Original Paper

Small Molecule TH-39 Potentially Targets Hec1/Nek2 Interaction and Exhibits Antitumor Efficacy in K562 Cells *via* G0/G1 Cell Cycle Arrest and Apoptosis Induction

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Key Words

TH-39 • Hec1/Nek2 • K562 cells • G0/G1 cell cycle arrest • Apoptosis

Abstract

Background: Cancer is still a major public health issue worldwide, and new therapeutics with anti-tumor activity are still urgently needed. **Methods:** The anti-tumor activity of TH-39, which shows potent anti-proliferative activity against K562 cells with an IC₅₀ of 0.78 μM, was investigated using immunoblot, co-immunoprecipitation, the MTT assay, and flow cytometry. **Results:** Mechanistically, TH-39 may disrupt the interaction between Hec1 and Nek2 in K562 cells. Moreover, TH-39 inhibited cell proliferation in a concentration- and time-dependent manner by influencing the morphology of K562 cells and inducing G0/G1 phase arrest. G0/G1 phase arrest was associated with down-regulation of CDK2-cyclin E complex and CDK4/6-cyclin D complex activities. Furthermore, TH-39 also induced cell apoptosis, which was associated with activation of caspase-3, down-regulation of Bcl-2 expression and up-regulation of Bax. TH-39 could also decrease mitochondrial membrane potential (ΔΨ_m) and increase reactive oxygen species (ROS) accumulation in K562 cells. The results indicated that TH-39 might induce apoptosis *via* the ROS-mitochondrial apoptotic pathway. **Conclusion:** This study highlights the potential therapeutic efficacy of the anti-cancer compound TH-39 in treatment-resistant chronic myeloid leukemia.

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Introduction

With increased incidence and mortality rates, cancer is still a major public health problem worldwide [1, 2]. Leukemia is one of the most common childhood cancers. In the development of leukemia, dysfunction in genes that coordinate accurate chromosomal

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alignment and segregation during mitosis is always observed [3, 4]. These significant molecular changes are controlled by mitotic spindle checkpoints, play an important role in leukemogenesis, and are also likely involved in apoptosis [3]. Anti-mitotic agents that target the mitotic apparatus through non-microtubule mitotic mediators have been designed [5]. Some chemotherapeutic drugs that interfere with mitosis are currently used in the clinic [6].

Highly Expressed in Cancer 1 (Hec1), a novel attractive non-microtubule target, was found to be an essential member of the Ndc80 complex along with Nuf2, Spc24, and Spc25 [7]. Hec1 plays an important role in mitotic processes as a mitotic regulator, including chromosome condensation, migration, and spindle assembly checkpoint (SAC) signaling [8-10]. Hec1 can directly interact with mitotic kinases NIMA-related kinase 2 (Nek2) and Aurora B [11]. Phosphorylation of Hec1 S165 by Nek2 is critical for Hec1 function in the modulation of chromosome segregation and cell survival [10, 12]. Hec1 is over-expressed in a variety of human cancers, including breast, colorectal, and gastric cancers [13-15]. Additionally, Hec1 over expression is associated with poor prognosis in primary breast cancer [16]. Consistently, depletion of Hec1 by RNA interference or small molecules targeting the Hec1/Nek2 interaction has been shown to effectively inhibit tumor growth in mouse models [17-19]. Altogether, these results suggest that inactivation of Hec1 and Nek2 by small molecules targeting their interaction is a potential therapeutic strategy for different types of cancer.

The first small molecule targeting the Hec1/Nek2 pathway was discovered by yeast two-hybrid screening [15]. The initial hit, INH1, and its analogues, INH6 (Fig.1), both disrupt the interaction of Hec1/Nek2 *via* direct binding to Hec1. INH1 and INH6 induced abnormal mitotic processes, as well as cell apoptosis [15, 20]. In our previous studies, we synthesized a series of novel N-(4-phenylthiazol-2-yl)cinnamamide derivatives that displayed anti-proliferative activity and induced apoptosis [21]. These novel anti-proliferative agents shared the same scaffold (4-aryl-N-arylarbony-2-aminothiazoles) with INH1 and INH6. Additionally, the most potent compound, 8f (TH-39), was much more effective than INH1 and INH6, with an IC_{50} as 0.78 μ M against the K562 cell line (Fig. 1). Thus, we selected TH-39 for further research. In this study, we explored the features and potential of TH-39 for preclinical development as a cancer therapeutic agent. The biological activity and mechanism of action were also investigated.

Materials and Methods

Materials

3-(4,5-dimethyl-2-thiazolyl)-2,5-di-phenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), DCFH-DA, Rhodamine-123 (Rh123), and propidium iodide (PI) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The Annexin V-FITC apoptosis detection kit was obtained from KeyGen Biotech (Nanjing, China). The primary antibodies against Hec1 (74 kDa), Nek2 (52 kDa) were purchased from Abcam (Cambridge, MA, USA). The primary antibodies against CDK2 (34 kDa), CDK4 (34 kDa), CDK6 (40 kDa), Cyclin D1 (34 kDa), Cyclin E (50 kDa), p21 (21 kDa), caspase-3 (17, 19, 35 kDa), Bcl-2 (26 kDa) and Bax (20 kDa) were obtained from Cell Signaling Technology Company (Beverly, MA). Antibody against β -actin was acquired from Beyotime (Beijing, China).

Preparation of TH-39

TH-39 (Fig. 2) was synthesized at the State Key Laboratory of Biotherapy, Sichuan University, Sichuan, China. The compound synthesis is described in detail in Fig. 2. Briefly, treatment of mesitylene 1 with bromoacetyl bromide in the presence of $AlCl_3$ and DCM under 0 °C afforded 2-bromo-1-mesitylethanone

Fig. 1. Structures of 4-aryl-N-arylarbony-2-aminothiazoles.

Fig. 2. The synthetic route for and the structure of TH-39 Reagents and conditions: (a) bromoacetyl bromide, AlCl_3 , DCM, 0 °C, 5 min; (b) thiourea, EtOH, reflux, 3h; (c) malonic acid, piperidine, pyridine, 115 °C; (d) EDCl, DMAP, DCM, r.t.

2. Then the key building block 4-mesitylthiazol-2-amine 3 was synthesized by treating 2-bromo-1-mesitylethanone with thiourea in EtOH with reflux for 3 h. Another key building block (*E*)-3-(4-(*tert*-butyl)phenyl) acrylic acid 5 was synthesized by treating it with 4-(*tert*-butyl) benzaldehyde 4 with malonic acid in the presence of piperidine and pyridine under 115 °C. Next, the final compound, TH-39, was synthesized by an amidation reaction.

TH-39 was determined by $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, and ESI-MS analysis. For the *in vitro* studies, TH-39 was prepared in DMSO at a stock concentration of 90 or 30 mM and diluted in the relevant medium at a final DMSO concentration of 0.1% (V/V). Medium with 0.1% DMSO served as vehicle control.

Cell lines and cell culture

Bel7402 and VERO cell lines were obtained from the China Centre for Type Culture Collection (CTCCC, Wuhan, China). Human chronic myeloid leukemia (CML) cell line K562 and other cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM or RPMI 1640 medium, containing 10% fetal bovine serum (FBS, Gibco, Auckland, N.Z.), 4 mM L-Glutamine, penicillin-streptomycin (Life Technologies), and cultured in a humidified atmosphere under 5% CO_2 at 37 °C.

Cell viability assay

The cell viabilities after treatment with TH-39 were measured by the MTT assay. Briefly, cells ($1\text{--}8 \times 10^3/100 \mu\text{L}$) were seeded in 96-well microplates and cultured for 24 h. After treatment with various concentrations of TH-39 for 96 h, 20 μL of the MTT solution (5 mg/mL) was added to each well and incubated for another 2–4 h at 37 °C. The formazan crystal formed by the living cells was dissolved with 150 μL of DMSO or 50 μL of SDS (20%) overnight. Then, the optical density was measured using the Spectra MAX M5 microplate spectrophotometer (Molecular Devices) at 570 nm. The data were processed in Excel and GraphPad Prism 5 (GraphPad Software, CA) to calculate the median inhibitory concentration (IC_{50}). For the effects of TH-39 on K562 cells with different treatment duration and concentrations, the cells were treated with TH-39 for 24, 48, 72 or 96 h and analyzed as described previously. The data were processed in Excel and GraphPad Prism 5 (GraphPad Software, CA) to determine the concentration-response curves for the relative concentration.

Immunoblot and Co-immunoprecipitation Assay

For immunoblotting, lysates were prepared in RIPA buffer (Beyotime, Beijing, China) on ice for 30 min and equalized by the BCA method before loading. The samples were separated on a sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and transferred onto polyvinylidene fluoride (PVDF) membranes (Amersham Bioscience, Piscataway, NJ). Following incubation with primary and horseradish peroxidase-conjugated secondary antibodies, the immunoreactive protein bands were detected using the ECL kit (Millipore, USA). A monoclonal β -actin antibody was used as a control.

For the co-immunoprecipitation, cells were lysed in NP-40 buffer (Beyotime, Beijing, China) containing 1 mM PMSF for 1 h and then incubated with a Hec1 antibody, or IgG as a control for 4 h on ice. The samples were collected by protein G agarose bead Beyotime (Beijing, China) and processed for immunoblotting.

Morphological analysis

For detecting the effect of TH-39 on cell morphology, cells were seeded in a six-well plate in specified numbers (1×10^5 cells/well) and were treated with various concentrations of TH-39 (0–30 μM). After incubation with TH-39 for 48 h, the cells were observed by light microscopy (Zeiss, Axiovert 200, Germany).

Analysis of cell cycle distribution by Flow Cytometry (FCM)

To analyze the cell cycle distribution, K562 cells were treated with TH-39 for the previously indicated time periods and the harvested cells were fixed with 75% ethanol overnight. Next, the cells were incubated with a 500 μL hypotonic solution containing 50 $\mu\text{g/mL}$ PI, 0.1% sodium citrate, and 0.1% Triton X-100 for 15 min in the dark, and then analyzed by FCM (Becton Dickinson, USA). Data were analyzed using Modfit 2.8 software.

Cell apoptosis Analysis by FCM

To further investigate the apoptosis inducing effects of TH-39, we analyzed the percentage of apoptotic cells by FCM with PI staining and Annexin V/PI dual labeling. After treatment with TH-39 for 72 h, the cells were harvested and stained with a PI solution or Annexin V-FITC/PI detection kit according to the manufacturer's instructions, and detected using a flow cytometer (Becton Dickinson, USA). The data were analyzed by Flow Jo software.

Measurement of ROS levels in cells

DCFH-DA was used to detect changes in ROS levels by FCM. After exposure to different concentrations of TH-39 for 48 h, K562 cells were incubated with DCFH-DA (10 μM) at 37 °C for approximately 20 min. The stained cells were washed in PBS and harvested, then measured by FCM.

Mitochondrial membrane potential ($\Delta\Psi\text{m}$) assay

We determined the changes of $\Delta\Psi\text{m}$ in K562 cells by FCM staining with Rh123 [22]. After treatment with 10 μM TH-39 for 72 h, the harvested cells were washed twice with cold PBS and then incubated with the Rh123 solution (5 $\mu\text{g/mL}$) at 37 °C for 30 min in the dark. Finally, the $\Delta\Psi\text{m}$ was measured by FCM after stained cells were washed with cold PBS.

Western blot analysis

To determine the effects of TH-39 on relevant signaling pathways, some proteins in K562 cells were evaluated using western blot. K562 cells were incubated with the previously indicated concentration of TH-39 for 48 h. Harvested cells were lysed in RIPA buffer (Beyotime, Beijing, China) on ice for 30 min and equalized by the BCA method before loading. Samples with about 30–40 mg of total protein were separated on a SDS-PAGE gel and transferred onto PVDF membranes (Amersham Bioscience, Piscataway, NJ). After incubation with primary and horseradish peroxidase-conjugated secondary antibodies, the immunoreactive protein bands were detected using the ECL kit (Millipore, USA). A monoclonal β -actin antibody was used as a control.

Statistical analysis

Cell culture-based experiments were performed in triplicate. Quantification of staining sections of was conducted using at least three different views. *P* values for comparison of two groups were determined by a 2-tailed Student's *t* test. *P* value < 0.05 was considered statistically significant.

Results

Effects of TH-39 on human cancer cells viability in vitro

To evaluate whether TH-39 possesses the potential to be an effective anti-cancer agent, a panel of 11 established cancer cell lines of different histotypes and 2 non-cancerous cell lines were treated with TH-39 for 96 h. Cell viability was evaluated by the MTT assay. The results indicated that TH-39 could decrease the viability of some cancer cell lines with IC_{50}

Fig. 3. The effects of TH-39 on the growth of human non-cancerous cells and cancer cells (A) Each cell line was treated with different concentrations (0–90 μ M) of TH-39 for 96 h and cell viability was measured by the MTT assay. The effects of TH-39 were represented as the half maximal inhibitory concentration (IC_{50} , μ M). (B) The expression levels of Hec1 and Nek2 in several cell lines. The protein levels were determined by western blot with special antibodies, and a monoclonal β -actin antibody was used as a control.

Fig. 4. The effects of K562 cells after treatment with TH-39 (A) CML cells K562 were treated with different concentrations of TH-39 for 24, 48, 72 h or 96 h and collected for determination of cell viability. The cell viability was measured by the MTT assay. Each point represents the mean $\pm$ SD for at least 3 independent experiments (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs vehicle control). (B) The effect of TH-39 on cell morphology by bright microscopy images. (C) K562 cells were incubated with DMSO (vehicle control) and 3.3 μ M TH-39 for 4 h, lysed, and lysates immunoprecipitated by Hec1 antibody to see co-immunoprecipitation Nek2. Rbt, rabbit. The expression levels of Hec1, Nek2 and β -actin in input lysates used for co-immunoprecipitation assay were also detected. (D) K562 cells were incubated with DMSO or TH-39 for 48 h and immunoblotted for the levels of Hec1 and Nek2 protein expression.

values ranging from 0.037 to 30.0 μ M while no apparent inhibition of the other cell lines was observed, including non-cancerous cell lines HEK293 and VERO (Fig. 3A).

To explore whether the activity of TH-39 is associated with protein expression, the levels of Hec1 and Nek2 were analyzed by western blot analysis in all cell lines. As shown in Fig. 3B, the expression of Hec1 and Nek2 were diverse among the different cell lines. The K562 cell line, which exhibited high expression of Hec1 and Nek2, was quite sensitive to TH-39, with an IC_{50} of 0.78 μ M. Thus, this cell line was selected for further study with respect to the potential antitumor mechanisms of TH-39.

Fig. 5. TH-39 induced G0/G1 phase arrest in K562 cells (A) K562 cells were treated with TH-39 for 72 h, and subjected to cell cycle analysis by FCM after incubated with a PI solution. (B) The cell cycle distributions were displayed in quantified histograms, including G0/G1, S, G2/M phase. (C) TH-39 induced G0/G1 phase arrest of K562 cells through down-regulation of CDK2-cyclin E complex and CDK4-cyclin D complex activities. After exposure of A375 cells to the indicated concentrations of TH-39 (0, 1.1, 3.3, 10, 30 μ M) for 48 h, the protein levels of CDK2, CDK4, CDK6, cyclin E, cyclin D and p21 were determined by western blot with special antibodies, and protein expressions were quantified.

TH-39 targeted the Hec1/Nek2 pathway and influenced the morphology of K562 cells

To verify the relationship of Hec-Nek2 pathway and TH-39, co-immunoprecipitation assay and western blot were performed to evaluate the interaction of Hec1 and Nek2 after TH-39 treatment. As shown in Fig. 4C, exposure of cells to TH-39 disrupted the binding of Nek2 to Hec1. In addition, treatment with TH-39 also resulted in the decrease of Hec1 and Nek2 expression. These results are consistent with the phenotypic consequences of Hec1 inhibitors (INH1 and INH6) in other cancers and indicate that TH-39 potentially targets the Hec1/Nek2 interaction.

Then, we examined the efficacy of TH-39 treatment against K562 cell proliferation. As shown in Fig. 4A, exposure of cells to various concentrations of TH-39 for 24 h, 48 h, 72 h, and 96 h resulted in decreased cell growth with increasing concentration and duration of exposure. Moreover, bright-field microscopy of K562 cells after incubation with TH-39 for 72 h was performed to assess the morphological effects. As shown in Fig. 4B, reduced proliferation and shrinking cell morphology was observed after treatment with TH-39 and these phenomena were more significant with the increased TH-39 concentration.

Fig. 6. Induction of cell apoptosis in K562 cells by TH-39 treatment (A) K562 cells were treated with indicated concentrations of TH-39 for 72 h, respectively, and then were analyzed by FCM after PI-staining. (B) The apoptosis of K562 cells after treated TH-39 for 72 h was analyzed using the Annexin V-FITC/PI dual-labeling technique. (C) Western blot was used to detect the levels of the typical apoptosis-related proteins cleaved caspase-3 and Bcl-2 family proteins. The expression of β -actin was used as the internal control. (D) Changes of the mitochondrial membrane potential ($\Delta\Psi_m$) in K562 cells were detected by FCM after treatment with 10 μ M TH-39 for 48 h with stain 5 μ g/mL Rh123.

TH-39 induced G0/G1 phase arrest of K562 cells

To examine whether the anti-viability activity of TH-39 in K562 cells was associated with cell cycle arrest, K562 cells were exposed to TH-39 at concentrations ranging from 0 to 30 μ M for 72 h, and cell cycle distribution was analyzed by flow cytometry (FCM). As shown in Fig. 5A, treatment with TH-39 for 72 h induced significant G0/G1 phase arrest in a concentration-dependent manner in K562 cells, with the percentage of G0/G1 fraction increased from 31.7% to 48.7%, 53.5%, 56.3%, 58.7%, and 60.1% in K562 cells treated with 0, 0.37, 1.1, 3.3, 10, and 30 μ M TH-39 for 72 h, respectively (Fig. 5B).

Effects of TH-39 on cell cycle-related proteins in K562 cells

To further elucidate the mechanism underlying TH-39 treatment on K562 cells, we investigated the expression levels of some key proteins involved in the G0/G1 phase transition by western blot in the K562 cell line. The results showed that TH-39 decreased the expression of CDK2, CDK4, CDK6, cyclin E and cyclin D in a dose-dependent manner (Fig. 5C), indicating that TH-39 inhibited the activity of the CDK2-cyclin E and CDK4/6-cyclin D complexes, which play important roles in the transition from G0/G1 to S phase. P21 could inhibit the activity of CDK-cyclin complex to regulate cell cycle progression [23], thus we also examined the expression of p21. Our results showed that p21 levels were increased in a concentration-dependent manner after TH-39 treatment. These results were consistent with the accumulation of the G0/G1 population in the FCM analysis.

Fig. 7. Effects of TH-39 on the pH values and ROS generation (A) pH values were detected after treated with TH-39. (B) Quantification of ROS change is shown (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with the vehicle group). (C) Treated with TH-39 in K562 cells induced ROS production. The harvested cells were loaded with DCFH-DA and measured by FCM.

TH-39 induced apoptosis in K562 cells

We next explored the induction of apoptosis after TH-39 treatment in K562 cells. First, the percentage of sub-G1 cells was detected by staining with PI solution. As shown in Fig. 6A, the percentage of sub-G1 K562 cells in the TH-39-treated group increased in a concentration-dependent manner. The apoptosis rate increased from 7.6% to 15.3%, 21.1%, 27.5%, and 33.4% after cells were treated with 0.37, 1.1, 3.3, 10 and 30 μM TH-39 for 72 h, whereas the proportion of apoptotic cells was merely 3.5 % in the vehicle control.

Then, we also confirmed the presence of apoptotic cells stained by Annexin V-FITC/PI dual-labeling with FCM. As shown in Fig. 6B, TH-39 induced apoptosis in a dose-dependent manner, which resulted in both early apoptotic (only Annexin V positive) and late apoptotic cells (Annexin V and PI-positive). After treatment with TH-39 for 72 h, the percentage of apoptotic K562 cells increased from 7.7% to 37.5% as the concentration was increased from 0 to 30 μM, respectively, and a nearly 30% change in apoptotic cells occurred between the control and the highest concentration (Fig.6B). Western blot also confirmed the induction of apoptosis, as an increased level of cleaved caspase-3 was observed after TH-39 treatment for 48 h (Fig. 6C).

Effects of TH-39 on the intrinsic apoptosis pathway

To further investigate which pathway was involved in TH-39-induced apoptosis, some related proteins were detected by western blot. The results indicated that the expression of Bcl-2 significantly decreased, whereas Bax increased in a concentration-dependent manner after exposure to TH-39 (Fig. 6C). Bcl-2 and Bax are the members of Bcl-2 family, which regulates the process of intrinsic apoptosis. These results suggested that apoptosis induced by TH-39 might be *via* the mitochondrial apoptotic pathway. To verify this hypothesis, we detected changes in mitochondrial membrane potential ($\Delta\Psi_m$) by FCM. As shown in Fig. 6D, treatment with 10 μ M TH-39 led to the loss of $\Delta\Psi_m$. These data indicated that TH-39 induced cell apoptosis through the mitochondrial-mediated apoptotic pathway.

Effects of TH-39 on ROS generation and pH value

Reactive oxygen species (ROS) are generated as by-products of cellular metabolism, especially in the mitochondria [24]. In this study, we detected ROS levels by FCM using DCFH-DA. The results showed that the ROS levels increased approximately twice after treatment with TH-39 (Fig. 7B and C). However, the increase did not occur in a concentration-dependent manner. We also found that the color of the medium after TH-39 treatment was not changed in a concentration manner either. The color of the medium always changed as its pH changed. We observed a significant color change in the medium after treatment with TH-39. Thus, we measured the pH of the medium using a pH-meter. As shown in Fig. 7A, the pH values declined in the TH-39 treated group and were consistent with the changes in ROS levels.

Discussion

CML is probably the most extensively studied human malignancy [4]. Currently, the modulation of protein function by specific signal transduction inhibitors is one of the therapeutic tools for CML [25-27]. In this study, we examined the effects of a small molecule, TH-39, which potentially targets the Hec1/Nek2 interaction, and our results indicated that TH-39 exhibited antitumor efficacy in K562 cells *via* G0/G1 cell cycle arrest and apoptosis induction.

The first compound (INH1) that specifically disrupts the Hec1/Nek2 interaction was found by a yeast two-hybrid screening [15]. Our compound, TH-39, was structurally similar to INH1, which is suggestive of a common mechanism of action for both compounds. The results of co-immunoprecipitation showed that TH-39 disrupted the binding of Nek2 to Hec1 (Fig. 4C). The degradation of Nek2 and Hec1 was also observed in TH-39-treated cells (Fig. 4D). These results are consistent with the consequences of Hec1 inhibitors (INH1 and INH6) in breast cancer cells and indicate that TH-39 potentially targets the Hec1/Nek2 interaction. From the levels of Hec1 and Nek2 in all cell lines analyzed by western blot, we also found the activity of TH-39 in K562 cells might be associated with the expression of Hec1 and Nek2 (Fig. 3).

Cell cycle deregulation plays an important role in modulating cell proliferation, and tumor-associated cell cycle defects are often mediated by alterations in cyclin-dependent kinase (CDK) activity [28]. CDK1, 2, 4 and 6 have been proven to drive cell cycle events. CDK4 and CDK6 are the two interphase cyclin dependent kinases that control cell cycle entry and progression through the G1 phase by forming CDK4/6-cyclin D1 complexes [29, 30]. These active complexes have the capacity to phosphorylate and partially inactivate the members of the retinoblastoma (RB) protein family including pRB and p107 [31]. Cyclin E is responsible for G1 to S phase progression by the CDK2-cyclin E complex [23, 32]. We found that treatment with TH-39 could inhibit the activity of CDK2-cyclin E and CDK4/6-cyclin D complexes, consistent with the arrest of G0/G1 phase (Fig. 5). P21 could regulate cell cycle progression by inhibiting the activity of CDK-cyclin complexes [33], and treatment with TH-39 did indeed increase the level of p21.

Apoptosis serves as a natural barrier to cancer development. Targeting apoptosis has been proved to be a promising strategy in anti-cancer drug discovery [34]. The intrinsic apoptosis pathway is one of the two classic apoptosis pathways. In this pathway, caspase-3 plays an important role and can be activated by upstream effector proteins and induce the apoptosis cascade. Cleavage of caspase-3 was observed after TH-39 treatment, and this result is consistent with the induction of apoptosis detected by FCM (Fig. 6). The Bcl-2 family, including anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax, is the central regulator in the mitochondrial apoptosis pathway [35]. The western blot result indicated that the expression of Bcl-2 significantly decreased, whereas Bax expression increased after exposure to TH-39 (Fig. 6C), suggesting that the apoptosis induced by TH-39 might be *via* the mitochondrial apoptotic pathway. This hypothesis was also verified by the loss of $\Delta\Psi_m$ (Fig. 6D).

ROS are generated as by-products of cellular metabolism, especially in the mitochondria [24]. ROS could lead to DNA damage, which could cause cell cycle arrest and apoptosis of tumor cells [36]. In this study, ROS levels were increased approximately twice after treatment with TH-39 (Fig. 7), which supports our conclusion. However, the change did not occur in a dose-dependent manner, but was consistent with the color of the medium. Cells always need energy metabolism to cell growth and division. Cancer cells can also process the glucose, first to pyruvate via glycolysis in the cytosol and then to carbon dioxide in the mitochondria [37]. Cell metabolism produces some material that alters the pH value. From the decline of pH values in TH-39 treated group (Fig. 7A), we speculated TH-39 could induce acid production. The changes in pH values did not occur in a dose-dependent manner, but this could be explained by cell number. When under the low concentration of TH-39, there are more cells that induced more acids production, which lowered the pH values. This acidic environment could potentially lead to oxidative stress, and consequently increase the level of ROS.

In conclusion, we assessed the anti-cancer activity of TH-39. Mechanism studies showed that TH-39 might act by disrupting the interaction between Hec1 and Nek2 in K562 cells. TH-39 also exhibited antitumor efficacy in K562 cells *via* G0/G1 cell cycle arrest and apoptosis induction. Therefore, this study highlights the potential of TH-39 as a treatment for chronic myeloid leukemia.

Acknowledgements

This research was supported by Zhejiang Apelo Medical Technology Co., Ltd. This research was supported by China Postdoctoral Science Foundation (No.2015M570790, No.2016T90860).

Disclosure Statement

The authors declare no conflict of interest.

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