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Thiamet-G-mediated inhibition of O-GlcNAcase sensitizes human leukemia cells to microtubule-stabilizing agent paclitaxel

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ABSTRACT

Although the microtubule-stabilizing agent paclitaxel has been widely used for treatment of several cancer types, particularly for the malignancies of epithelia origin, it only shows limited efficacy on hematological malignancies. Emerging roles of O-GlcNAcylation modification of proteins in various cancer types have implicated the key enzymes catalyzing this reversible modification as targets for cancer therapy. Here, we show that the highly selective O-GlcNAcase (OGA) inhibitor thiamet-G significantly sensitized human leukemia cell lines to paclitaxel, with an approximate 10-fold leftward shift of IC_{50}. Knockdown of OGA by siRNAs or inhibition of OGA by thiamet-G did not influence the cell viability. Furthermore, we demonstrated that thiamet-G binds to OGA in competition with 4-methylumbelliferyl N-acetyl-β-D-glucosaminide dehydrate, an analogue of O-GlcNAc UDP, thereby suppressing the activity of OGA. Importantly, inhibition of OGA by thiamet-G decreased the phosphorylation of microtubule-associated protein Tau and caused alterations of microtubule network in cells. It is noteworthy that paclitaxel combined with thiamet-G resulted in more profound perturbations on microtubule stability than did either one alone, which may implicate the underlying mechanism of thiamet-G-mediated sensitization of leukemia cells to paclitaxel. These findings thus suggest that a regimen of paclitaxel combined with OGA inhibitor might be more effective for the treatment of human leukemia.

1. Introduction

The antineoplastic activity of paclitaxel has been demonstrated for several types of cancer, particularly for the malignancies of epithelia origin, for instance breast and lung cancer [1,2]. Paclitaxel acts as a microtubule-stabilizing agent that interferes the microtubule depolymerization, resulting in cell cycle arrest at G2/M transition stage [3]. In contrast to those cancer types that are sensitive to paclitaxel, acute leukemia only responded modestly to paclitaxel treatment [4]. Thus, it has been suggested that paclitaxel might be administered in combination with other agents for the chemotherapy for hematologic malignancies [4].

O-GlcNAcylation refers to the posttranslational modification of O-linkage of N-acetyl-glucosamine moieties to serine and threonine residues on cytoplasmic and nuclear proteins [5]. The O-GlcNAc transferase (OGT) and the O-GlcNAcase (OGA), which transfers GlcNAc to and removes GlcNAc from proteins, respectively, are the key enzymes in the reversible reaction [6]. The roles of O-GlcNAcylation modification of proteins have been implicated in multiple aspects of cell physiology and pathobiology [7], such as nutrition metabolism [8], signaling in cardiovascular system [9,10], neurodegenerative disorders [11], and cancer [12,13]. It is noteworthy that O-GlcNAcylation is dynamically regulated [14,15]. In Alzheimer's disease, the reciprocal relationship between O-GlcNAcylation and phosphorylation of tau has been taken advantage for drug design [16,17]. Tau is a microtubule-associated protein and hyperphosphorylation of tau is a pathologic feature of Alzheimer's disease. The newly emerged O-GlcNAcase selective inhibitor thiamet-G has been shown to reduce the phosphorylation of tau at pathologically relevant serine and threonine sites in neuron cells [16], suggesting its potential value in the therapy for tau hyperphosphorylation-associated neurodegenerative disorders.

In light of that both paclitaxel and thiamet-G exert effects on microtubule organization directly or indirectly, we hypothesized that paclitaxel in combination with thiamet-G may give rise to more profound cytotoxicity to human leukemia cells than the

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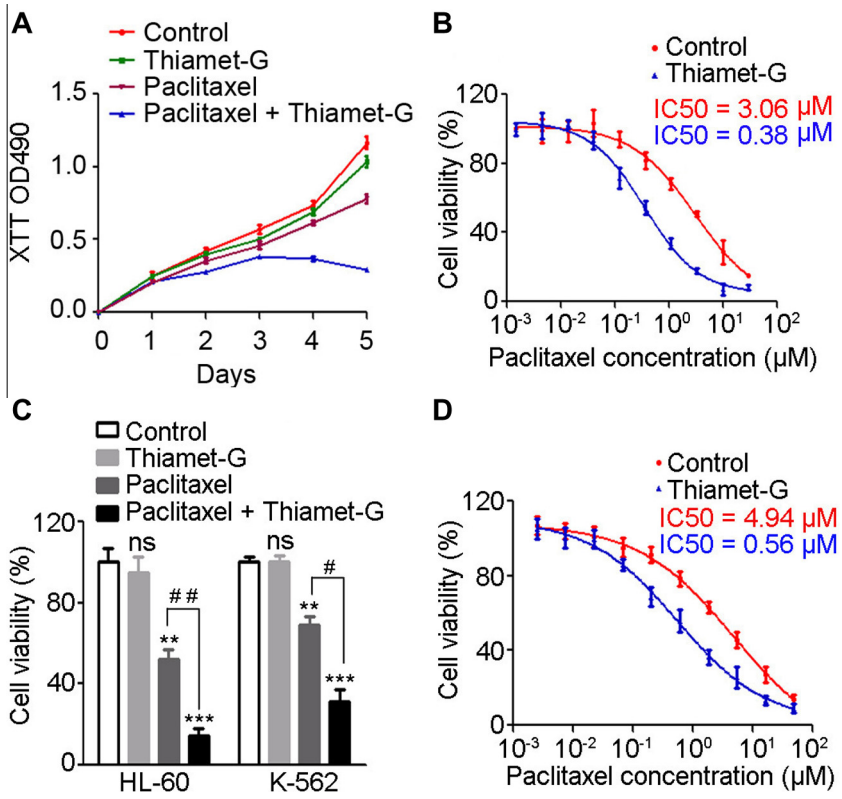

Fig. 1. Inhibition of O-GlcNAcase sensitizes leukemia cells to paclitaxel treatment. (A) Jurkat cells were treated with DMSO, paclitaxel (10 μM), Thiamet-G (10 μM), or paclitaxel (10 μM) in combination with Thiamet-G (1 μM) for the indicated time, and cell viability was analyzed by XTT assay. (B) Jurkat cells were treated with serially diluted paclitaxel in the presence or absence of Thiamet-G (1 μM), and cell viability was determined by XTT assay. (C) HL-60 cells and K-562 cells were treated with DMSO, paclitaxel (10 μM), Thiamet-G (10 μM), or paclitaxel (10 μM) in combination with Thiamet-G (1 μM) for 3 days, and cell viability was analyzed by XTT assay. (D) HL-60 cells were treated with serially diluted paclitaxel in the presence or absence of Thiamet-G (1 μM), and cell viability was determined by XTT assay. $^{**}P < 0.01$ versus control; $^{***}P < 0.001$ versus control; ns, not significant; $^{\#}P < 0.05$ versus paclitaxel; $^{\#\#}P < 0.01$ versus paclitaxel.

paclitaxel alone. Here, our results support the hypothesis by showing that the OGA-selective inhibitor thiamet-G significantly sensitized human leukemia cells to paclitaxel-mediated cytotoxicity. The underlying mechanism of sensitization could be due to synergistic effects of paclitaxel and thiamet-G on the perturbation of microtubule network.

2. Materials and methods

2.1. Reagents

Thiamet-G and paclitaxel were purchased from Selleck (TX, USA) and dissolved in DMSO. 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide dehydrate was obtained from Sigma (MO, USA). Purified O-GlcNAcase was purchased from Origene (MD, USA). siRNAs targeting OGA and luciferase (control) were synthesized by Invitrogen (Beijing, China). 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) was from Promega (WI, USA). Antibodies against O-GlcNAc (CTD110.6), Tau, and phosphorylated Tau were from Santa Cruz Biotech (CA, USA). Antibodies against β-actin and α-tubulin (DM1A) were obtained from Abcam (MA, USA). Horseradish peroxidase-conjugated secondary antibody and FITC-conjugated secondary antibody were obtained from Jackson ImmunoResearch Laboratories (PA, USA).

2.2. Cell culture and treatment

The human leukemia cell lines Jurkat E6-1, HL-60, and K-562 were purchased from the American Type Culture Collection (ATCC), maintained in RPMI1640 medium supplemented with 10% FBS, and incubated at 37 °C, 5% CO_2. siRNAs were transfected into cells by using lipofectamine 2000 (Invitrogen) according to the manufacture's protocol. For microtubule stability analysis, cells treated with DMSO, paclitaxel, thiamet-G, or paclitaxel combined with thiamet-G were incubated on ice for 20 min.

2.3. Enzymatic assay

All enzymatic assays were performed in triplicate at 37 °C using 4-methylumbelliferyl N-acetyl-β-D-glucosaminide dehydrate as substrate. 1 nM of purified OGA was incubated with the compounds for 5 min, and then 0.2 mM of the substrate was added. The liberation of 4-methylumbellifery was monitored by kinetic reading at excitation/emission 355/460 nm using a Tecan M200 plate in a mode of 60 s/cycle and 15 cycles in total.

2.4. Determination of inhibition mode of OGA

Purified OGA was incubated with thiamet-G for 5 min, and then different concentration of substrate was added to initiate the reaction. The reaction was monitored by kinetic reading at excitation/emission 355/460 nm. The inhibition mode was analyzed by Lineweaver–Burk plots.

2.5. Proliferation assay

Jurkat cells were seeded at 6000 cells/well in a 96-well plate, and 12 h later, cells were treated with compounds for the indicated

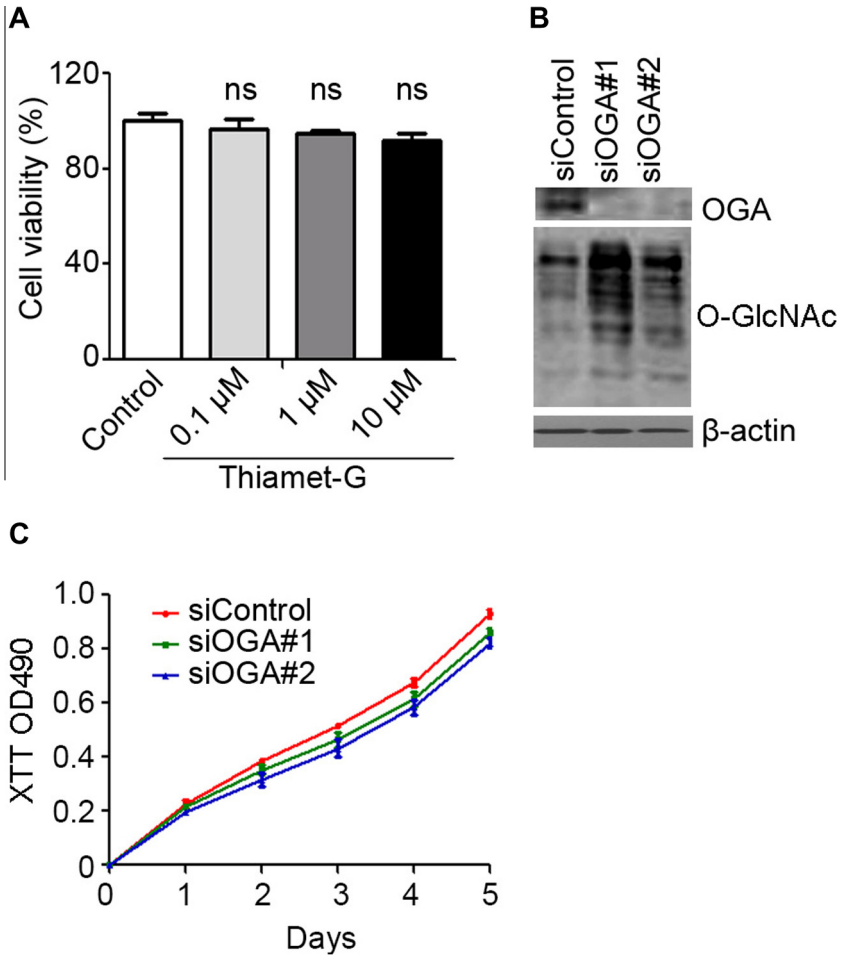

Fig. 2. Inhibition of O-GlcNAcase does not influence cell proliferation and survival. (A) The cell viability of Jurkat cells treated with thiamet-G was measured by XTT assay. ns, not significant. (B) Immunoblotting analysis was performed to examine the efficacy of siRNAs on knockdown of OGA and alterations of O-GlcNAcylation in Jurkat cells. The β-actin blot served as loading control for all samples. (C) Cell culture time course showed the proliferation of Jurkat cells transfected with OGA siRNAs or control siRNA.

time. Cell viability was determined by XTT assay according to the manufacturer's protocol.

2.6. Western blotting

Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked with Tris-buffered saline containing 0.02% Tween 20 and 5% fat-free dry milk for 1 h, and then incubated sequentially with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The blots were visualized with enhanced chemiluminescence detection reagent (Pierce) according to the manufacturer's protocol.

2.7. Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, and then permeabilized with 0.05% Triton X-100 in PBS for 20 min. Cells were blocked with 2% BSA in PBS for 1 h at room temperature, and then probed with primary antibodies and FITC-conjugated secondary antibodies. Nuclei were stained with DAPI for 10 min. Images were captured using a Zeiss fluorescence microscope.

2.8. Statistical analysis

All data were derived from three independent experiments, and expressed as means ± SD. Student's *t*-test and one-way analysis of variance (ANOVA) were performed for statistical analysis. P value < 0.05 was considered statistically significant.

3. Results

3.1. Thiamet-G sensitizes human leukemia cells to paclitaxel

XTT assay showed that paclitaxel alone modestly reduced the viability of Jurkat cells, whereas thiamet-G hardly affected the viability of Jurkat cells, when compared with the control (Fig. 1A). Surprisingly, the cell viability of Jurkat cells remarkably decreased in the presence of both paclitaxel and thiamet-G, compared with the control (Fig. 1A). By plotting the log concentration of paclitaxel versus cell viability, we determined an IC50 of 0.38 μM for paclitaxel in the presence of thiamet-G, in contrast to an IC50 of 3.06 μM for paclitaxel alone (Fig. 1B). Further, HL-60 and K-562 cells were examined for thiamet-G induced sensitization to paclitaxel. We observed that paclitaxel in combination with thiamet-G more potently decreased the viability of HL-60 and K-562 cells than did the paclitaxel alone (Fig. 1C). The sensitivity of HL-60 cells to paclitaxel increased approximately by 10-fold in the presence of thiamet-G, compared with that in the presence of paclitaxel alone

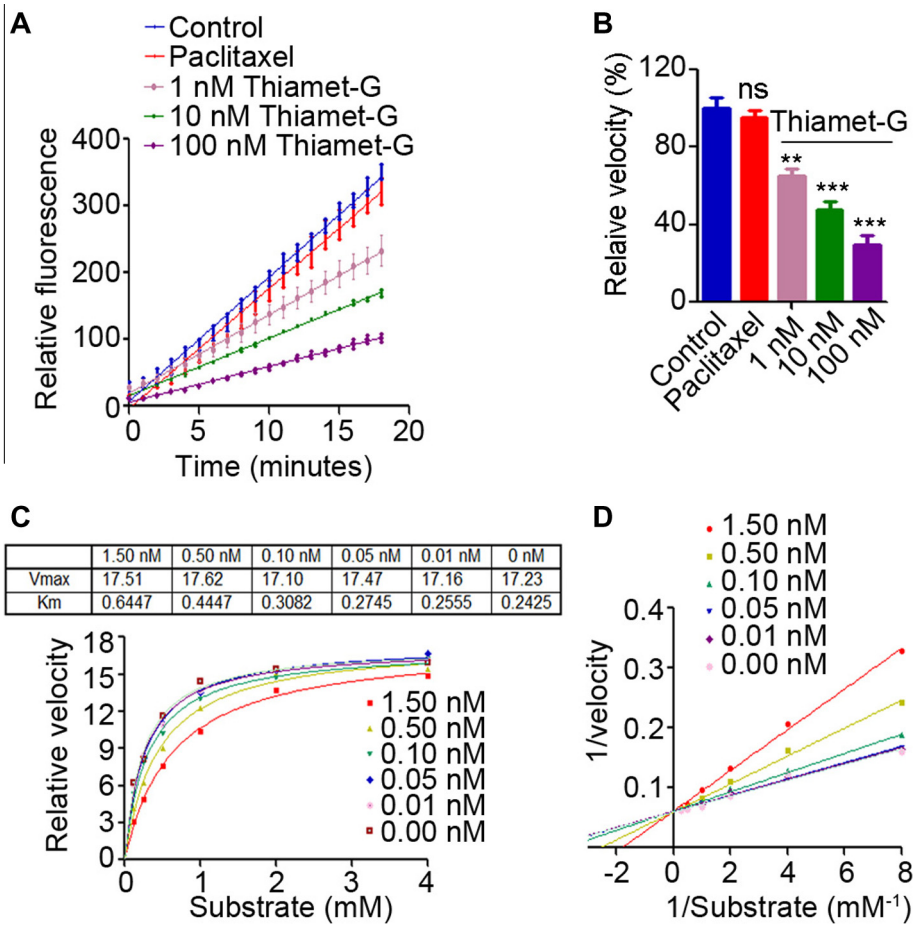

Fig. 3. Thiamet-G is a competitive inhibitor of O-GlcNAcase. (A) Purified OGA was incubated with the substrate 4-methylumbelliferyl N-acetyl-β-D-glucosaminide dehydrate in the presence of DMSO, paclitaxel, or different concentration of Thiamet-G, respectively to monitor the activity of OGA by kinetic reading of fluorescent intensity of 4-methylumbelliferyl. (B) Experiments were performed as in panel A, and the relative velocity was determined. (C) Substrate concentration versus velocity curves in the presence of indicated concentration of thiamet-G. (D) Experiments were performed as in panel C, and Lineweaver–Burk plots were employed to analyze the inhibition mode. $^{**}P < 0.01$ versus control; $^{***}P < 0.001$ versus control; ns, not significant.

(Fig. 1D). These results reveal that inhibition of OGA activity enhances the sensitivity of human leukemia cells to paclitaxel.

3.2. Thiamet-G caused minimum cytotoxicity to Jurkat cells

To explore the mechanism of thiamet-G-mediated sensitization of leukemia cells to paclitaxel, we first examined the potential cytotoxicity of thiamet-G to Jurkat cells. Result showed that thiamet-G in a wide range of concentration (0.1–10 μM) did not significantly affect the cell viability, compared with the control (Fig. 2A). Further, we knocked down the endogenous OGA by using siRNA oligonucleotides. Western blot analysis showed that two siRNAs almost completely suppressed the expression of OGA at protein level in contrast to the control siRNA (Fig. 2B, *OGA blot*). As a result, the level of O-GlcNAcylation obviously increased (Fig. 2B, *O-GlcNAc blot*). Interestingly, knockdown of endogenous OGA did not significantly decrease the cell proliferation either, as observed in a time course curve (Fig. 2C).

3.3. Thiamet-G binds to OGA in competition with the substrate of OGA

Enzyme kinetic analyses were used to describe the characteristics of the inhibition of OGA by thiamet-G. The reaction time course showed that accumulation of fluorescence intensity of 4-methylumnelliferyl liberated from the substrate 4-methylumbelliferyl N-acetyl-β-D-glucosaminide dehydrate by OGA was significantly inhibited in the presence of thiamet-G, compared with that of control. The inhibition of OGA by thiamet-G was dose-dependent (Fig. 3A). Paclitaxel hardly affected the enzymatic activity of OGA, compared with the control (Fig. 3A). The relative velocities of substrate cleavage under each condition were derived from fluorescence intensity-time courses, showing slower substrate cleavage velocity of OGA in the presence of higher concentration of thiamet-G (Fig. 3B). To determine the binding of thiamet-G to OGA is competitive or non-competitive, we recorded the relative velocity versus serially diluted substrate concentration under a series of thiamet-G concentrations (Fig. 3C). Lineweaver–Burk plots revealed that maximum velocities under all concentrations of thiamet-G were almost the same, as all plots had the same *Y*-axis intercept; the *km* value of OGA, as reflected by the *X*-axis intercept ($-1/km$), increased with the increasing concentration of thiamet-G (Fig. 3D), suggesting that thiamet-G competes with the substrate for the binding to OGA.

3.4. Thiamet-G decreases phosphorylation of tau and alters the microtubule dynamics

Western blot analysis showed that phosphorylation of microtubule-associated protein tau significantly decreased in Jurkat cells upon the treatment of thiamet-G (Fig. 4A). Semi-quantitative

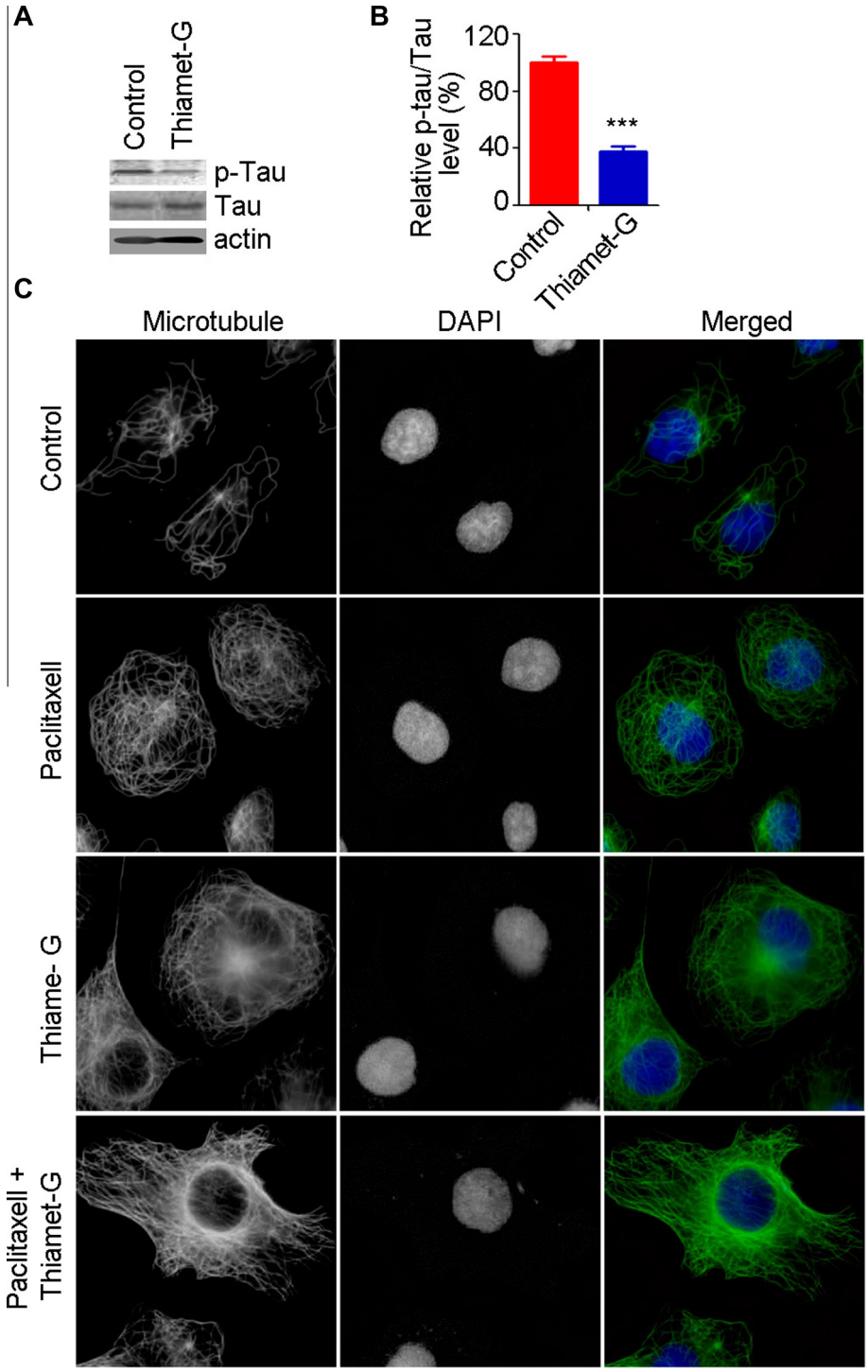

Fig. 4. Thiamet-G mediated inhibition of O-GlcNAcase decreases phosphorylation of Tau and alters the microtubule stability. (A) Immunoblotting analysis of Tau phosphorylation with/without thiamet-G treatment. (B) Experiments were performed as in (A), and relative ratio of phosphorylated Tau to total Tau was quantified. (C) Jurkat cells were treated with DMSO, paclitaxel, thiamet-G or paclitaxel plus thiamet-G for 3 h, and then cells were incubated on ice for 20 min, microtubule (green) and DAPI (blue) were immunostained and shown. $^{***}P < 0.001$ versus control.

analysis showed that the phosphorylation of tau in the presence of thiamet-G was about 40% of that in control (Fig. 4B). We next investigated the effect of thiamet G and paclitaxel on microtubule network. Jurkat cells treated with compounds were incubated on ice, and then immunostained with tubulin antibody. Immunofluorescence studies showed that the microtubule stabilizer paclitaxel alone significantly restrained the microtubule depolymerization induced by ice, in contrast to the control (Fig. 4C, *paclitaxel* panel). Interestingly, the thiamet-G also caused the similar phenomenon (Fig. 4C, *thiamet-G* panel). The combination of paclitaxel with thiamet-G contributed to profound alterations of microtubule network, with more accumulation of thick microtubule filament in the cytoplasm and condensed microtubule embracing the nucleus (Fig. 4C, *paclitaxel* + *thiamet-G* panel).

4. Discussion

The O-GlcNAcylation is a posttranslational modification that is involved in a wide range of cellular processes. The alterations of O-GlcNAcylation level in a diverse set of cancer types have been observed, which brings up the idea that OGT and OGA, the two key catalyzing enzymes could be targeted for cancer therapy [12]. Thiamet-G is an OGA selective inhibitor that was developed in recent years, and its emerging roles in neurodegenerative disorders have been greatly concerned [16–19]. In contrast, few studies have explored the therapeutic potential of thiamet-G for cancers. The limited efficacy of paclitaxel on hematological malignancies prompted us to test whether paclitaxel in combination with thiamet-G will have synergistic effects on the cytotoxicity to human leukemia cells.

In the present study, we showed that thiamet-G markedly sensitized the human leukemia cell lines to paclitaxel as indicated by XTT assay for cell viability (Fig. 1). Interestingly, the sensitization was not due to thiamet-G mediated cytotoxicity, because thiamet-G in a wide range of concentration did not influence the cell survival (Fig. 2A). Furthermore, we showed that knockdown of endogenous OGA by siRNA oligonucleotides did not affect the cell viability either (Fig. 2C). Thus, the underlying mechanism for the enhanced cytotoxicity of paclitaxel in the presence of thiamet-G may not be directly associated with OGA. It is possible that the alterations of O-GlcNAcylation of certain key proteins synergistically contributed to paclitaxel induced cell apoptosis.

Thinking of the reciprocal relationship between O-GlcNAcylation and phosphorylation of microtubule-associated protein tau in neurodegenerative disorders [16], we hypothesized that the OGA inhibitor thiamet-G might also affect microtubule organization in human leukemia cells. Surprisingly, we found that thiamet-G treatment decreased the phosphorylation of microtubule-associated protein tau (Fig. 4A), which may be due to the occupancy of phosphorylation sites in tau by elevated O-GlcNAcylation upon the inhibition of OGA [16]. It has been shown that hyperphosphorylation of tau causes disassociation of tau from microtubule and a loss of tau-induced microtubule stabilization [20]. In light of this, we speculated that the decreased phosphorylation of tau could promote the binding of tau to the microtubule and enhance the tau-induced microtubule stabilization. In support of our hypothesis, immunofluorescence studies showed that thiamet-G alone to some extent altered the microtubule structure in Jurkat cells, which is characterized by accumulation of thick microtubule filament as well as unstructured and condensed microtubule (Fig. 4C, *thiamet-G* panel). When paclitaxel was used in combination with thiamet-G to treat Jurkat cells, it resulted in more extensive alterations on cytoskeleton than did the paclitaxel alone (Fig. 4C, *paclitaxel + thiamet-G* panel). Although thiamet-G can interfere microtubule dynamics as shown in this study, other mechanisms that are possibly involved in thiamet-G-mediated sensitization of Jurkat cells cannot be excluded, because OGA regulates the O-GlcNAcylation of a large number of targets in many biology processes [21,22].

In the present study, we also investigated the inhibition mode of OGA by thiamet-G. With the enzyme kinetic analysis, we determined that thiamet-G was bound to OGA in competition with the OGA substrate (Fig. 3). This finding is in accordance with the report that thiamet-G takes up the active center of OGA in the crystal structure of OGA-thiamet-G complex [16].

Taken together, we first reported here the synergistic cytotoxic effects of paclitaxel and thiamet-G on three human leukemia cell lines, which might be helpful to the improvement of chemotherapy for hematological malignancies.

Author contributions

JZ, YS and ND designed the whole study and review the final manuscript. ND, LP, FYS, LF and XZ performed experiments, analyzed the data and contributed to the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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