5'-GTG GGT TCC CTA GTT AGC CA-3'; GAPDH 5'-AGG TGA AGG TCG GAG TCA AC-3' and 5'-CGC TCC TGG AAG ATG GTG AT-3'. PCR conditions include an initial denaturing step at 92℃ for 2 min and then 40 (for qRT-PCR) cycles of amplification. Each cycle consists of a 92℃ segment of 30 sec, a 57℃ segment of 30 sec and then a 68℃ segment of 30 sec. For qRT-PCR, the values were normalized to those of GAPDH to obtain the relative folds of induction. All reactions were run in triplicates.

1.7. MS Data Analysis

SILAC and MS data analysis was performed as previously described [31][32].

1.8. Statistical Analysis

Student's t tests were used for statistical comparisons between two groups. Multiple group means were compared by one-way ANOVA. Asterisks above a column indicate comparisons between a specific genotype and wild type, whereas asterisks above a bracket denote comparisons between two specific genotypes. Data are presented as mean ± standard error of the mean (SEM). P-values < 0.05 were considered statistically significant.

3. Results

3.1. Triptolide inhibits HIV transcription more potently than other *Tripterygium wilfordii* extracts

Previous studies reported that some compounds derived from *Tripterygium wilfordii* have potential as antivirals[9]. We first compared the effects of several compounds extracted from the traditional Chinese medicine *Tripterygium wilfordii*, including Triptolide (TPL), Triponide, Triptophenolide, Celastrol, Wilforlide A, and Demethylzeylasteral, on HIV transcription inhibition (Fig.1A). HIV-LTR driven luciferase activity was most effectively suppressed by TPL. Other substances had little to no effect on HIV-LTR activity at the same dose as nanomolar TPL, which resulted in a 90% decrease (Fig.1B).

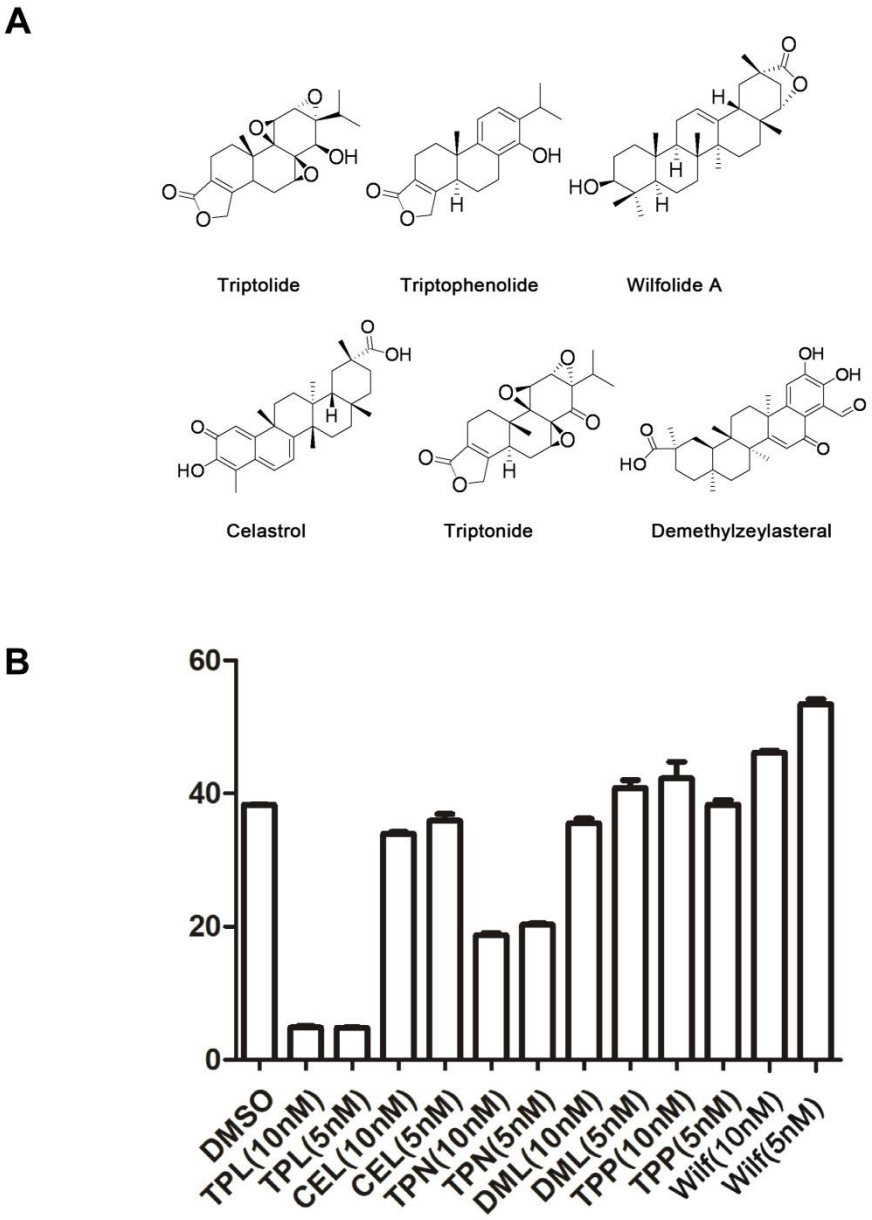

Fig. 1. TPL has a good effect in HIV-1 transcription inhibition compared with other compounds. (A) Chemical structure of several ingredient derived from Tripterygium wilfordii. (B) Comparison of different tested molecule with TPL in HIV-1 transcription. Luciferase assay was performed 24h after drug treatment in NH2 cells. NH2 cells were incubated with different compound in dosage-dependent. NH2, Hela based HIV-1 LTR-luciferase reporter gene stably expressing cells. Each experiment was repeated for at least three times. No asterisk denotes p>0.05, *, p<0.05, Student's t-test. Data are presented as means ± SEM.

3.2. Triptolide inhibits HIV transcription elongation in a time- and dose-dependent manner

Next, we set out to investigate whether TPL affects transcription elongation, which is a key step that regulates HIV gene expression. The inhibition of HIV-LTR luciferase activity appeared somewhere between 12 h and 16 h and continued toward 24 h (Fig.2A). Treatment by a serial concentration of TPL showed that the inhibition was dose-dependent, which coincide with the mRNA level of luciferase gene, indicating that it was transcription rather than protein level that was affected by TPL (Fig.2B&C). Treatment of TPL didn't cause the cleavage of PARP1, suggesting no apoptosis occurred under those conditions (Fig.2D). In

addition, a reduction in the level of phosph-Serine2 of Pol II CTD, which reflects the status of the transcription elongation, was observed upon the treatment of TPL (Fig.2D). We next conducted a qRT-PCR-based assay that measured the relative abundance of HIV transcripts at several different locations downstream of the viral transcription start site (Fig.2E). TPL treatment displayed minor inhibitory effect on short transcripts, whereas its inhibition of the synthesis of longer transcripts at downstream locations was much more pronounced, indicating that the process of elongation was impaired (Fig.2F). Taken together, these data revealed that TPL most likely inhibited the elongation stage of HIV transcription.

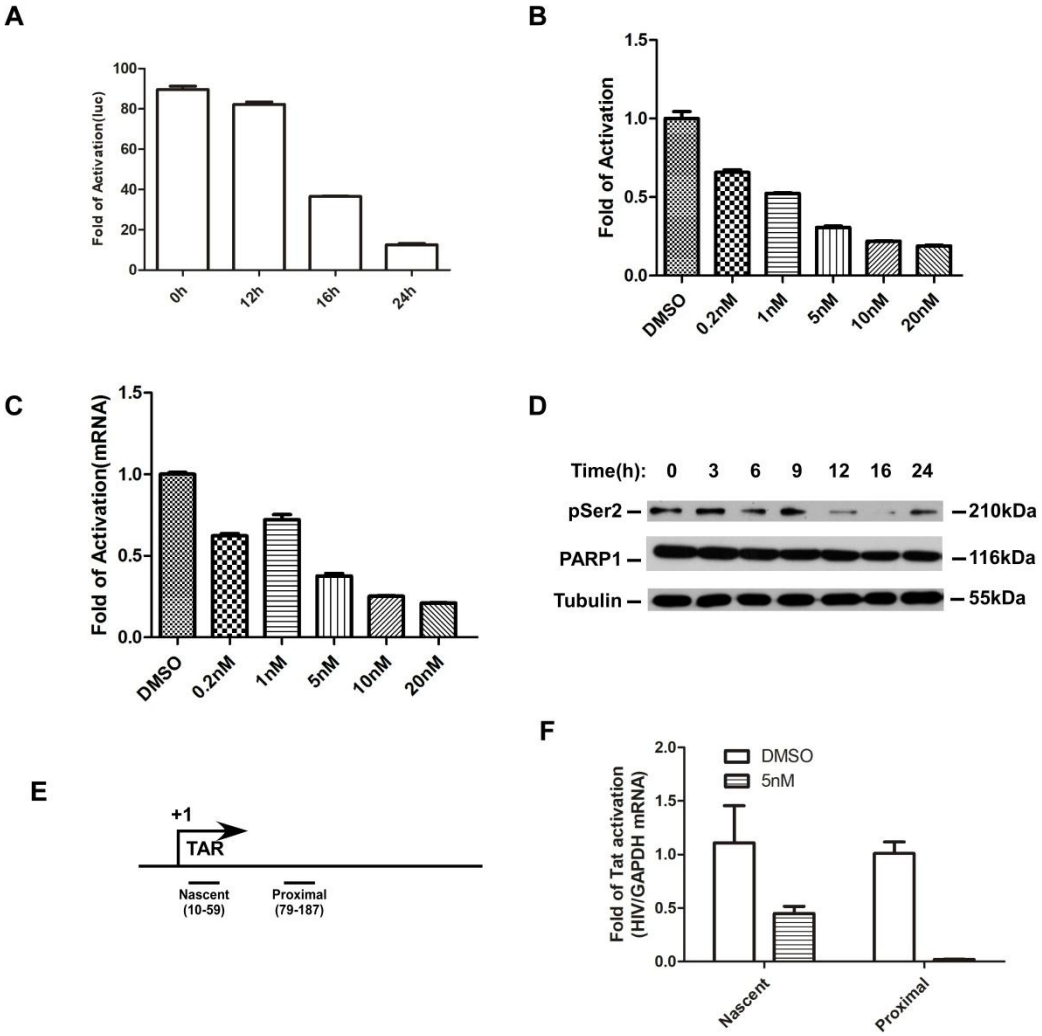

Fig. 2. TPL inhibits HIV-1 transcription in time and dose dependent manner. (A) Representation of luciferase expression treated with different time of TPL. NH2 cells were incubated with 10 nM TPL for 0, 3h, 6h, 9h, 12h, 16h, and 24h. (B) TPL induces Pol II phosphorylation downregulation. Samples were lysed for further analysis by western blotting with indicated antibodies. Pol II, polymerase II. (C) Detection of luciferase activity treated with different concentrations of TPL. NH2 cells were collected at 24h with TPL treatment in 0, 0.2nM, 1nM, 5nM, 10nM and 20nM. Luciferase activities were measured in extracts of NH2 cells containing the integrated HIV-1 LTR-luciferase reporter gene and expressing the indicated proteins. (D) Level of luciferase mRNA treated with different concentrations of TPL. mRNA transcripts from integrated HIV-1 LTR-luciferase reporter gene were analyzed by qRT-PCR with indicated primers and normalized to GAPDH. Each experiment was repeated for at least three times. No asterisk denotes $p>0.05$, *, $p<0.05$, Student's t-test. Data are presented as means ± SEM.

3.3 Triptolide induces HIV Tat degradation but not host P-TEFb associated proteins

P-TEFb exists in different protein complexes that include super elongation complex (SEC), 7SK snRNP and Brd4/P-TEFb, which further form a dynamic network[10]. Considering that P-TEFb is indispensable in HIV transcription, we then checked whether TPL affected the levels of P-TEFb and its associated proteins. Surprisingly, P-TEFb itself (CDK9 and CycT1), as well as SEC (ELL2), 7SK snRNP (HEXIM1 and LARP7) and Brd4, remained unchanged after different concentrations of TPL treatment (Fig.3A). As reported, TPL decreased the viral Tat protein, which is required for full HIV transactivation (Fig.3B). The mRNA level of Tat remained constant in the presence of TPL, indicating the reduction of Tat occurred solely on protein level (Fig.3C). Given that the formation of the Tat-SEC leads to activation of HIV transcriptional elongation, the reduction of Tat would result in less SEC on viral TAR RNA and inhibited HIV transcription.

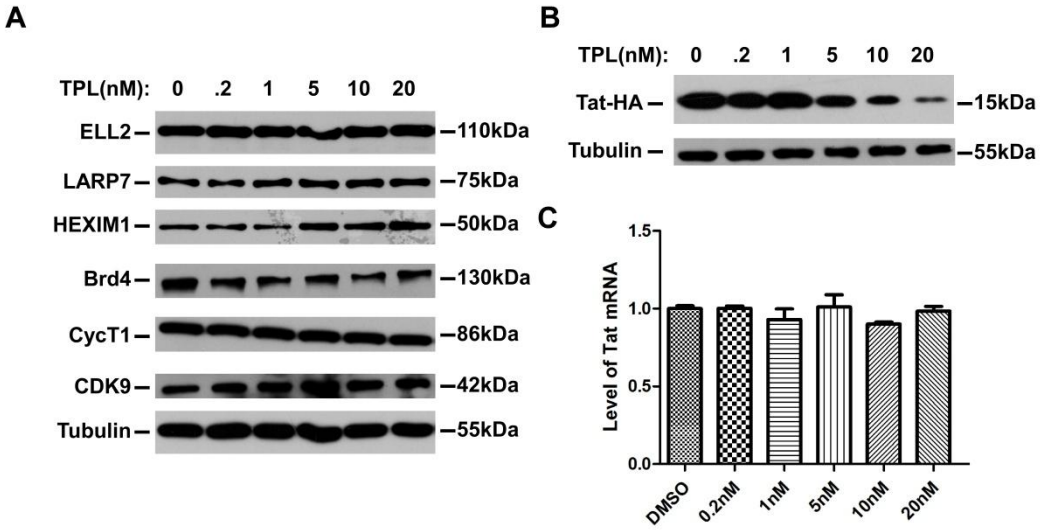

Fig. 3. TPL promotes Tat degradation while not affecting mRNA. (A) TPL does not affect expression of other P-TEFb associated factors. Cells were incubated with TPL in 0, 0.2nM, 1nM, 5nM, 10nM, 20nM for 24h. Samples were lysed for further analysis by western blotting with indicated antibodies. Tubulin was used as an internal control. (B) TPL promotes Tat degradation in dose dependent manner. 250 ng/well Tat-HA was transfected to NH1 cells in 12 well plate. Protein expression were measured in extracts of NH1 cells containing the integrated HIV-1 LTR-luciferase reporter gene. (C) Tat mRNA level was not changed with TPL treatment. Cells were collected for mRNA extraction and reverse transcription. Tat mRNA were analyzed by qRT-PCR with indicated primers and normalized to GAPDH. Each experiment was repeated for at least three times. No asterisk denotes $p>0.05$, *, $p<0.05$, Student's t-test. Data are presented as means ± SEM.

3.4. Triptolide induces Tat degradation regardless of CycT1 binding

It is reported that interacting with CycT1 promotes Tat stability[11], [12]. Wild-type Tat binds to CycT1 on its Tat/TAR recognition motif [13]. With a point mutation in the region, Tat-C22G has impaired binding to CycT1, which means C22G is unstable with less protection from CycT1[14]. Indeed, under normal condition, there was less C22G expression despite same amount of plasmids transfected (Fig.4A, compare lane 1 and lane 3). However, regardless of CycT1 binding ability, TPL caused both WT and C22G degradation (Fig.4A). As a result, TPL suppressed WT Tat mediated HIV transcription as well as C22G activity (Fig.4B).

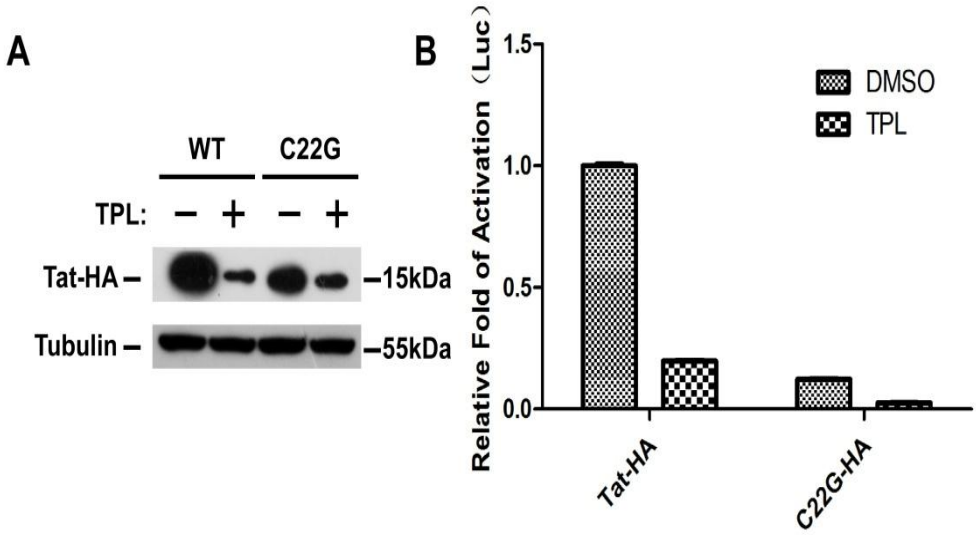

Fig. 4. TPL promotes Tat breakdown not through blocking CycT1 stabilization to Tat. (A) TPL accelerates Tat(C22G) degradation. Protein expression were determined using NH1 cells with Tat-HA and Tat(C22G)-HA transfected respectively. Cells were incubated with 10 nM TPL for 24h. (B) TPL inhibits Tat(C22G)-induced HIV-1 transcription activation. Luciferase activities were analyzed after 10 nM TPL treatment. Each experiment was repeated for at least three times. No asterisk denotes p>0.05, *, p<0.05, Student's t-test. Data are presented as means ± SEM.

3.5. Triptolide induces ubiquitin-dependent proteasomal degradation of Tat

To determine whether TPL decreases Tat protein level by proteasomal degradation, we treated cells with proteasome inhibitor MG132. While TPL alone decreased Tat protein level, treatment with MG132 blocked this process, suggesting that Tat degradation was likely mediated by proteasome (Fig.5A). To determine whether this process is ubiquitin dependent or not, we observed that PYR-41, an E1 ubiquitin-activating enzyme inhibitor, also rescued the degradation of Tat by TPL, further confirming this was a ubiquitin-dependent proteasomal degradation (Fig.5B).

Previous studies showed that E3 ligase CUL4B has been reported to be involved in Tat degradation, we asked whether CUL4B played a role in TPL induced Tat degradation[15]. First, three siRNAs that target different regions of CUL4B mRNA were tested for their knock-down efficiency. siCUL4B #3 was picked for subsequent experiments (Fig.5C). Knock-down of CUL4B indeed increased Tat protein in normal condition (Fig.5D, lane 1 vs lane 2). However, Tat was still degraded by TPL even when most CUL4B was depleted, indicating CUL4B was not involved in TPL induced Tat degradation (Fig.5D, lane 2 vs lane 4).

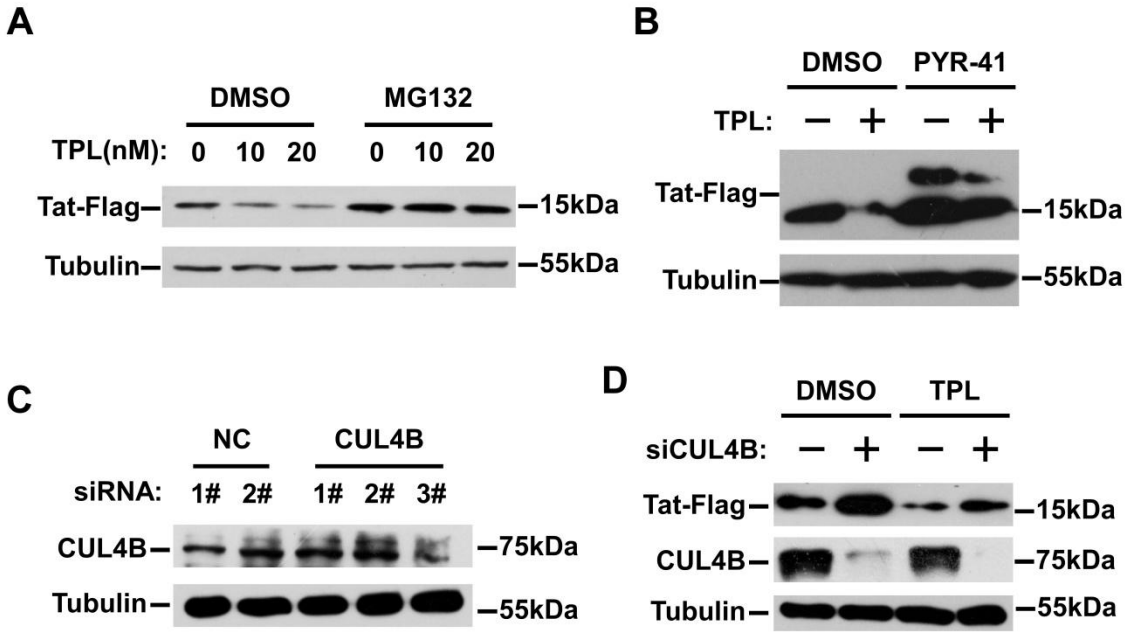

Fig. 5. TPL promotes Tat degradation in ubiquitin proteasome pathway. (A & B) Western blot assay was performed with MG132 or PYR-41 treatment. Cells transfected with Tat-flag were treated with indicated MG132 or PYR-41, and then collected after TPL treatment. The protein level was determined by indicated antibodies. (C) Comparison of different CUL4B siRNA knockdown efficiency. Protein expression were analyzed using cells transfected with different siRNA targeting to CUL4B. (D) Detection of Tat degradation with TPL treatment after CUL4B knockdown. Protein were subjected to western blotting with the indicated antibodies.

3.6. More than one ubiquitination sites on Tat are responsible for its degradation induced by TPL

The data presented so far have revealed that ubiquitination is important in TPL induced Tat degradation. To identify potential ubiquitination sites, Tat protein was purified and subjected to mass spectrometry (MS) analysis. Two lysine residues on Tat, namely K19 and K71, were found to be readily ubiquitinated with TPL treatment (Fig.6B). However, single site mutation of either of the two lysine to arginine didn't affect TPL-induced degradation (Fig.6C, right). In order to determine which lysine residue on Tat was responsible for Tat degradation, we generated a series of mutants where every single lysine (K) was substituted to arginine (R), respectively. While K50R retained as much activity as WT Tat, most K/R mutants displayed certain levels of inhibition (Fig.6A). However, the suppression by TPL remained effective on all mutants (Fig.6A). Compared to WT Tat, most K/R mutants displayed similar levels of degradation after TPL treatment except for K41R, which showed some resistance to TPL induced degradation, suggesting that ubiquitination on K41 partly contributed to Tat degradation (Fig.6B).

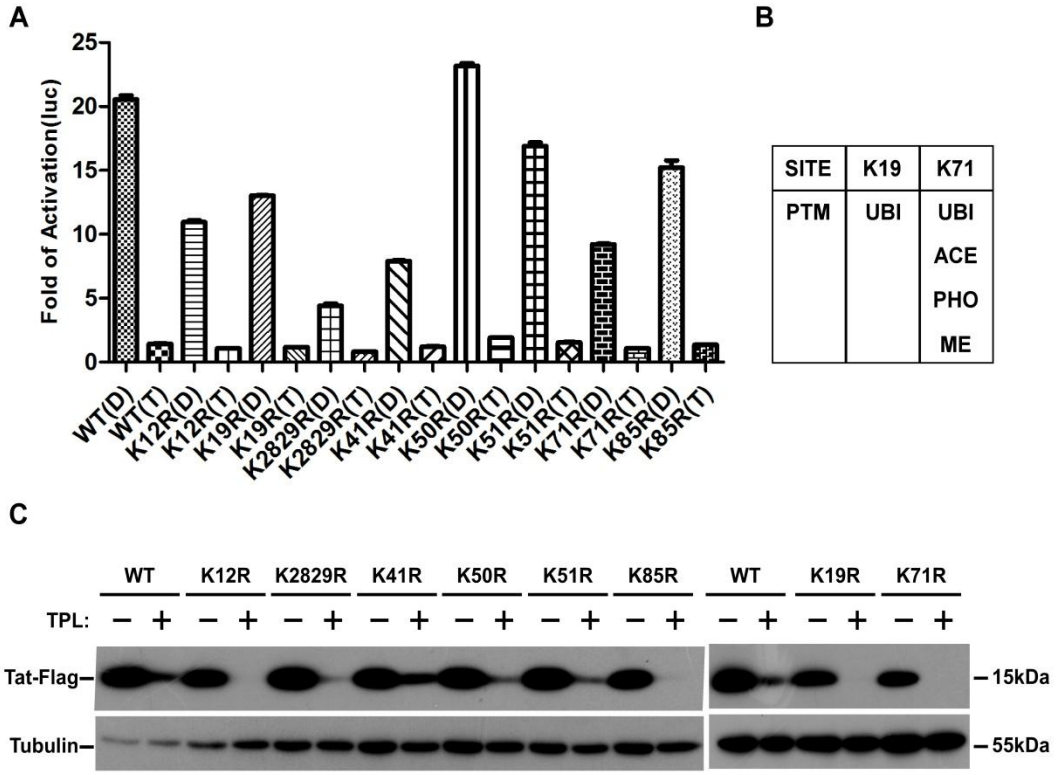

Fig. 6. TPL induces Tat breakdown not in mono site ubiquitination. (A) Luciferase activity of different Tat mutant with TPL treatment. Luciferase activities were measured in extracts of NH1 cells containing the integrated HIV-1 LTR-luciferase reporter gene and transfected with Tat or Tat mutant. The folds of Tat activation were calculated. (B) Stability of different Tat mutant with TPL treatment. Samples were lysed for further analysis by western blotting with indicated antibodies. Each experiment was repeated for at least three times. No asterisk denotes p>0.05, *, p<0.05, Student's t-test. Data are presented as means ± SEM.

3.7. MS identifies a list of E3 ligase candidates that might be responsible for TPL-induced Tat degradation

The ubiquitin-dependent proteasome degradation starts with the attachment of ubiquitin to a substrate protein, which requires E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase[16]. E3 ligase is critical because it determines the substrate specificity of the cascade[17]. In order to identify specific E3 ligases responsible for TPL induced Tat degradation, we performed SILAC-based quantitative mass spectrometry (MS) to compare the E3 ligases associated with Tat before and after TPL treatment. Assuming that E3 ligase and substrate interaction occurs ahead of protein degradation, we first examined Tat protein level after TPL treatment for different period of time. Tat protein level decreased slowly around 11 to 13 h after TPL treatment and started to accelerate at 17 h (Fig.7A). Therefore, we decided to harvest cells after 12 h of TPL treatment. Tat-flag was transfected into 293T cells and anti-Flag IP experiments were performed. IP elutes of control and TPL-treated samples were subject to MS analysis. Among all the Tat-associated E3 ligases identified by MS, 17 of which were significantly changed after TPL treatment (Fig.7B). Further investigation needs to be done to identify specific E3 ligase among these 17 candidates that is involved in TPL induced Tat degradation.

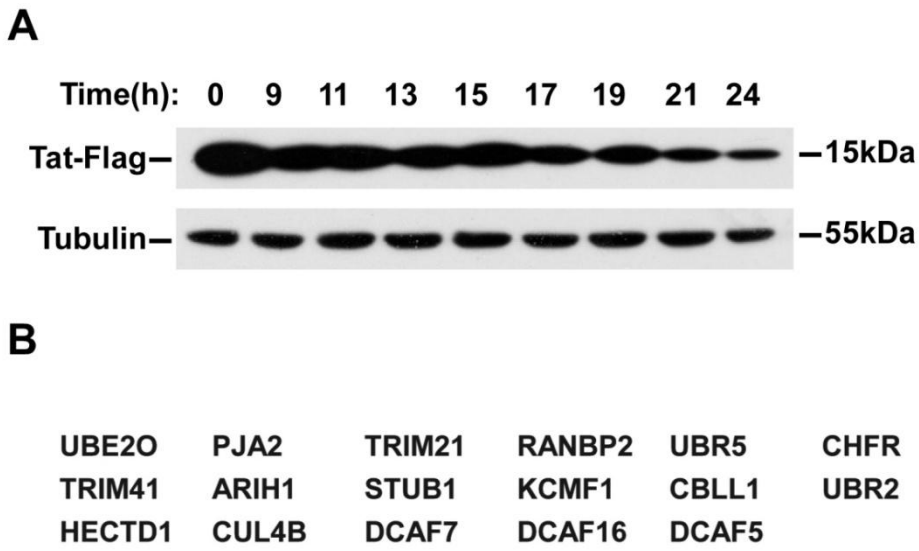

Fig. 7. Several E3 ligase candidates is identified that might be responsible for TPL-induced Tat degradation. (A) Western blot assay was performed with TPL treatment. Tat protein was detected with TPL treatment in time dependent manner. (B) A list of E3 ligase candidates were identified that might be involved in TPL-induced Tat degradation by MS.

4. Discussion

HIV/AIDS remains an ongoing worldwide public health issue despite decades of intensive investigation. Currently, ART has been recognized as the most effective way to restrain HIV infection. However, problems like side effects, lifelong medication, drug resistance remain challenging. It is always necessary and helpful to add new weapons that can fight against HIV.

Upon infection, the viral gene expression from the integrated proviral DNA depends largely on host transcription machinery[18]. Tat is a viral encoded protein that is crucial for HIV transcription activation. The effect of Tat inhibition is restricted to HIV transcription but not for the general host gene expression, which makes it a perfect target for HIV suppression[19]. As such, effort has been put into research on Tat degradation. For example, proteins, including host p14(ARF) and HIV nucleocapsid protein, inhibited Tat transactivation of HIV LTR by promoting Tat degradation via a ubiquitin-independent pathway[20], [21]. Zhang et al showed that lncRNA NRON directly linked Tat to the ubiquitin/proteasome components including CUL4B and PSMD11, which facilitated Tat degradation[15]. Moreover, Curcumin inhibited HIV activity likely by promoting the degradation of intrinsically unfolded Tat protein[22].

TPL is a component extracted from the famous traditional Chinese herbal medicine *Tripterygium wilfordii* that has been promoted for use in rheumatoid arthritis and psoriasis[23]. The use of TPL for HIV treatment is currently under clinical trials (NCT02219672 and NCT03403569). Whether other *Tripterygium wilfordii* components are effective in HIV inhibition remains to be elucidated. By screening a panel of *Tripterygium wilfordii* extracts, we confirm that TPL is most effective in HIV inhibition (Fig.1). It has been reported that TPL suppressed HIV replication by inducing Tat degradation[7]. Here we demonstrate that TPL-mediated Tat degradation is a ubiquitin-dependent process by employing an E1 inhibitor PYR-41 (Fig.5B).

Ubiquitination is a post-translational modification in which one single or multiple ubiquitin molecules are attached to lysine residues on substrate proteins. Mapping ubiquitination sites has been challenging. One of the reasons is that mutation of preferred ubiquitination sites may result in another site on the substrate being ubiquitination alternatively[24], which is very likely the case for Tat ubiquitination. Single site

mutation of all the lysine residues on Tat does not protect it from being degraded upon TPL treatment (Fig.6B). To determine whether one lysine is preferred, future studies may use an approach of adding back[25] individual lysine to a lysine-free Tat mutant, then check if the newly added lysine is sufficient to induce the degradation of Tat.

The human genome encodes more than six hundred E3 ubiquitin ligases, which are the most important determinant of substrate specificity in ubiquitination of proteins[26]. Although CUL4B has been shown to be a Tat-associated E3 ligase, it does not play a role in Tat degradation by TPL treatment (Fig.5D). By employing affinity purification of Tat-interacting proteins and SILAC quantitative MS, we narrow down the number of E3 ligases with different association with Tat after TPL treatment to 17, which makes it easier for future investigations aiming to search for the right E3 ligase (Fig.7B).

In summary, we report that TPL inhibits HIV transcription on the elongation stage. Among *Tripterygium wilfordii* extracts, TPL is the most effective compound to suppress HIV. TPL-induced Tat degradation is ubiquitin dependent. Our study further reveals the mechanism of Tat degradation promoted by TPL, and provide some candidate E3 ligases involved in this process. Focusing on function of Tat regulation is important for elucidating principle of HIV-1 gene transcriptional elongation and developing new drug target for AIDS therapy. Although the universal therapeutic useage of triptolide may be limited due to safety concepts, unveiling the mechanism Tat degradation promoted by TPL provides a pivotal tool to understand action mode of Tat degradation, which may serve potential novel strategy for AIDS remedy.

Credit Authorship Contribution Statement

Y. W. designed the research and analyzed the data. Y. W. performed the experiments. Y. W., Y. Z., Yun W. and J. S. wrote the manuscript. Y. W. conceived and directed the project. We thank Dr. Rongdiao Liu, Dr. Xiang Gao, Dr. Yuhua Xue for helpful discussion on the manuscript. All the authors discussed the results and commented on the manuscript."

Data Availability Statement

All data in this study are available from the corresponding author upon reasonable request.

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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