

Inhibitory effect of the anthelmintic drug pyrvinium pamoate on T315I BCR-ABL-positive CML cells

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Abstract. Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by a chromosome translocation that generates the BCR-ABL oncogene, which encodes a constitutively activated tyrosine kinase. Despite progress in controlling CML at the chronic phase by first and second generations of BCR-ABL tyrosine kinase inhibitors (TKIs), effective drugs with good safety are not available for CML patients harboring T315I BCR-ABL and those in advanced stages of CML. Therefore, there is an urgent requirement for the development of effective therapies against T315I BCR-ABL. In the present study, it was demonstrated that pyrvinium pamoate, an anthelmintic drug approved by the Food and Drug Administration had potent inhibitory effects on growth and survival in CML cells with T315I BCR-ABL. In addition, this agent was equally effective in inhibiting the Wnt/ β -catenin signaling in wild-type and T315I BCR-ABL CML cells. Thus, the clinical efficacy of pyrvinium pamoate in treating patients with CML bearing T315I BCR-ABL should be further investigated.

Introduction

Chronic myeloid leukemia (CML) is a type of hematopoietic disorder arising from chromosomal abnormality between chromosomes 9 and 22 (1). This chromosomal abnormality, known as the Philadelphia chromosome, leads to myeloid

proliferation (2). Although tyrosine kinase inhibitor (TKI) imatinib mesylate (Gleevec) has been demonstrated to possess clinical benefit for patients with chronic phase CML harboring wild-type BCR-ABL, the CML patients harboring BCR-ABL mutants (e.g., E255V, Y253H, M351T and T315I) do not respond to imatinib (3). The majority of imatinib resistance is caused by BCR-ABL mutants, however other mutants can be overcome by the second-generation TKIs including dasatinib, nilotinib and bosutinib (4). For T315I mutation resistance to imatinib, effective therapeutics remain to be developed. The third-generation TKIs, such as ponatinib, show activity against CML with T315I BCR-ABL (5), however, the Food and Drug Administration (FDA) suspended its clinical use in 2013 due to its cardiovascular toxicity (6). Thus, agents effective against T315I BCR-ABL mutation would be valuable in treating refractory CML patients.

Pyrvinium (6-(dimethylamino)-2-[2-(2,5-dimethyl-1-phenylpyrrol-3-yl)ethenyl]-1-methyl-quinolinium) pamoate (Fig. 1A) is a FDA-approved drug used for the treatment of pinworm infections (7). Previous studies have demonstrated that pyrvinium pamoate can effectively inhibit the growth of tumor cells of diverse tissue origins, including the pancreas, colon, breast, brain, myeloma and erythroleukemia (8-14). It also has been demonstrated that pyrvinium pamoate exhibits increased cytotoxicity in tumor cells under hypoglycemic conditions and enhances the effects of conventional chemotherapeutic drugs in tumor xenografts (10,12,15). A previous study reported that pyrvinium pamoate was active against CML patients harboring wild-type BCR-ABL (16). However, whether it is against T315I BCR-ABL CML cells remains to be elucidated.

In the present study, the activity of pyrvinium pamoate was determined in CML cells with T315I BCR-ABL. It was identified that pyrvinium pamoate potently suppressed proliferation and induced apoptosis, and inhibited the Wnt/ β -catenin signaling pathway in the CML cells with T315I BCR-ABL. These results suggest that pyrvinium pamoate may be an effective drug for the treatment of CML with T315I BCR-ABL mutation.

Materials and methods

Reagents and antibodies. Pyrvinium pamoate (CAS: 3546-41-6; chemical structure, Fig. 1A) was purchased

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Abbreviations: CML, chronic myeloid leukemia; TKIs, tyrosine kinase inhibitors; COX II, cytochrome *c* oxidase subunit II; AIF, apoptosis-inducing factor

Key words: chronic myeloid leukemia, T315I, imatinib resistance, pyrvinium pamoate, apoptosis, β -catenin

from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and was prepared as a 20 mM stock solution in dimethyl sulfoxide (DMSO) and stored at -20°C . Antibodies and their sources were as follows: Mouse monoclonal antibodies against poly ADP-ribose polymerase (PARP; clone 4C10-5; cat no. 51-6639GR; dilution, 1:4,000), X-linked inhibitor of apoptosis protein (XIAP; cat no. 610762; dilution, 1:1,000), c-Myc (cat no. 51-1485GR; dilution, 1:1,000), β -catenin (cat no. 610154; dilution, 1:1,000), cytochrome *c* (clone 6H2.B4; cat no. 556432; dilution, 1:1,000), caspase-3 (cat no. 610322; dilution, 1:1,000) and rabbit polyclonal antibodies against active caspase-3 (cat no. 557038; dilution, 1:500) were from BD Pharmingen (San Diego, CA, USA). Antibodies against B-cell lymphoma (Bcl)-2-associated X (Bax; cat no. sc-493; dilution, 1:1,000), Bcl-X_L (S-18) (cat no. sc-634; dilution, 1:1,000), glycogen synthase kinase (GSK)3 β (cat no. sc-9166; dilution, 1:1,000), cyclin D1 (cat no. sc-753; dilution, 1:1,000) and mouse monoclonal antibodies against apoptosis-inducing factor (AIF; cat no. sc-55519; dilution, 1:500), were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); antibodies against β -actin (clone AC-15; cat no. A5441; dilution, 1:4,000) and α -tubulin (clone B-5-1-2; cat no. T5168; dilution, 1:1,000) were from Sigma-Aldrich; Merck KGaA; antibodies against caspase-8 (cat no. 9746S; dilution, 1:1,000), caspase-9 (cat no. 9508S; dilution, 1:1,000), phosphorylated (p)-GSK3 β (Ser9; cat no. 9336S; dilution, 1:1,000) and Mcl-1 (cat no. 4572S; dilution, 1:500) were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-survivin (cat no. NB 500-201; dilution, 1:1,000) was from Novus Biologicals, Bcl-2 (cat no. 05-729; dilution, 1:1,000) was from EMD Millipore (Billerica, MA, USA) and cytochrome *c* oxidase subunit II (COX II; dilution, 1:1,000) was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The secondary Alexa Fluor[®] 680CW goat anti-mouse (cat no. 926-68070; dilution, 1:10,000) and 800CW goat anti-rabbit (cat no. 926-32211; dilution, 1:10,000) antibodies were purchased from LI-COR Biosciences (Lincoln, NE, USA).

Cell culture. K562 cells were purchased from American Type Culture Collection (Manassas, VA, USA), and grown in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% heat inactivated fetal bovine serum (FBS; Biological Industries USA, Cromwell, CT, USA), maintained in a humidified atmosphere with 5% CO₂ at 37°C. KBM5 and KBM5-T315I cells were provided by Professor Sai-Ching J. Yeung (Department of Emergency Medicine, University of Texas MD Anderson Cancer Center, Houston, TX, USA) and grown in Iscove's modified Dulbecco's medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% heat inactivated FBS as previously described (17). The 32D myeloid cells stably expressing either 210 kDa wild-type BCR-ABL (32D-BCR-ABL) or T315I BCR-ABL [32D-T315I; both provided by Dr Lin Qiu (Harbin Institute of Hematology and Oncology, Harbin, Heilongjiang, China)] were established and maintained in RPMI-1640 with 10% FBS as described previously (18,19).

Cell viability assay. Cell viability was determined by MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation assay; Promega Corporation, Madison, WI, USA) (20). Briefly,

2x10⁴ cells in 100 μl were exposed to various concentrations of pyrvinium pamoate for 72 h. Control cells were treated with medium containing <0.1% DMSO. The absorbance/optical density were read on a 96-well plate reader at wave length 490 nm. The drug concentration resulting in 50% inhibition of cell growth (IC₅₀) was determined (21).

Colony formation assays. The K562, KBM5, KBM5-T315I, 32D-BCR-ABL and 32D-T315I cells were treated with pyrvinium pamoate or DMSO (control) for 24 h, then cells were harvested and washed with PBS, and 2,000 cells were seeded in RPMI-1640 or Iscove's medium containing 0.3% agar and 20% FBS in the absence of drug treatment. Following incubation for another 10–14 days at 37°C and 5% CO₂, colonies with >50 cells were counted using an inverted phase-contrast microscope (22).

Trypan blue exclusion assay. The K562, KBM5, KBM5-T315I, 32D-BCR-ABL and 32D-T315I cells were cultured at 2x10⁵ cells/well in 6-well plates, then cells were treated with increasing concentrations of pyrvinium pamoate, or with the fixed concentration for different time periods. Viable and dead cells were assessed by counting with a hemocytometer following trypan blue exclusion assay (23).

Preparation of cell lysates and cytosolic fractions. Whole lysates were prepared with radioimmunoprecipitation assay buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with freshly added 10 mM β -glycerophosphate, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and Roche Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Basel, Switzerland) (24). The cytosolic fraction was prepared with digitonin extraction buffer to detect the level of cytochrome *c* and AIF in the cytosol, as described previously (25).

Preparation of cytoplasmic and nuclear fractions. Control or drug-treated cells were pelleted by centrifugation at 300 x g for 5 min at room temperature and rinsed with PBS. Pellets were then resuspended in 200 μl ice-cold lysis buffer (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.4% NP-40 with 1 mM DTT, 0.5 mM PMSF, 1 mM sodium orthovanadate and the Roche Complete Mini Protease Inhibitor Cocktail) by pipetting up and down (without bubbling) ~10 times (22,26). Following incubation on ice for 10 min, the lysates were centrifuged at 10,000 x g for 10 min at 4°C. The supernatants were transferred to fresh tubes and referred to as cytoplasm ice extracts. Following washing with the lysis buffer, the pellets were resuspended in nuclear protein extraction buffer with inhibitors (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA with 1 mM DTT, 0.5 mM PMSF, 0.2 mM sodium orthovanadate and the Roche Complete Mini Protease Inhibitor Cocktail) and centrifuged at 10,000 x g for 10 min at 4°C. The resultant supernatants were kept as nuclear fractions (22,26).

Western blotting analysis. The whole cells were lysed in radioimmunoprecipitation assay buffer on ice for 30 min. Equal volumes (20 μl) of lysate were electrophoresed with 10%

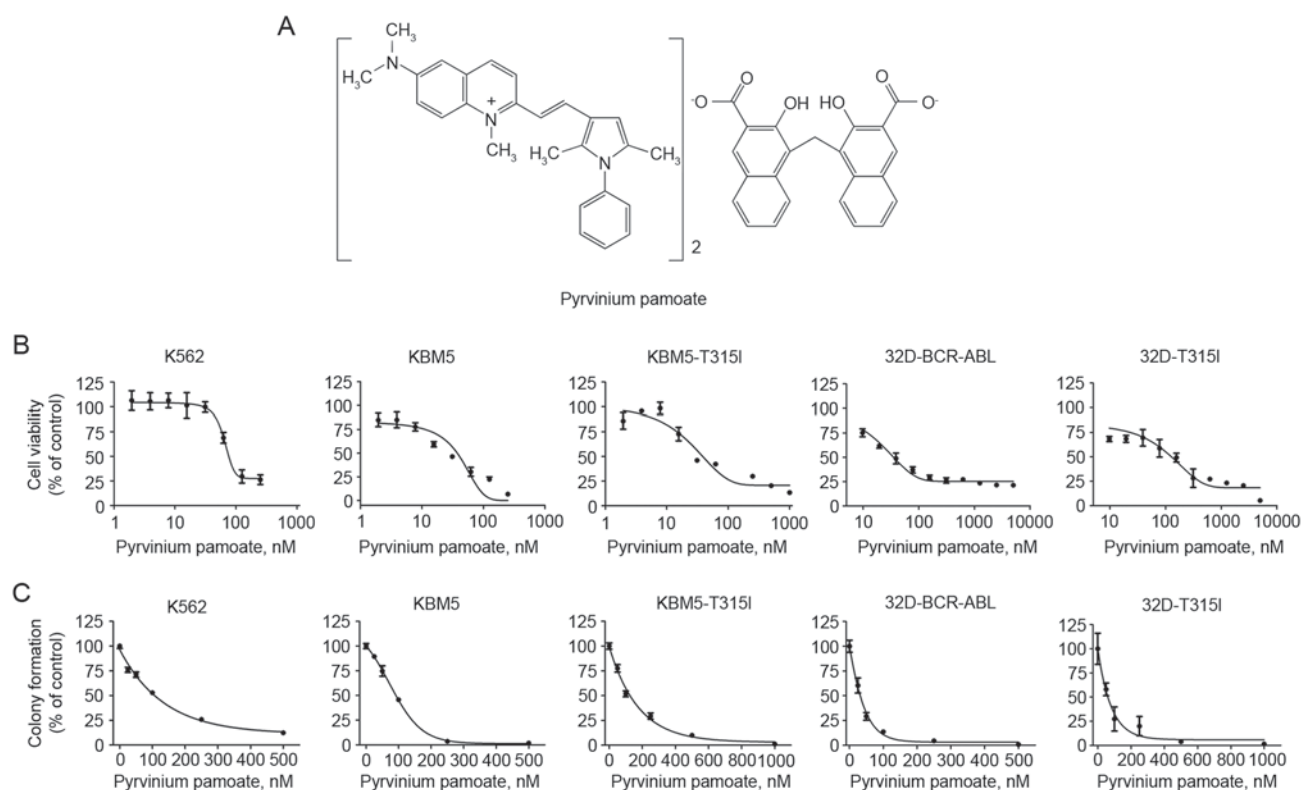


Figure 1. Pyrvinium pamoate reduced the growth of CML cells with T315I or wild-type BCR-ABL. (A) The chemical structure of pyrvinium pamoate. (B) Five lines of BCR-ABL-expressing cells were cultured with escalating concentrations of pyrvinium pamoate for 72 h, followed by MTS assay to test the cell viability. Log dose-response curves with mean \pm standard deviation are presented. (C) Pyrvinium pamoate inhibited the clonogenicity of five lines of BCR-ABL-expressing cells in a dose-dependent manner. CML cells were treated with pyrvinium pamoate at the indicated concentrations for 24 h and then seeded in the drug-free soft agar for another 10-14 days of incubation, colony-forming units were counted. CML, chronic myeloid leukemia.

SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes. The membranes were blocked in PBS with 0.05% Tween-20 containing 5% skimmed milk and incubated with the primary antibody at 4°C overnight, and then incubated with the secondary 680/800 antibodies. Protein expression was assessed by the Odyssey infrared imaging system (LI-COR Biosciences). β -actin was used as a protein loading control.

Statistical analysis. All experiments were performed a minimum of three times, and data are presented as the mean \pm standard deviation. Differences between multiple groups were analyzed by one-way analysis of variance, with post-hoc intergroup comparisons made using Tukey's test, unless otherwise stated. GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Pyrvinium pamoate effectively inhibited the proliferation of CML cells harboring T315I BCR-ABL. Firstly, the effect of pyrvinium pamoate on cell viability was examined. A total of three lines of human CML cells were incubated with increasing concentrations of pyrvinium pamoate (Fig. 1A) for 72 h. The MTS assay indicated that K562 and KBM5 cells, both of which harbor wild-type BCR-ABL, were sensitive to pyrvinium pamoate with IC_{50} values of 14.2 and 26.2 nM,

respectively (Fig. 1B), KBM5-T315I cells harboring T315I BCR-ABL exhibited a similar sensitivity to pyrvinium pamoate with an IC_{50} value 29.2 nM. The ability of pyrvinium pamoate to inhibit the viability was further confirmed in a pair of murine 32D myeloid cells stably transfected with either the wild-type or T315I BCR-ABL, the IC_{50} values were 49.7 and 133 nM, respectively (Fig. 1B). In an additional independent set of experiments, similar results were obtained in colony formation experiments, which indicated that pyrvinium pamoate inhibited the clonogenicity of CML cells bearing either wild-type BCR-ABL or T315I BCR-ABL (Fig. 1C).

These results demonstrated that pyrvinium pamoate could be equally effective in inhibiting the proliferation of CML cells with T315I BCR-ABL mutation as to that of wild-type CML cells.

Pyrvinium pamoate induced the apoptosis in CML cells harboring T315I BCR-ABL. Whether pyrvinium pamoate was able to induce cell death was investigated. The cells were cultured with increasing concentrations of pyrvinium pamoate for 36 h, and cell death was measured by trypan blue exclusion assay. Pyrvinium pamoate led to marked increases in cell death in a dose- and time-dependent manner in the CML cells harboring either wild-type or T315I BCR-ABL (Fig. 2A and B). In addition, pyrvinium pamoate induced a dose- and time-dependent cleavage of PARP and caspase-3 in all five lines of BCR-ABL-expressing cells (Fig. 2C and D). The levels of caspase-9 and -8 were also

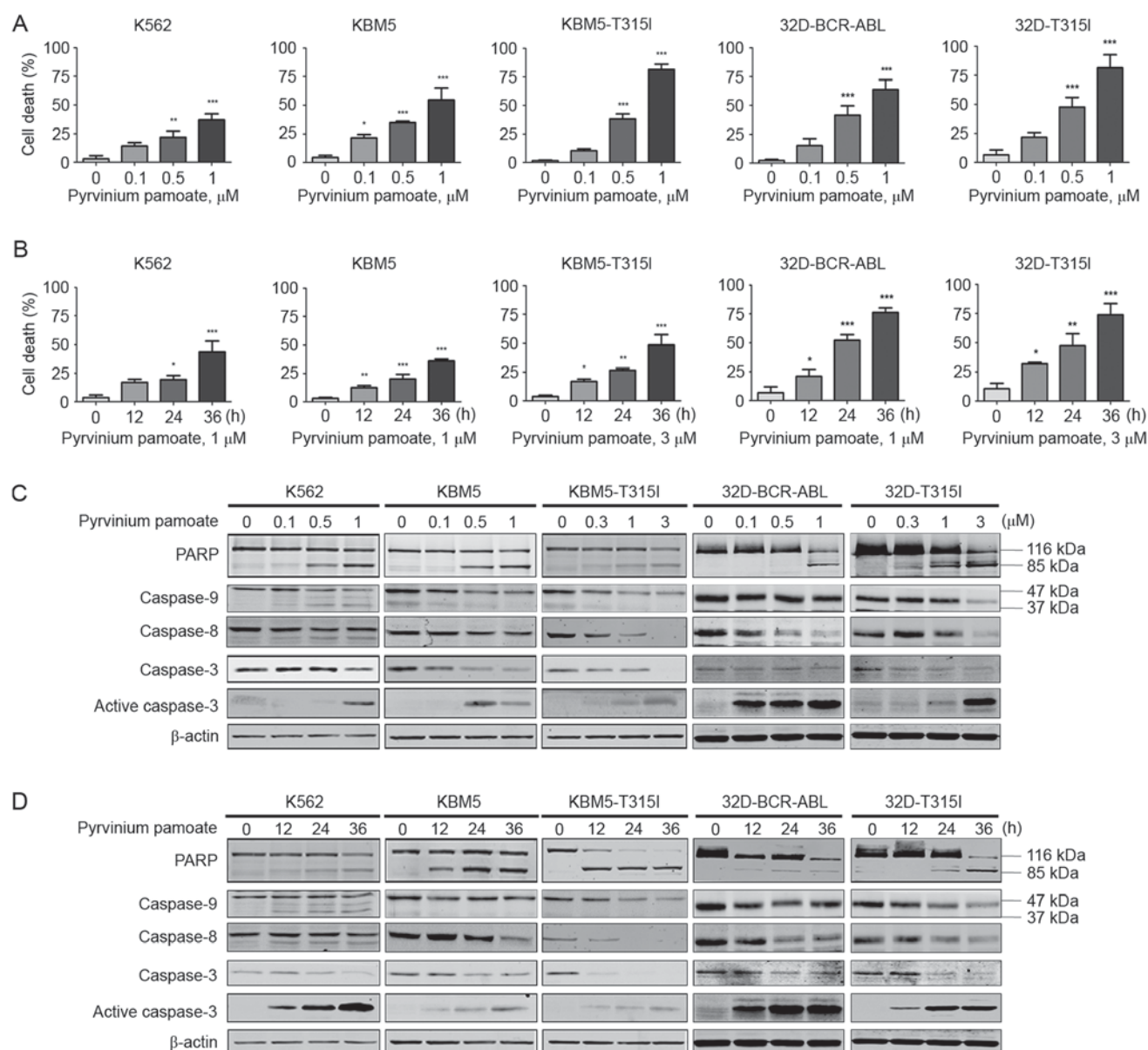


Figure 2. Pyrvinium pamoate induced apoptosis in CML cells with T315I BCR-ABL. (A and B) Cell death was evaluated by trypan blue exclusion assay. (A) K562, KBM5 and 32D-BCR-ABL cells were treated with pyrvinium pamoate (0, 0.1, 0.5 and 1 μ M) for 36 h, KBM5-T315I and 32D-T315I cells were treated with pyrvinium pamoate (0, 0.3, 1 and 3 μ M) for 36 h. (B) K562, KBM5 and 32D-BCR-ABL cells were treated with 1 μ M pyrvinium pamoate for 0, 12, 24 and 36 h; KBM5-T315I and 32D-T315I cells were treated with 3 μ M pyrvinium pamoate for 0, 12, 24 and 36 h. Trypan blue exclusion assay was used to measure the cell death of the five cell lines. * P <0.05, ** P <0.01, *** P <0.001, one-way analysis of variance with post hoc intergroup comparison by the Tukey's test. (C and D) Dose- and time-course western blotting analysis of PARP cleavage, caspase-3, -8, and -9 in the whole-cell lysates of CML cells were shown. β -actin was used as a loading control. CML, chronic myeloid leukemia; PARP, poly adenosine diphosphate-ribose polymerase.

decreased in a dose- and time-dependent manner, suggesting their activation by pyrvinium pamoate (Fig. 2C and D). Pyrvinium pamoate additionally induced a time-dependent release of AIF and cytochrome *c* into cytosol (Fig. 3A). Additionally, in all the drug-treated BCR-ABL-expressing cells, a decrease in the protein levels of anti-apoptotic proteins Mcl-1, survivin and XIAP was observed (Fig. 3B and C) but not of other Bcl-2 family members. Taken together, pyrvinium pamoate effectively induced apoptosis in five lines of BCR-ABL-expressing cells regardless of their BCR-ABL status.

Pyrvinium pamoate effectively inhibited the Wnt/ β -catenin signaling in CML cells harboring T315I BCR-ABL. It was

investigated whether pyrvinium pamoate affected BCR-ABL expression, and western blotting analysis indicated no change in BCR-ABL expression in the CML cells treated with pyrvinium pamoate (Fig. 4A), suggesting that the antineoplastic effect of pyrvinium pamoate on CML cells is BCR-ABL independent. Previous reports have demonstrated that pyrvinium pamoate inhibits Wnt/ β -catenin signaling pathway in breast and lung cancer (13,27). It was therefore investigated whether pyrvinium pamoate inhibited the Wnt/ β -catenin signaling in CML cells. The levels of β -catenin were markedly decreased in two pairs of CML cells (KBM5 and KBM5-T315I cells or 32D-BCR-ABL and 32D-T315I cells)(Fig. 4B). Subsequently, the upstream and downstream proteins of the Wnt/ β -catenin signaling pathway were

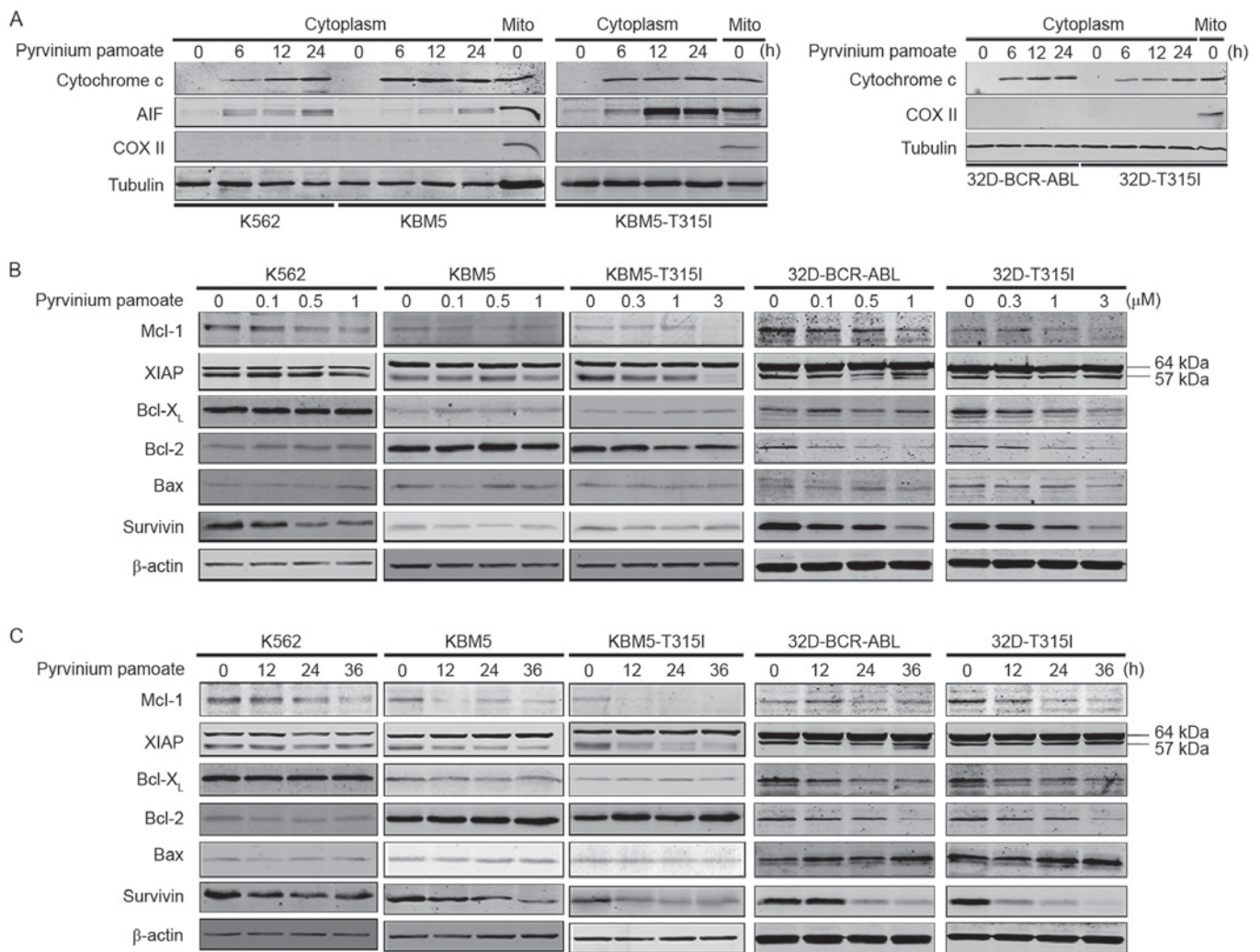


Figure 3. Pyrrvinium pamoate regulated the expression of the apoptosis-associated proteins in CML cells with T315I BCR-ABL. (A) Pyrrvinium pamoate induced release of AIF and cytochrome *c* from the mitochondria. COX II served as a mitochondrial indicator. (B and C) The expression of apoptosis-associated proteins in whole cell lysates was detected by western blotting analysis. The cells were treated with (B) different concentrations of pyrrvinium pamoate for 36 h or (C) 1 μ M pyrrvinium pamoate for K562, KBM5 and 32D-BCR-ABL cells; or 3 μ M pyrrvinium pamoate for KBM5-T315I and 32D-T315I cells for the indicated time periods. β -actin was used as a loading control. CML, chronic myeloid leukemia; AIF, apoptosis-inducing-factor; COX II, cytochrome *c* oxidase subunit II; XIAP, X-linked inhibitor of apoptosis protein; Bcl, B-cell lymphoma; Bax, Bcl-2-associated X; Mcl-1, myeloid cell leukemia sequence 1.

measured. The results indicated that the levels of p-GSK3 β (Ser9), cyclin D1 and c-Myc were decreased (Fig. 4B), suggesting that pyrrvinium pamoate may block the activation of canonical Wnt/ β -catenin signaling.

Due to the fact that nuclear translocation of β -catenin is required for its functions (i.e., to activate T-cell factor/lymphoid enhancer factor), the effects of pyrrvinium pamoate on the subcellular distribution of β -catenin were measured. The results indicated that the levels of β -catenin in nuclear and cytosolic fractions were reduced by pyrrvinium pamoate in a dose-dependent manner (Fig. 4C).

Discussion

The present study aimed to investigate the anti-tumor activity of pyrrvinium pamoate, an anthelmintic drug, in CML cells harboring the T315I mutation. The results demonstrated that this agent is equally effective in inhibiting proliferation and inducing apoptosis in CML cells harboring either wild-type

or T315I BCR-ABL. Due to the fact that pyrrvinium pamoate is an FDA-approved drug, the results of the present study may lead to a clinical trial for imatinib-resistant patients with T315I BCR-ABL.

Pyrrvinium pamoate is a quinolone-derived cyanine dye, and the FDA approved its anthelmintic property in the 1950s (28). This agent has been previously investigated in solid tumors and hematopoietic malignancy (8-14). It was identified that pyrrvinium pamoate potently inhibited the viability and induced apoptosis in both wild-type and T315I BCR-ABL-expressing cells at low nanomolar concentrations. The results were consistent with the report by Xiang *et al* (16) regarding the effect of pyrrvinium pamoate on CML cells with wild-type BCR-ABL. Of note, pyrrvinium pamoate was reported to be active against primary CD34⁺ blast cells from 8 patients with CML who harbored BCR-ABL point mutations (E255K, E453K and E255V). The results of the present study were in agreement with those from the literature (16), suggesting that this agent may be a promising potential therapy

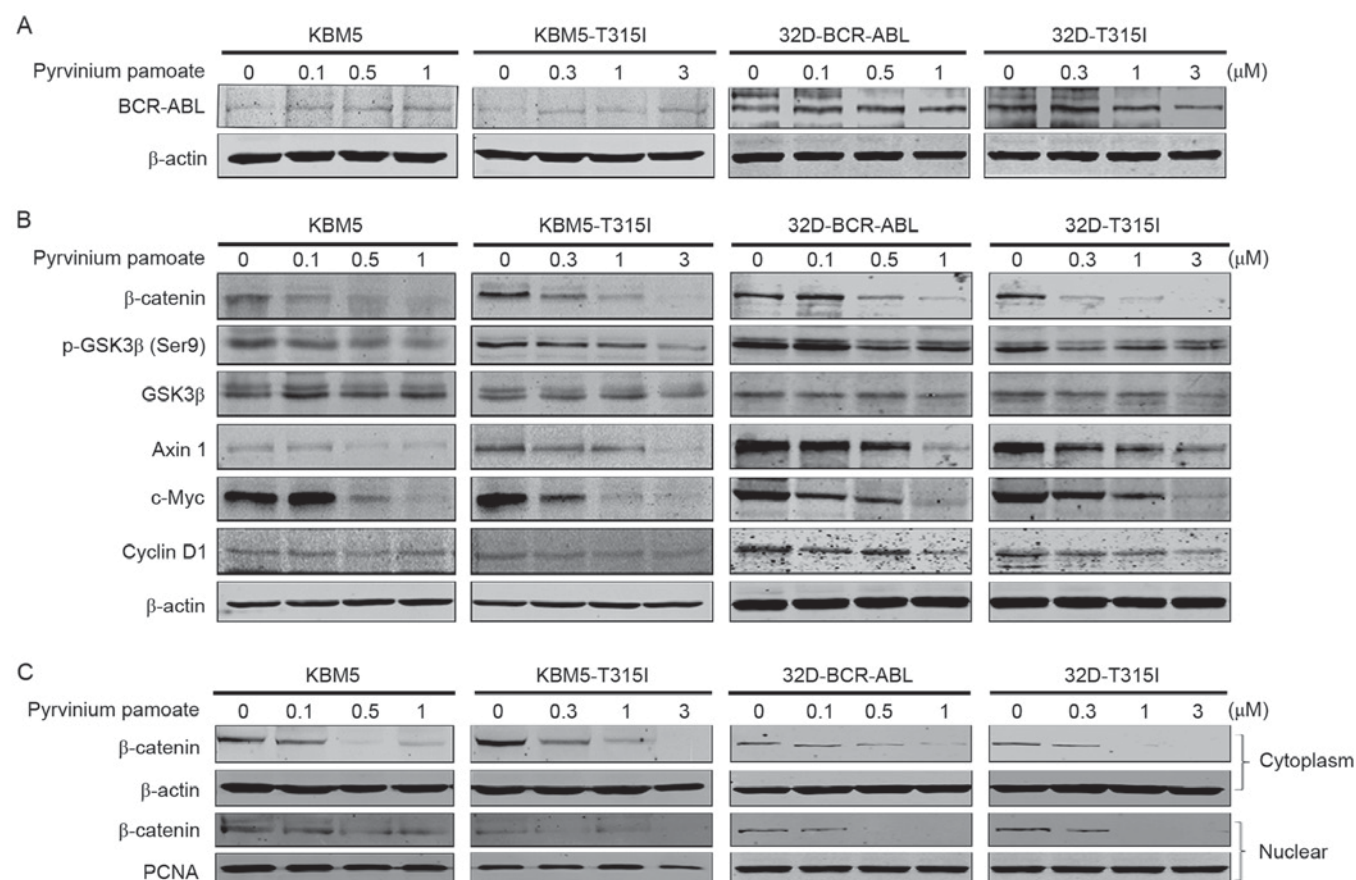


Figure 4. Pyrvinium pamoate inhibited Wnt/β-catenin signaling in CML cells with T315I BCR-ABL. (A) Western blotting was performed to examine the levels of BCR-ABL in CML cells treated with pyrvinium pamoate. (B) CML cells were incubated with incremental dose of pyrvinium pamoate for 36 h, cell lysates were subjected to western blotting analysis. (C) The levels of β-catenin in cytoplasm and nucleus were determined by western blotting analysis. CML, chronic myeloid leukemia; p-, phosphorylated; GSK, glycogen synthase kinase; PCNA, proliferating cell nuclear antigen.

for patients with CML who harbor wild-type and different point mutations of BCR-ABL including T315I.

Although it has been used for more than 50 years, the molecular mechanisms of pyrvinium pamoate remain unclear. It has been reported that in different types of cancer that pyrvinium pamoate may target different signaling pathways (8-11). We observed that the inhibitory effects of pyrvinium pamoate on mutant CML cells might be associated with its apoptotic action. Apoptosis, which is programmed cell death, can occur via the cell death receptor-mediated extrinsic pathway or the mitochondria-mediated intrinsic pathway (29). Reduction of apoptosis is believed to contribute to cancer development. In the present study, it was observed that pyrvinium pamoate induced apoptosis in both the wild-type and T315I BCR-ABL mutant CML cells (Figs. 2 and 3). The apoptosis may be caused by the mitochondrial-dependent pathway, as evidenced by the release of AIF and cytochrome *c* to the cytosol was increased following treatment with pyrvinium pamoate (Fig. 3A).

Previous studies indicated that pyrvinium pamoate could inhibit the growth and differentiation of different cancer cells through the Hedgehog signaling pathway (14), or Wnt/β-catenin signaling pathway (13,27,30). Consistent with those results, decreased levels of β-catenin were observed, and the upstream and downstream proteins of Wnt/β-catenin pathway, including phospho-GSK3β (Ser 9) and c-Myc in

CML cells either with wild-type or T315I BCR-ABL mutations following pyrvinium pamoate treatment (Fig. 4B). Thus, it is suggested that pyrvinium pamoate inhibits the growth of CML cells by inhibiting the Wnt/β-catenin signaling pathway. Taken together, it may be beneficial to investigate the clinical efficacy of pyrvinium pamoate in treating patients with CML bearing T315I BCR-ABL.

In summary, it was reported that pyrvinium pamoate effectively inhibits the proliferation and induces apoptosis in the wild-type or T315I BCR-ABL-expressing cells, suggesting that pyrvinium pamoate is a promising agent that may be used for treatment of patients with imatinib-resistant T315I BCR-ABL CML in addition to imatinib-sensitive wild-type CML cells.

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