INNO-406 inhibit the growth of chronic myeloid leukemia and promote its apoptosis via targeting PTEN

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Research

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Abstract

Background Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm. INNO-406 is a novel tyrosine kinase inhibitor (TKI) that possess specific Lyn kinase inhibitory activity with no or limited activity against other sarcoma (Src) family member kinases. The present study aimed to confirm the anti-tumor effect of INNO-406 on CML cells, and elucidate the molecular mechanism underlying its effect. Methods The cell proliferation and apoptosis were detected by MTT, western blot and flow cytometry respectively. Results As suggested by the findings, INNO-406 significantly inhibited the proliferation and induced apoptosis of CML cells. In addition, INNO-406 promoted the expression level of PETN. Rescue experiment revealed that PTEN knockdown reversed the effect of INNO-406 which indicated the correlation between INNO-406 and PTEN. Further study determined that PTEN inhibited the phosphorylation of AKT and 4EBP1 and subsequently altered the expression of apoptotic protein expressions including bax, cytochrome c (cyto-c), cleaved caspase3 and bcl-2. In vivo study further confirmed that INNO-406 inhibited the growth of CML cells in vivo by targeting PTEN. Conclusion Based on the above findings, this work extended our understanding of INNO-406 in the chemotherapy of CML and its molecular mechanism.

Background

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm characterized by the formation of the fusion gene consist of Abelson murine leukemia (ABL) gene and breakpoint cluster region (BCR) which encodes a constitutively active Bcr-Abl tyrosine kinase [1–3]. Tyrosine kinase inhibitors (TKIs) targeting BCR-ABL1 tyrosine kinase have made great development in the treatment of CML over the decades which improved 10 year overall survival from approximately 20% to 80–90% [4, 5]. However, about 25% of the children and half of the adults are still insensitive to chemotherapy or will relapse. Moreover, side effects, such as skin toxicity and allergic reaction are still clinical challenges which limited application of TKI [6, 7]. Thus, a better understanding of the molecular mechanisms involved in the progression of CML is urgently needed.

Bafetinib (INNO-406) is a novel tyrosine kinase inhibitor designed based on the chemical structure of imatinib for treating Bcr-Abl + leukemia [8, 9]. It is a potent dual function Bcr-Abl kinase and Src family kinase Lyn inhibitor which is developed to overcome imatinib resistance [10, 11]. According to the previous study, INNO-406 exerts a higher potency in inhibiting Abl than imatinib, moreover, it inhibits Lyn with higher selectivity than other SFK/Abl inhibitors. INNO-406 may be effective in the CML treatment with possible application to central nervus system leukemia and also be less liable to cause unfavorable side effects than other therapeutic agents that target multiple kinases, such as SFK inhibitors [12, 13].

The limitation of chemoresistance on the therapeutic effect of chemotherapy drugs makes the research on enhancing drug sensitivity or inhibiting drug resistance as important as the development of new drugs.

In the present study, we tried to elucidate the effect and the underlying mechanism of Bafetinb on the proliferation and apoptosis of CML cells. We found that Bafetinb is capable of inhibiting proliferation and

inducing apoptosis of CML cells. In addition, it notably promotes the expression of PTEN and modulates the downstream AKT/4EPB1 signal pathway.

Methods

Cell culture and reagents

The human chronic myeloid cell lines (K562 and KCL22) were purchased from the Chinese Academy of Sciences. The culturing of cells was carried out in Dulbecco's INNO-406ered Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA). Each cell line was cultured at 37 ℃ with 5% CO_2. In addition, the cells were treated with the indicated concentration of INNO-406 (Selleck, USA), whereas the control cells received an equivalent amount of DMSO. The final concentration of DMSO is below 0.1%. The adenovirus knocking down PTEN was established by Geenpharma (Shanghai, China).

MTT assay

The detection of the cell viability was carried out with MTT assay (Sigma-Aldrich, USA). The K562 and KCL22 cells were seeded onto 96-well plates and followed by treating with 5, 25, 50 and 100 μM of INNO-406 (Bio-techne Corporation, MN, USA) for a period of 24 h. Thereafter, the addition of 10 μl MTT to the cells was done, followed by incubating at a temperature of 37 ℃ for another period of 2 h. Optical density was measured with the use of a microplate reader at 450 nm. Moreover, the calculation of the inhibition rates was carried out as well. Each trial was conducted at least 3 times.

Cell apoptosis

After getting treated with different concentrations of INNO-406, the collection of both K562 and KCL22 cells was done, which were further washed with PBS 3 times. Concerning the cell apoptosis analysis, cells were stained with annexin V-FITC/propidium iodide following the guidelines of the manufacturer. Cell apoptosis was detected in a BD FACS Calibur flow cytometry system (Becton Dickinson, NJ, USA). Each trial was conducted at least 3 times.

Western blot analysis

Total proteins were extracted using RIPA-Buffer supplemented with 10 mM PMSF (Beyotime, Shanghai, China). A bicinchoninic acid assay (BCA) was carried out to quantify protein concentrations. The separation of 40 μg proteins was carried out on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to polyvinylidene fluoride (PVDF) membranes. Thereafter, the membrane was blocked with 5% non-fat milk in Tris-buffered saline and 0.1% Tween 20. Subsequently, the membranes were incubated with primary antibodies at 4 ℃ overnight. Together with washing using TBST buffer 5 times, the membranes were incubated with the secondary antibody for another 2 h. An enhanced chemiluminescence (ECL) system kit (Beyotime, Shanghai, China) was employed for the detection of bands. The optical densities (OD) value was analyzed by ImageJ software (NIH, Bethesda, MD, USA). Each trial was conducted at least 3 times.

Immunofluorescence

The tumor tissues were fixed in 4% paraformaldehyde for 24 h, followed by dehydrating in a graded alcohol series and embedding in paraffin, followed by cutting into 5 μm sections. The sections were deparaffinized, rehydrated with a graded alcohol series and then incubated in 96 ℃ with 0.01 mol/l sodium citrate buffer for the antigen retrieval. Following the incubation in 5% H2O2 for a period of 2 h, the sections were incubated using primary antibodies overnight at 4 ℃. Immunostaining was carried out with the use of streptavidin-peroxidase following the manufacturer's instructions (Beyotime, Shanghai, China). Finally, the sections were not only observed under a fluorescence microscope (Leica, Wetzlar, Germany) but also imaged. Each trial was conducted at least 3 times.

Xenograft model

A number of 3×10^6 K562 cells in 100 μl PBS were subcutaneously injected into the posterior flank region of the nude mice. The long diameter and short diameter were detected every 2 days, together with calculating with the use of the formula, which is as follows: tumor volume = 0.5 × long diameter × short diameter^2. The mice were injected intraperitoneally with 5 mg/kg INNO-406 (Bio-techne Corporation, MN, USA) every day since the cell injection. Finally, the mice were sacrificed followed by excising the tumors. All animal procedures were approved by the Animal Research Committee of Affiliated Hospital of Qingdao University and were performed according to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Statistical analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze all data for statistical significance. All the data are presented as the means ± SD. One-way ANOVA was used to assess the difference between multiple groups. Differences between two groups were analyzed by the Student’s t-test. $P < 0.05$ was considered as statistical significance.

Results

INNO-406 inhibited the proliferation of chronic myeloid leukemia cells

To investigating the effect of INNO-406 on the growth of CML cells including K562, and KCL22, MTT assay was carried out to detect the proliferation of those cells following the treatment of INNO-406 at the dose of 5, 25, 50 and 100 μM and for a period of 24 h. As revealed by Fig. 1, INNO-406 of 25, 50 and 100 μM significantly inhibited the proliferation of K562, and KCL22 cells (Fig. 1A). Moreover, we evaluated the growth curve of K562 and KCL22 cells during 72 h under INNO-406 treatment. It was suggested by the findings that INNO-406 notably inhibited the growth of both K562 and KCL22 cells (Fig. 1B, C).

INNO-406 induced apoptosis of CML cells

Apoptosis is well known to be involved in the antitumor effects. We next investigated whether INO-406 induces apoptosis of CML cells. Annexin V/PI dual staining and flow cytometry were used to evaluate the apoptosis of CML cells. We found that INNO-406 of 25 and 50 μM induced the apoptosis of CML cells while 5 μM INNO-406 did not influence the apoptosis of CML cells (Fig. 2A, B). In addition, to confirm our observation, we detected the expression of apoptotic proteins. Accordingly, INNO-406 of 25 and 50 μM promoted the expression of bax, cyto-c and cleaved caspase3 while inhibited that of bcl-2 (Fig. 2C).

INNO-406 promoted the expression of PTEN

To elucidate the molecular mechanism underlying the effect of INNO-406 on CML cells. We evaluated the dysregulated expression of proteins. PTEN is a superstar in anti-cancer research. We detected the level of PTEN under INNO-406 treatment. Interestingly, qPCR (Fig. 3A), western blot (Fig. 3B) and immunofluorescence assay (Fig. 3C) demonstrated that INNO-406 notably increased the mRNA and protein level of PTEN.

PTEN konckdown reversed the effect of INNO-406 on proliferation and colony formation of CML cells

We then performed knockdown of PTEN along with INNO-406 treatment to check out whether PTEN is involved in the effect of INNO-406 on CML cells. Accordingly, INNO-406 up-regulated the expression of PTEN while the transduction of siRNA designed for PTEN notably reduced the level of PTEN (Fig. 4A). INNO-406 inhibited the proliferation and induced apoptosis of CML cells while PTEN knockdown reversed these effects of INNO-406 (Fig. 4B, C).

INNO-406 modulated the phosphorylation of PTEN/AKT/4EBP1

As we know, PTEN was involved in the regulation of PI3K/AKT pathway. We speculated that INNO-406 might regulate the progression of apoptosis through modulating PTEN mediated PI3K/AKT signaling pathway. Western blot assay demonstrated that INNO-406 inhibited the phosphorylation of AKT as well as 4EBP1 in CML cells (Fig. 5A-B). PTEN knockdown reversed the effect of INNO-406 significantly (Fig. 5A). Then, apoptotic protein expression was evaluated, we found that PTEN knockdown reversed the effect of INNO-406 on promoting the expression of bax, cyto-c, cleaved caspase3 and inhibiting the expression of bcl-2 (Fig. 5B).

INNO-406 inhibited the CML cell growth in vivo

In vivo study was carried out to further investigate the impact of INNO-406 on CML. INNO-406 significantly suppressed the tumor growth and reduced the weight of tumors in comparison with that in the control group. Interestingly, this effect could be reversed by knockdown of PTEN by tail vein injection of si-PTEN adenovirus (Fig. 6A-C).

Discussion

As a new tyrosine kinase inhibitor for the treatment of CML, studies evaluating the effect and molecular mechanism of INNO-406 are quite limited. In this research work, we explored the anti-tumor effect of INNO-406 on K562 and KCL22 cells in vitro and in vivo and found that INNO-406 could target PTEN to inhibit the proliferation and induce apoptosis of CML cells. Mechanically, INNO-406 promotes the expression of PTEN and inhibits the phosphorylation of AKT and 4EBP1, subsequently promotes apoptotic protein expression such as bax, cyto-c and cleaved caspase3.

Being different from the second-generation TKIs, INNO-406 demonstrates specific Lyn kinase activity with no or limited activity against other Src-family member kinases [14–16]. However, the potential chemoresistance of INNO-406 will be the key factor limiting its application. Elucidating the molecular mechanism underlying its biological effects will help to alleviate the influence brought by chemoresistance.

In order to find out the mechanism underlying the effect of INNO-406 on proliferation and apoptosis, we focused on PTEN. PTEN/ PI3K/ Akt is a well known critical signaling axis to regulate the signal transduction of many biological processes including apoptosis, metabolism, and proliferation [17–19]. PTEN is a protein phosphatase that can dephosphorize serine and threonine residues [20, 21]. It has been previously confirmed that PTEN is capable of inhibiting phosphorylation of AKT [22, 23]. In our study, we confirmed that up-regulated PTEN expression by INNO-406 treatment inhibited the phosphorylation of AKT. 4EBP1 is a downstream protein of AKT/mTOR pathway which is crucial for protein synthesis due to its effect of binding to eIF4E and stoping the formation of the cap-dependent translation initiation complex [24, 25]. 4EBP1 has been indicated to participate in the regulation of apoptosis by targeting various genes. For instance, 4EBP1 modulate Bcl-2 expression to change apoptosis of the penumbral cortex in cerebral infarction injury [26]. 4EBP1/Mcl-1 Pathway inhibition is involved in the inhibition of the cytotoxic effects of proteasome inhibitors on FLT3-ITD-positive AML cells [27].

In the present study, we determined that PTEN inhibited the phosphorylation of AKT and 4EPB1 and influenced the expression of apoptotic proteins such as bax, cyto-c, cleaved caspase3 and bcl-2. However, in order to elucidate the precise mechanism, the evaluation of mTOR and s6k should be carried out which will be studied in our future research.

Conclusions

Taken together, we indicated that INNO-406 was capable of inhibiting the proliferation of CML cells through inducing apoptosis. The underlying mechanism was involved in the regulation of PTEN/AKT/4EPB1 signal pathway. These findings may extend out understanding of INNO-406 in the chemotherapy of CML and its molecular mechanism. We hope that it will provide help in avoiding drug resistance or solve the problem of drug resistance.

Abbreviations

Chronic myeloid leukemia (CML)

Abelson murine leukemia (ABL)

breakpoint cluster region (BCR)

tyrosine kinase inhibitors (TKIs)

bicinchoninic acid assay (BCA)

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

enhanced chemiluminescence (ECL)

optical densities (OD)

Declarations

Ethics approval and consent to participate

All animal procedures were approved by the Animal Research Committee of Affiliated Hospital of Qingdao University and were performed according to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Consent for publication

All authors have read and approved the publication of the present manuscript.

Availability of data and materials

All data supporting the results is available on the request from the corresponding author.

Competing interests

No conflicts of interest exist in the manuscript.

Funding

Not appliable.

Authors' contributions

SUN J aquired the data, WANG Y helped analysis data, SUN L supervised the project.

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Not applicable.

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Figures

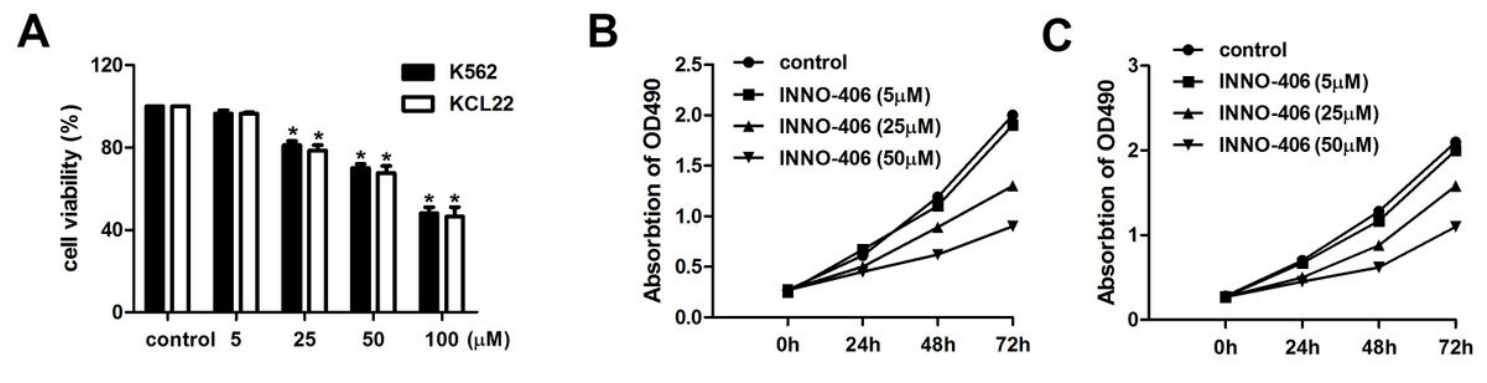

Figure 1

INNO-406 inhibited the proliferation of chronic myeloid leukemia cells. (A) MTT was performed to evaluate the cell viability of CML cell lines including K562 and KCL22 cells under different concentrations of INNO-406 treatment for 24h. (B, C) MTT was carried out to evaluate the cell viability of K562 and KCL22 cells under the treatment of INNO-406 for different times and concentration.*p□0.05 vs control.

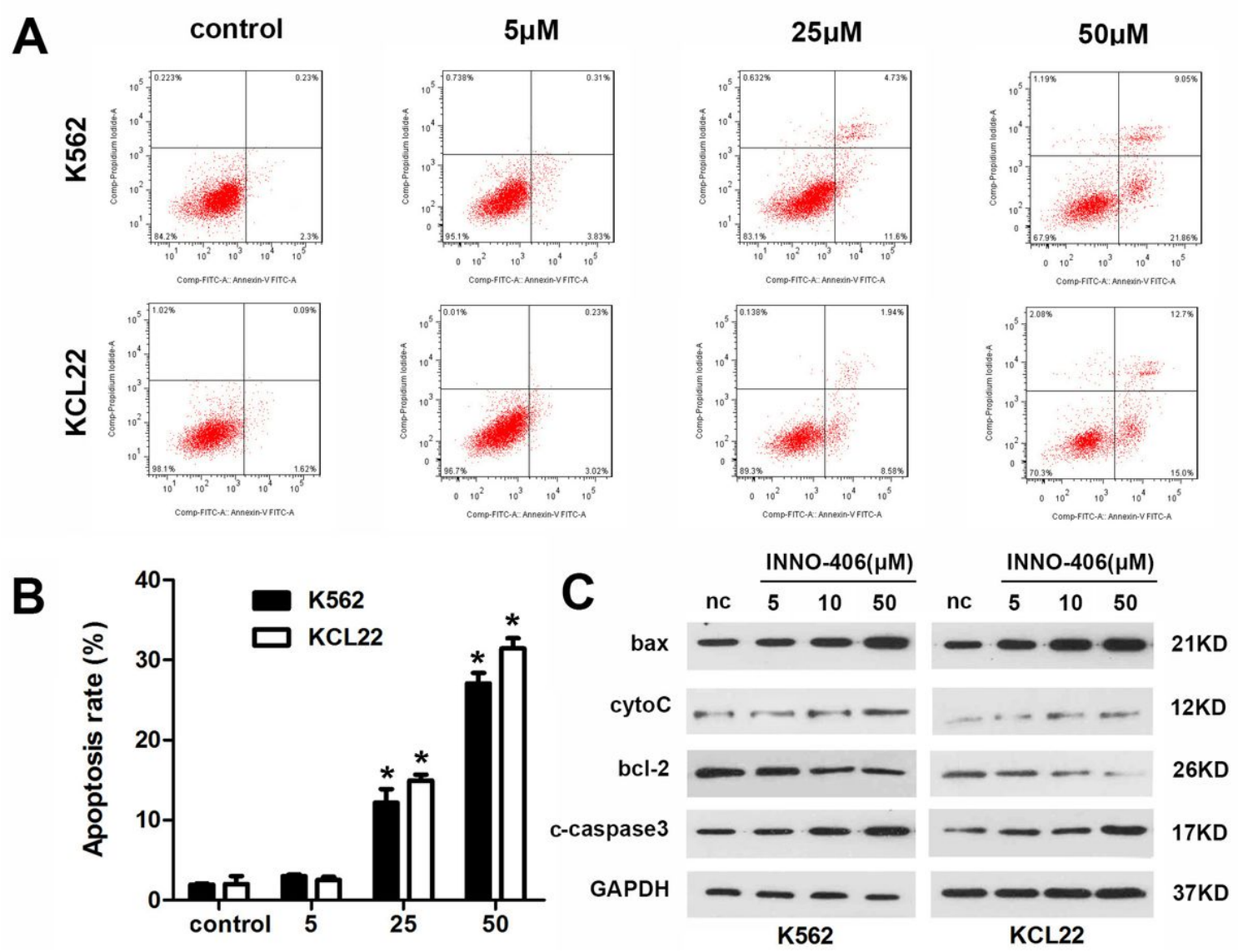

Figure 2

INNO-406 induced apoptosis of chronic myeloid leukemia cells. (A, B) Flow cytometry was performed to evaluate the cell apoptosis of CML cell lines including K562 and KCL22 cells under different concentrations of INNO-406 treatment for 24h. (B, C) Western blot was carried out to evaluate the expression of apoptotic proteins in K562 and KCL22 cells under the treatment of INNO-406. *p□0.05 vs control.

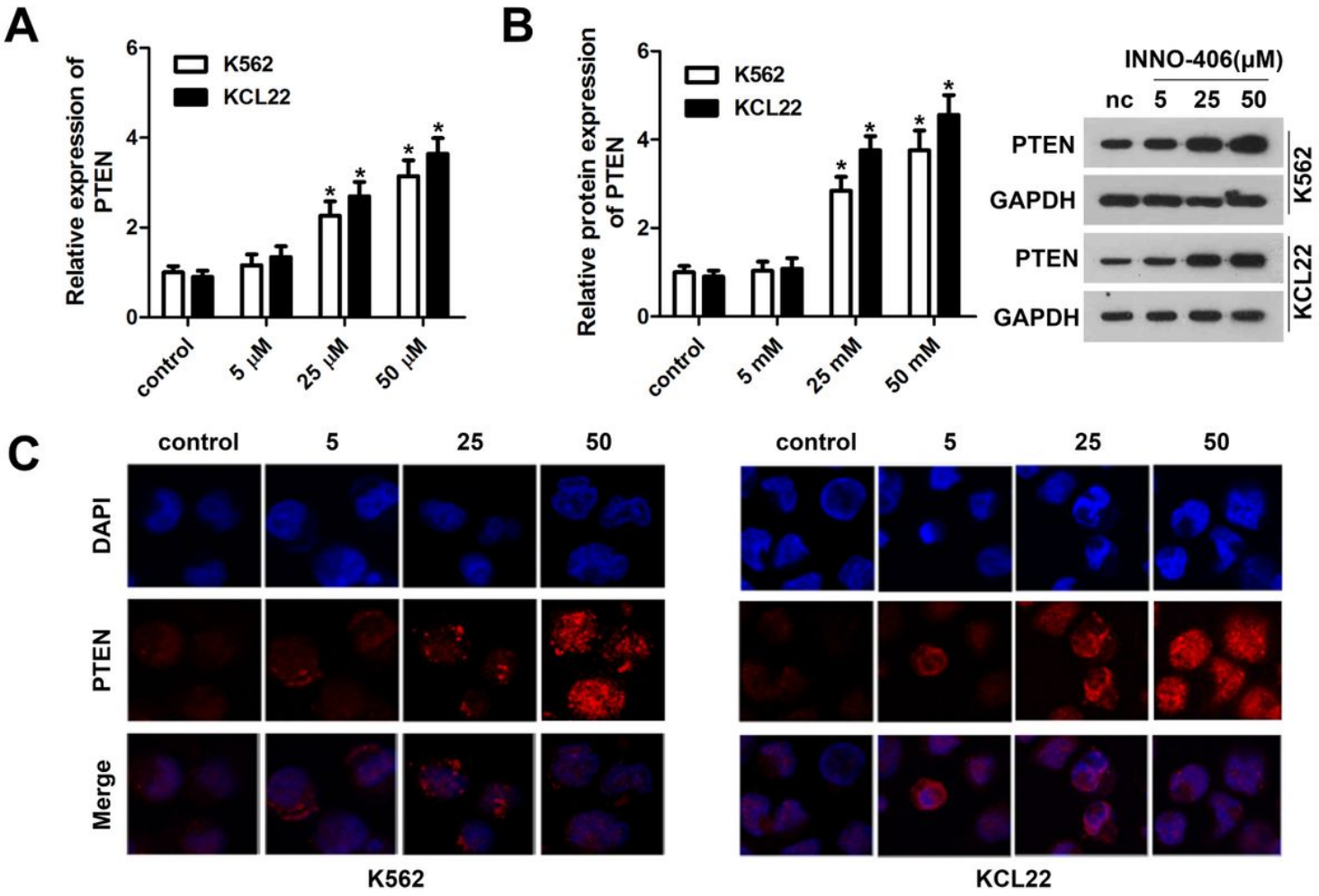

Figure 3

INNO-406 promoted the expression level of PTEN. (A) qPCR and (C) western blot were used to evaluate the mRNA and protein expression levels of PTEN in CML cells. (C) Immunofluorescence was further carried out to evaluated the expression level of PTEN in CML cells. *p□0.05 vs control.

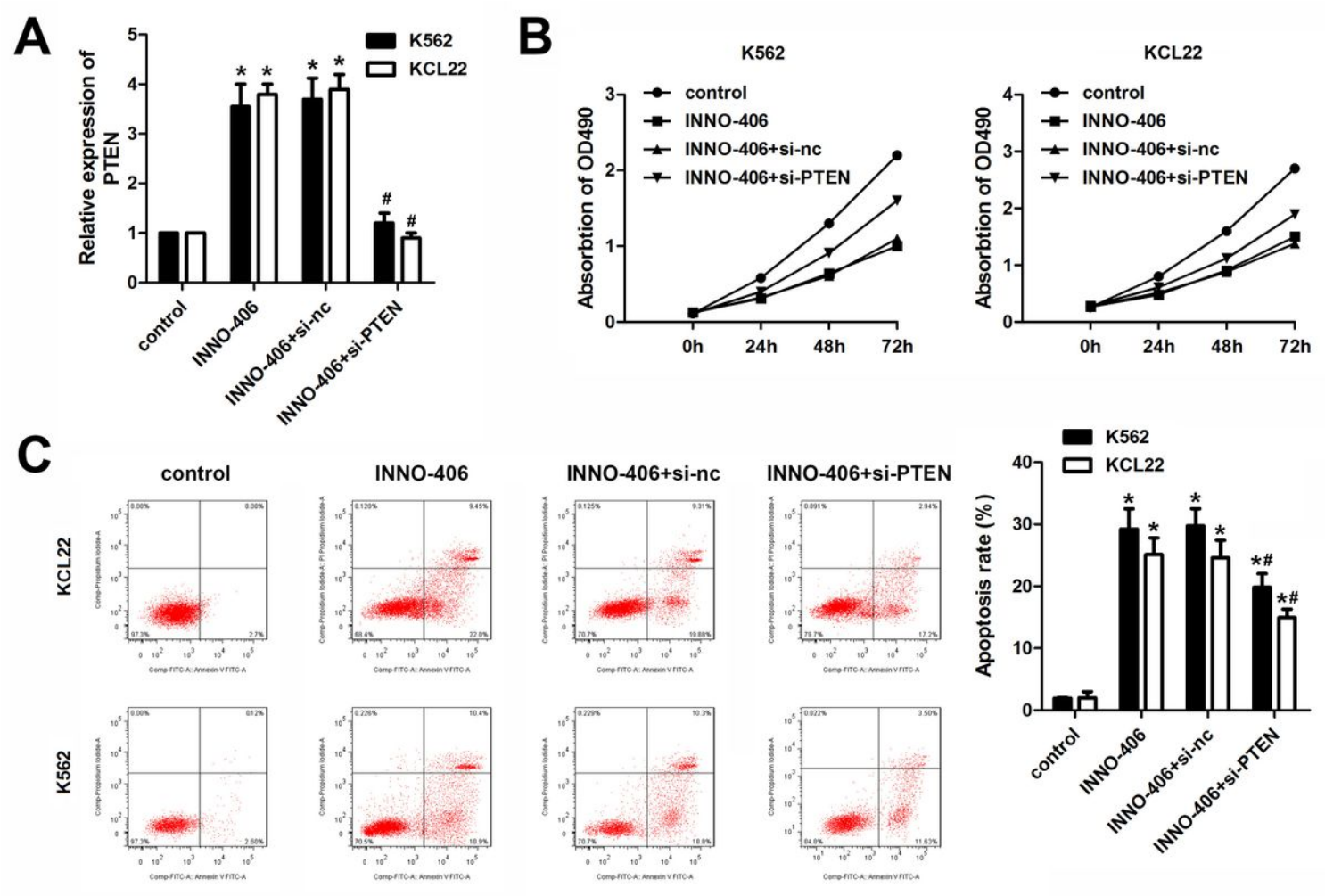

Figure 4

PTEN knockdown reversed the inhibitory effect of INNO-406 on CML cell proliferation and apoptosis. (A) qPCR was performed to evaluate the expression level of PTEN after the transfection of si-PTEN and treatment of INNO-406. (B) MTT was used to detect the cell proliferation of CML cells. (C) Flow cytometry assay was performed to investigate the colony formation ability of INNO-406. *p□0.05 vs control , #p□0.05 vs INNO-406+si-nc group.

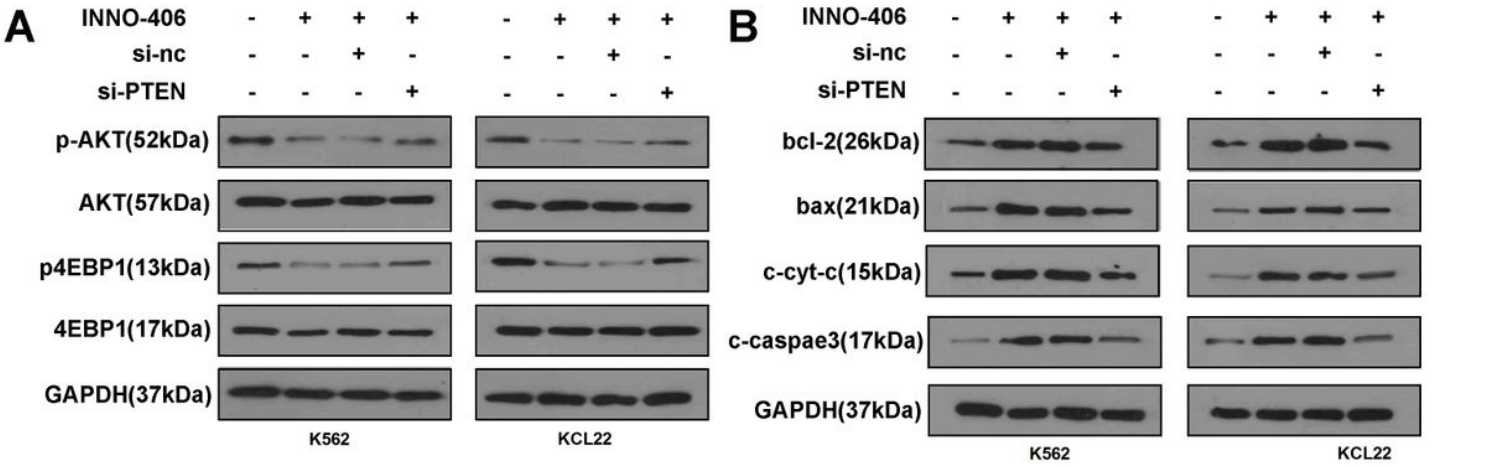

Figure 5

INNO-406 altered the phosphorylation of AKT/4EBP1. Western blot was performed to evaluate the phosphorylation and expression of (A) AKT and 4EBP1 and (B) the apoptotic proteins such as bax, bcl-2, cyto-c, and cleaved caspase3.

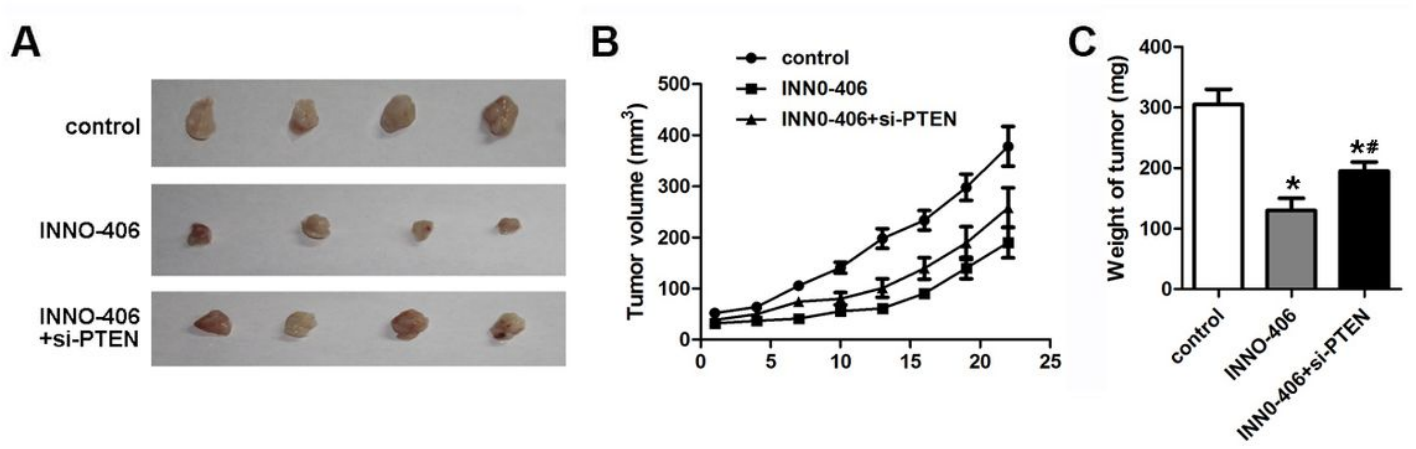

Figure 6

INNO-406 inhibited the chronic myeloid leukemia growth in vivo. (A) The subcutaneous xenograft tumors generated from CML cells in nude mice. (B) Tumor growth in nude mice was indicated by the curves represented the trend of the tumor size increase. (C) The weight of the tumors was showed. *p□0.05 vs control, #p□0.05 vs INNO-406 group.