European Journal of Pharmacology 876 (2020) 173064

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

Pseudolaric acid B induces mitotic arrest and apoptosis in both imatinib-sensitive and -resistant chronic myeloid leukaemia cells

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ARTICLE INFO

Keywords:
Chronic myeloid leukaemia cells
PAB
BCR-ABL
Apoptosis
Cell cycle
Drug resistance

ABSTRACT

The selective BCR-ABL tyrosine kinase inhibitor imatinib is one of the first-line therapies in the management of chronic myeloid leukaemia (CML). However, acquired resistance to this inhibitor, which is especially conferred by the T315I point mutation in BCR-ABL, impedes the efficacy of imatinib therapy. Therefore, the discovery and development of novel agents to overcome imatinib resistance is urgently needed. *Pseudolaric acid B* (PAB), a small molecule isolated from the traditional Chinese medicine *Cortex pseudolaricis*, has been reported to be a potential candidate for immune disorders and cancer treatment. However, its effects on CML and the involved molecular mechanism have not been reported. In the current study, by performing both *in vitro* and *in vivo* experiments in CML cells, we showed that PAB blocked the cell cycle at G_2/M phase and subsequently activated the caspase pathway, cleaved the BCR-ABL protein and inhibited the BCR-ABL downstream pathways, ultimately leading to cell proliferation inhibition, cytotoxicity and apoptosis. These events were observed in both imatinib-sensitive and imatinib-insensitive CML cell lines. Moreover, PAB decreased the viability of primary blood mononuclear cells from CML patients and induced apoptosis in these cells. Our findings suggest that PAB could be used as a novel agent to sensitize imatinib-resistant CML.

1. Introduction

Chronic myeloid leukaemia (CML) is a myeloproliferative haematologic neoplasm characterized by overexpression of the p210 BCR-ABL fusion gene due to a chromosomal translocation termed t (9; 22) (q34; q11), also known as the Philadelphia (Ph) chromosome (Ren, 2005). The tyrosine kinase activity of c-ABL is activated in this fusion protein, contributing to its malignant transformation (Melo and Barnes, 2007). BCR-ABL triggers several mitogenic signalling pathways that are involved in cell proliferation and survival, such as the MAPK/ERK cascade, PI3K/Akt/mTOR and STAT pathways (Lugo et al., 1990; Modi et al., 2011; O'Hare et al., 2011). Therefore, targeting BCR-ABL is a primary strategy for treating CML. Imatinib (IM), a first generation BCR-ABL tyrosine kinase inhibitor (TKI), specifically binds to BCR-ABL's ATP-binding site and thereby inhibits its autophosphorylation; hence, it is the first-line therapy in the management of CML (Modi et al., 2011; Schindler et al., 2000). However, acquired resistance to IM therapy is due to the amplification and point mutation of BCR-ABL frequently and significantly limits the prognosis of CML patients (Apperley, 2007; Vaidya et al., 2011; Hochhaus et al., 2002). To overcome this acquired resistance, second generation TKIs such as nilotinib, dasatinib and bosutinib, which are effective against all mutations except the T315I mutation, have been developed (Redaelli et al., 2009; Laneuville et al., 2010). Although a third generation TKI, ponatinib, was explored to overcome the T315I point mutant, it has its own side effects, and there is emerging resistance to the drug. Thus, the discovery of novel compounds to overcome resistance caused by the T315I mutant is needed to improve the current CML therapies.

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https://doi.org/10.1016/j.ejphar.2020.173064
Received 2 February 2020; Received in revised form 29 February 2020; Accepted 10 March 2020
Available online 14 March 2020

Cortex pseudolaricis, an effective component of *Pseudolaric acid B* (PAB), has been regarded as a Traditional Chinese Medicine (TCM) for the treatment of fungal skin infections, eczema, and other skin diseases for centuries (Liu et al., 2017). Previous studies have demonstrated that PAB has antimicrobial, antifungal, antifertility and antiangiogenic properties (Chiu et al., 2010; Liu et al., 2012; Yu et al., 2012; Zhang et al., 2018). More recently, PAB was found to suppress cancer progression via induction of apoptosis, autophagy, proliferation inhibition and cell cycle arrest in various tumor models, such as liver cancer, gastric cancer, cervical cancer, lung cancer, breast cancer and melanoma. Additionally, PAB has been reported to overcome multiple drug resistance (MDR) in gastric and liver cancer cell lines (Wong et al., 2005). Results from Wen, who is one of our research members, also reported that PAB overcomes 5-Fu-resistance in colorectal cancer via CDK1-involved cell mitotic arrest (Wen et al., 2016a). However, the antineoplastic effect of PAB on CML, especially IM-resistant CML, has not been explored.

In our present study, we investigated the antineoplastic effects of PAB in IM-sensitive and IM-insensitive CML cell lines. PAB showed excellent cytotoxic effects in cell lines with wild-type BCR-ABL and BCR-ABL harbouring the T315I mutant. Significantly, primary blood mononuclear cells from CML patients were also sensitive to PAB treatment. The specific mechanisms of action of PAB involves disturbance of the cell cycle, activation of caspases, and downregulation of BCR-ABL and its downstream cascade. Moreover, PAB also inhibited tumour growth in mouse xenograft models. We propose that PAB could be used as a novel agent to overcome IM-resistance caused by the BCR-ABL-T315I mutation in CML.

2. Materials and methods

2.1. Cell cultures

KBM5 cell line, which expresses wild-type p210 BCR-ABL, was derived from a female myeloid CML patient in blast crisis, as described previously (Shi et al., 2009). KBM5-T315I cell line was derived from the KBM5 cell line through exposure to increasing concentrations of imatinib and subsequent selection of surviving clones harbouring the T315I mutation, as previously described (Shi et al., 2009; Melo and Chuah, 2008). These two cell lines were cultured in IMDM (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), but KBM5-T315I cells were provided with 1 μM imatinib in order to maintain the IM-resistant phenotype. Murine BaF3 cells stably expressing either 210-kDa wild-type BCR-ABL (BaF3-p210-WT) or T315I BCR-ABL (BaF3-p210-T315I) were kindly provided by Dr. B.Z. Carter (University of Texas M.D. Anderson Cancer Center, Houston, TX, USA) (Mak et al., 2009). K562 cell line was purchased from ATCC. BaF3-p210-WT, BaF3-p210-T315I and K562 cell lines were cultured in RPMI 1640 medium (Gibco-BRL) with 10% FBS as described previously (Shi et al., 2009).

Blood samples from patients with CML were obtained from discarded materials utilized for routine laboratory tests at the Department of Hematology, Guangzhou First Municipal People's Hospital (Guangzhou, Guangdong, China). Total 8 CML patients were recruited. Primary blood mononuclear cells were prepared from healthy donors. The use of these materials in the current study was approved by the institution, along with the permission of the patients and volunteers. Mononuclear cells were isolated by Ficoll-Paque and cultured in RPMI-1640 medium with 15% FBS as described previously (Shi et al., 2009).

2.2. Reagents

Pseudolaric acid B and z-VAD-FMK were purchased from Selleck Chemicals Co., Ltd. (Shanghai, China). The purities of these compounds were > 99%, as determined by high-performance liquid chromatography (HPLC). Both reagents were dissolved in dimethyl sulfoxide (DMSO), and 20 mM stock solutions were prepared and stored at −20 °C. Rabbit monoclonal antibodies (Abs) against PARP, caspase 3, active-caspase 3, Mcl-1, Bcl-xl, Bax, Survivin, XIAP, c-abl, phospho-c-abl (Y245), Stat 5, phospho-Stat 5A/B (Y694, Y699), ERK1/2, phospho-ERK1/2 (T202/Y204), AKT, phospho-AKT, Crkl, phospho-Crkl (Y207) and mouse monoclonal Abs against caspase 8 and BCL-2 were obtained from Cell Signalling Technology (Beverly, MA, USA) and diluted 1:1000. Antibodies against phospho-histone H3 (Ser10) and phospho-cdc2 (Thr16) were purchased from Signalway Antibody (Baltimore, MD, USA). Rabbit monoclonal Abs against caspase 9, cytochrome c, cyclin B1, and β-actin were obtained from Proteintech Group (Chicago, IL, USA). Ki-67 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G secondary antibodies were obtained from Merck Millipore (Darmstadt, Germany).

2.3. Cell viability assay

Cell viability assays were performed using the MTS kit (Promega, Madison, WI, USA). Briefly, 2×10^4 cells in 100 μl were seeded in each well of a 96-well plate. The cells were treated with a gradient concentration of an indicated drug, and then the MTS assay was performed after 48 h. The absorbance was detected using a plate reader (Varioskan Flash 3001, Thermo, Waltham, MA, USA) at 490 nm.

2.4. EdU incorporation assay

Cell proliferation was evaluated by a Cell-Light EdU DNA cell proliferation kit (RiboBio, Guangzhou, Guangdong, China) according to the manufacturer's instructions. KBM5, KBM5-T315I and K562 cell lines were cultured in 6-cm dishes at a density of 2×10^5 cells per millilitre and stimulated with the indicated dose of PAB for 24 h. Then, the cells were exposed to 50 μM EdU for 2 h and fixed in 4% formaldehyde for 30 min and 0.5% Triton X-100 for 10 min. After being washed with PBS, the cells were cultured for 30 min with 100 μl 1 × Apollo reaction cocktail. These cells were analysed by flow cytometry (Beckman Coulter, Inc., CA, USA).

2.5. Cell cycle analysis

Cells were stimulated for the indicated times and then collected and washed with PBS. They were then fixed in 70% cold ethanol overnight at 4 °C. Afterwards, the cells were washed twice in PBS and stained with propidium iodide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cell cycle distribution was detected by flow cytometry (Beckman Coulter, Inc.).

2.6. Western blot analysis

After each treatment, cells were collected and washed with PBS. The cell pellet was lysed in RIPA buffer (1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with freshly added 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 × Roche Complete Mini Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). The cytosolic fraction was prepared with digitonin extraction buffer (10 mM PIPES (pH 6.8), 0.015% (wt/vol) digitonin, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 5 mM EDTA and 1 mM PMSF) to detect the cytosolic levels of cytochrome c and AIF. Western blotting was performed as previously described (Shi et al., 2009).

2.7. Cellular apoptosis assay

The CML cells were treated as shown in the indicated figure. Cell apoptosis was evaluated by Annexin V/PI double staining according to the manufacturer's instructions (Sungene Biotech, Tianjin, China).

Apoptotic cells were identified by flow cytometry (Beckman Coulter, Inc.). Annexin V-FITC-positive and Annexin V-FITC-plus-PI-positive cells were considered apoptotic cells.

2.8. Mitochondrial membrane potential measurement

The mitochondrial membrane potential (ΔΨm) of CML cells was measured using rhodamine-123 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) staining. After PAB treatment, the cells were cultured with rhodamine-123 (2.5 μg/ml) for an additional 30 min at 37 °C. After washing with PBS, the membrane potential was measured with a BD FACSCanto II flow cytometer (Franklin Lakes, NJ, USA).

2.9. Tumour xenograft experiments

Four-week-old male nu/nu BALB/c mice were bred at the Experimental Animal Center at Guangzhou Medical University. After one week of adaptation, KBM5 or KBM5-T315I cells (2×10^7) were inoculated subcutaneously into the flanks of the mice. When the tumours grew to 50 mm^3, the mice were grouped and injected intraperitoneally with PAB (50 mg/kg/day) or imatinib (50 mg/kg/day) or PAB (50 mg/kg/day) plus imatinib (50 mg/kg/day), or an equal volume of vehicle (10% DMSO, 20% Cremophor and 70% NaCl). Tumour size and body weight were measured every day. Tumour volume was calculated with the following formula: $a^2 \times b \times 0.4$, where “a” is the smallest diameter and “b” is the diameter perpendicular to “a”. The mice were killed on the twelfth day, and the tumours were removed, weighed, dissected and stored. All animal studies were conducted with the approval of the Guangzhou Medical University Institutional Animal Care and Use Committee (GY 2019-109).

2.10. Immunohistochemical staining

Tumour sections were fixed in 4% formaldehyde for 36 h and then processed into paraffin sections using a standard technique. The sections were immunostained for c-ABL and Ki67 or as indicated, while HE staining was used to visualise the morphology of the tumour tissue. MaxVision reagent (MaixinBiol, Fuzhou, China) was applied to each slide (5 μm) according to the manufacturer's instructions. The detailed protocol was described previously (Shi et al., 2009).

2.11. Statistical analysis

All experiments were repeated at least 3 times, and the results are presented as the mean ± S.D. where applicable. Statistical analysis was performed by one-way analysis of variance followed by Tukey's test with GraphPad Prism software 5.0 (San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. PAB decreases the viability of both IM-sensitive and IM-resistant CML cell lines

To evaluate the cytotoxic effect of PAB on CML cells, we performed an MTS assay. Here, we used five CML cell lines: the IM-sensitive CML cell lines KBM5, K562 and Baf 3, the IM-resistant CML cell lines KBM5-T315I and Baf3-T315I. Fig. 1A shows the difference in the sensitivity of the IM-sensitive and IM-resistant CML cell lines to imatinib treatment; the IC_{50} values of IM-sensitive CML cell lines KBM5 and K562 were 0.11 μM and 0.66 μM, while the KBM5-T315I cell line was 5.96 μM. Interestingly we found that PAB significantly inhibited the viability of both IM-sensitive and -resistant CML cell lines (Fig. 1B). The IC_{50} values of PAB in the IM-sensitive CML cell lines KBM5, Baf3-p210-WT and K562 were 0.35 μM, 0.48 μM and 0.64 μM, respectively, and the IC_{50} values of this compound in the IM-resistant CML cell lines KBM5-T315I and Baf3-p210-WT were 0.27 μM and 0.31 μM, respectively. These results indicate that the IM-resistant CML cells were at least equally sensitive to PAB treatment as their IM-sensitive counterparts. Additionally, the viability of primary blood mononuclear cells from CML patients was significantly inhibited by PAB treatment (Fig. 1C), while the viability of cells from healthy donors was remarkably unaffected (Fig. 1D). The detailed information of CML patients was shown in Supplemental Table 1. This result indicates that PAB has good selectivity for CML cells. Moreover, we evaluated the combination of PAB and imatinib in KBM5 and KBM5-T315I cells. The result in Fig. 1E illustrates that PAB and imatinib has no synergy effect on both IM-sensitive CML cell line (KBM5) and IM-resistant CML cell line (KBM5-T315I). We also performed an EdU incorporation assay, and the results showed that PAB inhibited proliferation in a dose-dependent manner in both IM-sensitive and IM-resistant CML cell lines (Fig. 1F).

3.2. PAB induces mitotic arrest in both IM-sensitive and IM-resistant CML cells

As PAB was previously reported to be a cell cycle inhibitor, we then determined whether the inhibitory effect of PAB on the proliferation of CML cells was due to the suppression of cell cycle progression. We first performed the flow cytometry assay, and the results showed that PAB induced G_2/M arrest in a time-dependent manner in both IM-sensitive and IM-resistant KBM5 - cell lines as well as K562 cells (Fig. 2A). Consistently, treatment of these three cell lines with PAB increased the protein expression of cyclin B1, a well-known biomarker of G_2/M arrest, as shown in the Western blot assay (Fig. 2B). PAB-induced cyclin B1 expression occurred mainly at approximately 12 h and then decreased at 24 h. To further determine whether PAB-mediated blockade occurs in G_2 or M phase, we detected the expression level of phospho-histone H3 since the phosphorylation of histone H3 is a mitosis-specific marker. Our results showed that the levels of phospho-histone H3 were increased after the three cell lines were treated with PAB (Fig. 2B). Additionally, we observed the upregulation of p53 and p-cdc2, which was associated with mitosis progression, in primary blood mononuclear cells from CML patients after PAB treatment (Supplemental Fig. B). Taken together, our data suggest that PAB induces M phase blockade in both IM-sensitive and IM-resistant CML cells.

3.3. PAB induces apoptosis in IM-sensitive and IM-resistant CML cell lines

It has been suggested that cells arrested in M phase might be sensitive to the induction of apoptosis. To determine whether PAB induces apoptosis in IM-sensitive and IM-resistant cells, we analysed the levels of apoptosis by flow cytometry. The results demonstrated that PAB induced dose- and time-dependent apoptosis in IM-sensitive and IM-resistant CML cell lines (Fig. 3A, Supplemental Fig. A). Furthermore, apoptosis was also observed in primary mononuclear cells from CML patients after PAB treatment (Fig. 3B). To determine whether apoptosis is associated with alterations in the mitochondrial pathway, we measured the change in the mitochondrial membrane potential and found that PAB downregulated the mitochondrial membrane potential (Fig. 3C). Moreover, we detected increased levels of cytoplasmic AIF and cytochrome C in both IM-sensitive and IM-resistant CML cells treated with PAB (Fig. 3D).

3.4. PAB-induced apoptosis in CML cells is caspase-dependent

The caspase-dependent pathways play an important role in cellular apoptosis. Under cellular stress, cytochrome C is released from mitochondria into the cytoplasm and binds APAF-1 to recruit and activate caspase 9. Activated caspase 9 then cleaves caspase 3 and leads to the degradation of cellular components. In our study, PAB induced the cleavage of caspase 3, 8, 9 and PARP in a dose- and time-dependent manner in both IM-sensitive and IM-resistant CML cell lines (Fig. 4A).

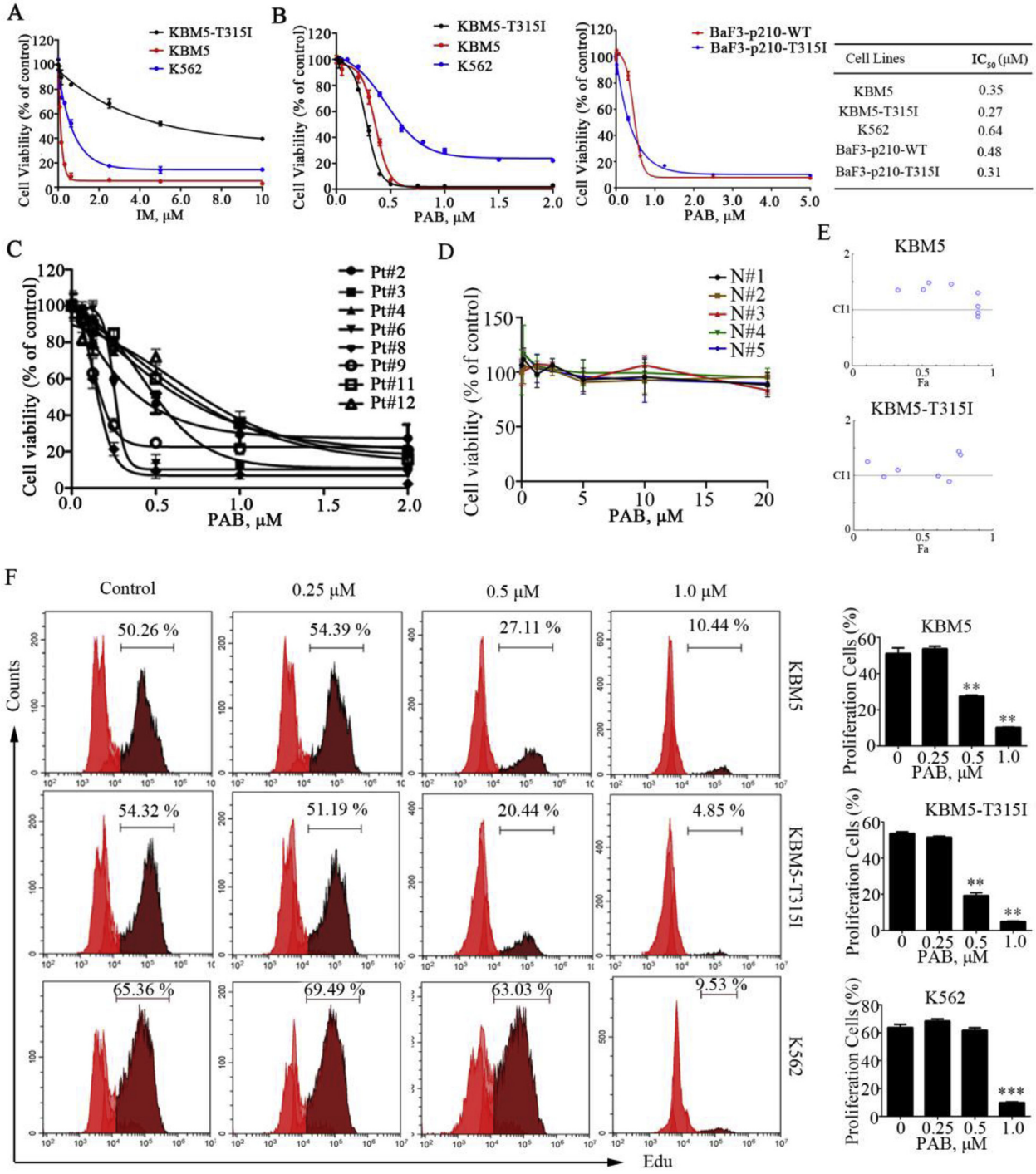

Fig. 1. PAB inhibits the viability of CML cell lines. (A) IM-sensitive KBM5 and K562 cell lines and IM-resistant KBM5-T315I cell line showed individual cytotoxicity to imatinib treatment. The cell viability was measured using MTS assay after incubated with IM for 48 h. (B) PAB decreased the viability of KBM5, KBM5-T315I, K562, BaF3-p210-WT, BaF3-p210-T315I cells. The cells were cultured with increasing concentrations of PAB for 48 h and then analysed using the MTS assay. The IC_{50} values are shown in table. (C) The viability of primary blood mononuclear cells isolated from CML patients was inhibited by PAB incubation. Isolated cells from eight patients were subjected to increasing concentrations of PAB over 48 h and then analysed by the MTS assay. (D) After 48 h of treatment, PAB showed no cytotoxicity in primary blood mononuclear cells from healthy donors. (E) The CI value of PAB combining with imatinib were equal or greater than one, which indicated no synergy effect on PAB with imatinib. (F) PAB inhibits the proliferation of IM-sensitive and IM-insensitive CML cells. KBM5, KBM5-T315I, and K562 cells were treated with the indicated doses of PAB for 24 h and then measured by the EdU assay. The graph represents data from three replicates. Mean ± S.D. (n = 3). **P < 0.01, ***P < 0.001 versus the control group.

Consistent with caspase activation, the precursors of caspase 3, 8, 9 and PARP were decreased after PAB treatment (Fig. 4A). These results indicate that PAB activates the caspase-dependent pathway to trigger apoptosis in CML cells. It is known that mitochondrial-related apoptosis is associated with decreased levels of anti-apoptotic Bcl-2 and IAP family proteins. We found a decrease in the expression of Bcl-2, Mcl-1, Bcl-xl, XIAP and survivin in a PAB dose- and time-dependent manner in both IM-sensitive and IM-resistant CML cells (Fig. 4B). Taken together, these results demonstrate that PAB induces apoptosis in IM-sensitive and IM-resistant CML cells through the activation of caspase-dependent

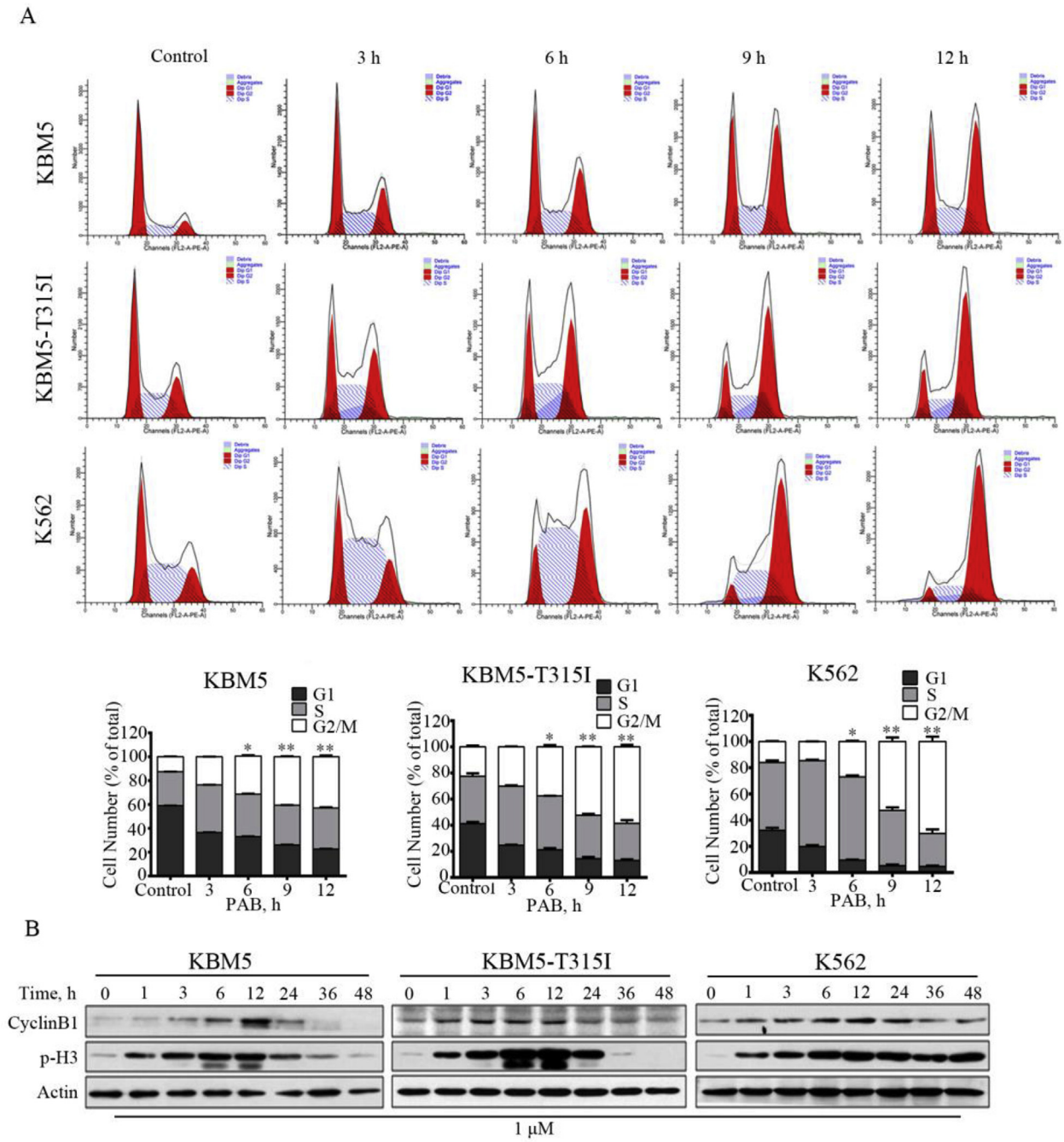

Fig. 2. PAB induces mitotic arrest in CML cell lines. (A) PAB induces cell cycle arrest at G_2/M phase. CML cells stimulated with 1 μM PAB were collected at 0 h, 3 h, 6 h, 9 h and 12 h. Cell cycle assays were performed by flow cytometry after PI staining. The graph represents data from three replicates. Mean ± S.D. (n = 3). *P < 0.05, **P < 0.01 versus the control group. (B) The Western blot shows the expression of cyclin B1 and phospho-histone h3 (Ser10) at the indicated time point.

pathways.

3.5. PAB downregulates the protein expression of BCR-ABL

The BCR-ABL fusion protein causes abnormal activation of tyrosine kinase, which is essential in the pathogenesis of CML. We found that PAB downregulated the protein level of BCR-ABL and inhibited BCR-ABL Y245 phosphorylation in a dose- and time-dependent fashion in both IM-sensitive and IM-resistant CML cell lines (Fig. 5A and B). Further examination revealed that PAB inhibited the expression of BCR-ABL downstream pathway-related proteins, as evidenced by dose- and time-dependent decreases in the levels of phospho-ERK, phospho-AKT, phospho-Stat 5 and phospho-Crkl in IM-sensitive and IM-resistant CML cells after treatment with PAB (Fig. 5A and B). Moreover, we found declined BCR-ABL expression in primary mononuclear cells from CML patients after PAB treatment (Supplemental Fig. B). We then investigated the mechanisms of the PAB-mediated inhibition of BCR-ABL. Then we investigated both the transcription and the degradation of BCR-ABL. We found that PAB treatment neither altered levels of total ubiquitinated proteins (Supplemental Fig. C) nor affected the mRNA level of BCR-ABL in CML cells (Supplemental Fig. D). Our previous studies have demonstrated that the decreased protein levels of BCR-ABL during apoptosis are likely due to cleavage by activated caspases (Shi et al. 2009, 2014). Since PAB was able to activate the caspase pathway (Fig. 4), we then investigated whether the downregulation of BCR-ABL protein by PAB in CML cells was due to caspase cleavage. To verify this,

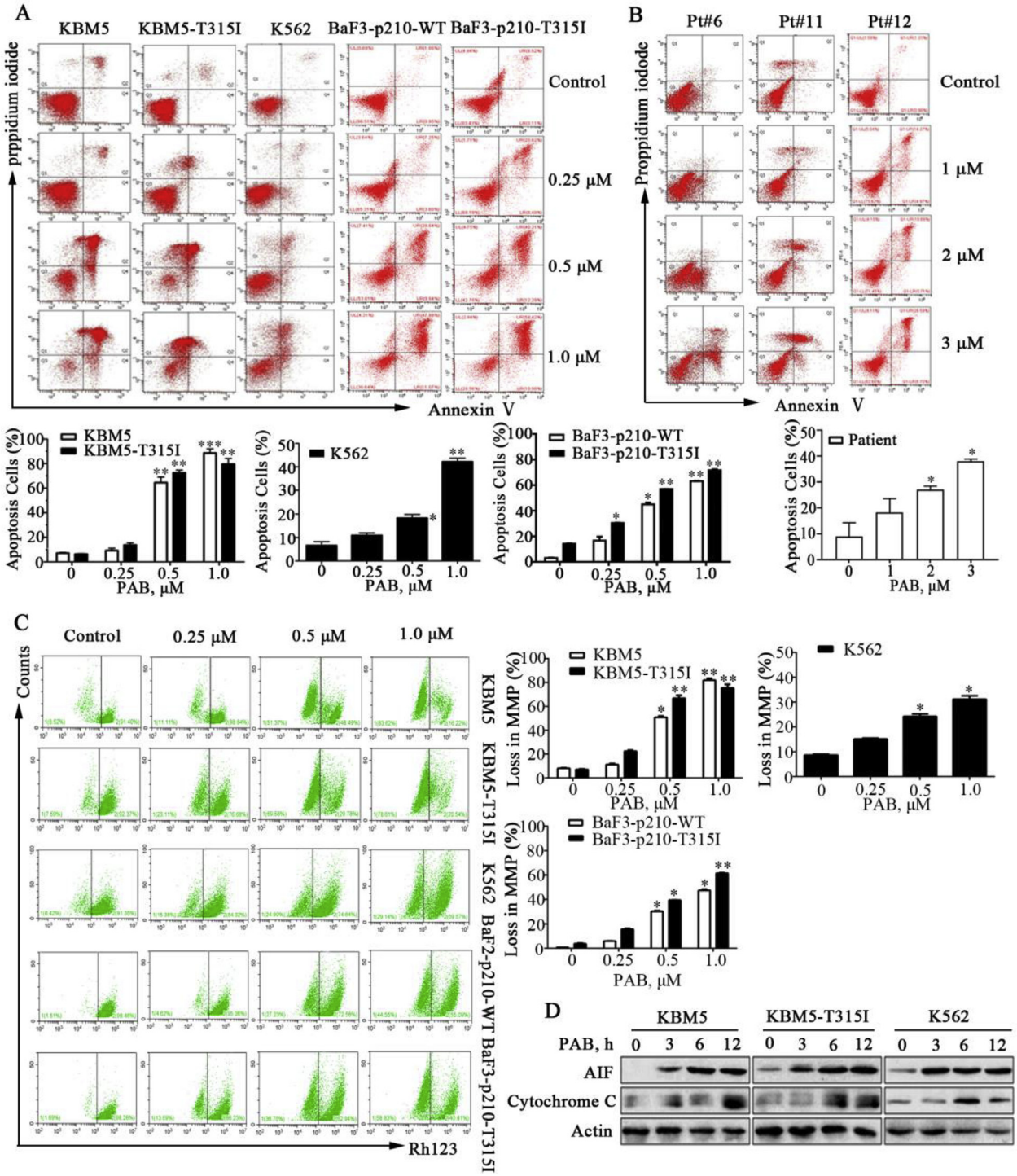

Fig. 3. PAB induces apoptosis in CML cells. (A) KBM5, KBM5-T315I, BaF3-p210-WT, BaF3-p210-T315I cell were cultured with the indicated concentrations of PAB for 48 h, while K562 cells were treated with the indicated doses for 72 h. Apoptotic cells were detected by Annexin V/PI staining and flow cytometry. The graph represents data from three replicates. Mean ± S.D. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 versus the control group. (B) Primary mononuclear cells from CML patients were apoptotic under PAB treatment. The cells were stained with Annexin V/PI after 48 h of culture in various doses of PAB and measured by flow cytometry. The graph represents data from two patients. Mean ± S.D., *P < 0.05 versus the control group. (C) PAB decreases the mitochondrial membrane potential in CML cells. KBM5, KBM5-T315I, BaF3-p210-WT, BaF3-p210-T315I cells were treated with various doses of PAB for 48 h, and K562 cells were treated with various doses of PAB for 72 h. The mitochondrial membrane potential was evaluated using flow cytometry after staining with rhodamine-123. The graph represents data from three replicates. Mean ± S.D. (n = 3). *P < 0.05, **P < 0.01 versus the control group. (D) AIF and cytochrome C were released from the mitochondria into the cytoplasm under treatment with 1 μM PAB. The expression levels of AIF and cytochrome C in the cytoplasm were analysed by Western blotting. Actin was used as a loading control.

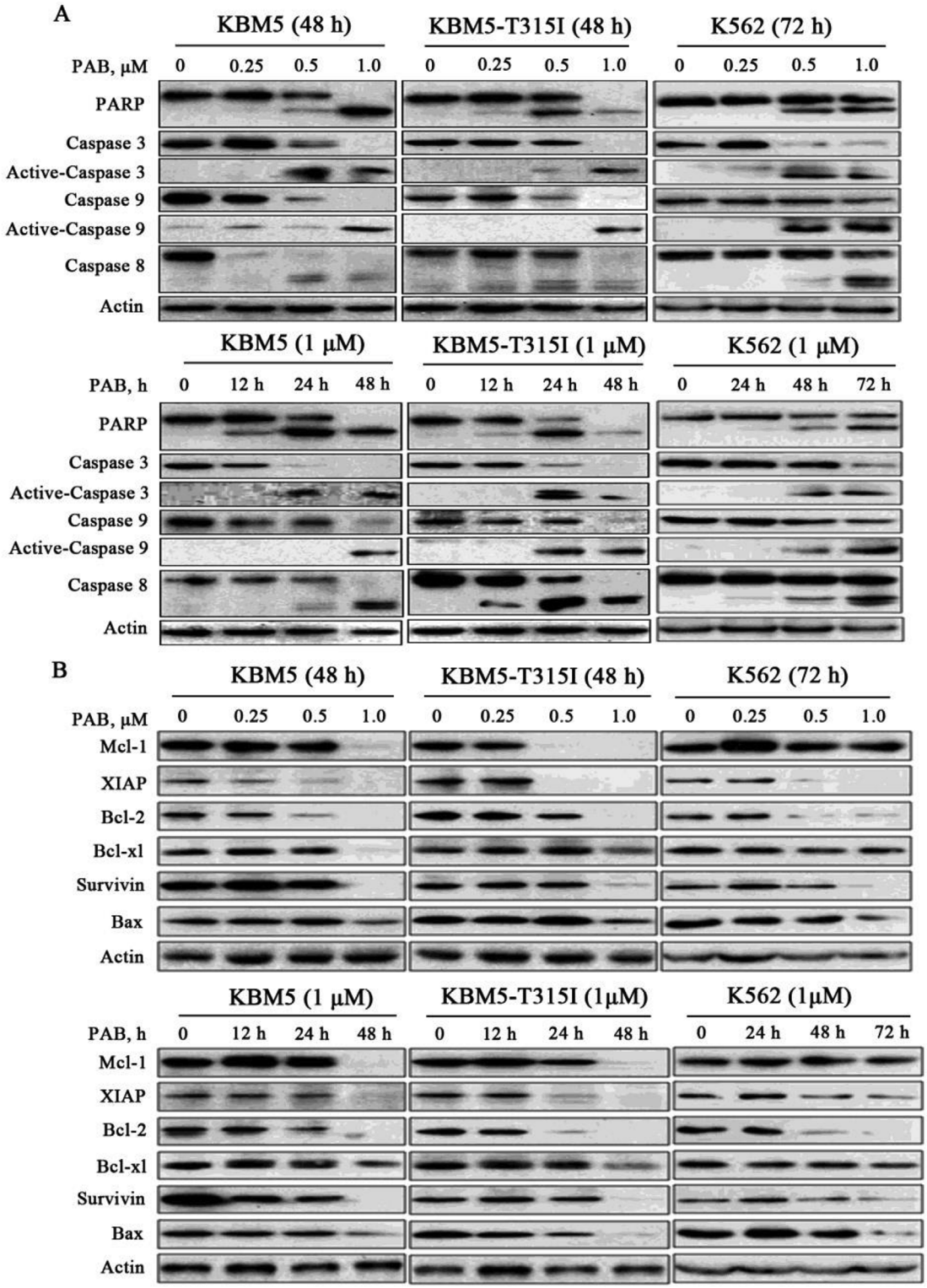

Fig. 4. PAB triggers apoptosis through caspase activation in CML cells. (A) KBM5, KBM5-T315I and K562 cells were cultured with the indicated doses of PAB for the indicated times. Cell lysates were immunoblotted to analyse the expression of PARP, caspase 3, active caspase 3, caspase 8, caspase 9 and active caspase 9. Actin was used as a loading control. (B) PAB decreased the expression of anti-apoptosis proteins. KBM5, KBM5-T315I and K562 cells were exposed to PAB under the indicated conditions. The expression of Mcl-1, Bcl-2, Bcl-xl, Bax and Survivin were detected by Western blot analysis. Actin was used as a loading control.

KBM5-T315I and KBM5 cells were incubated with PAB in the absence or presence of the pan-caspase inhibitor z-VAD-fmk, first we performed the MTS assay which revealed that PAB suppressed cell viability could be rescued by ZVAD (Fig. 5C). And then the levels of total BCR-ABL protein, phospho-BCR-ABL, and phospho-Stat 5 were measured (Fig. 5D). We found that z-VAD-fmk partially inhibited PAB-mediated decreases in BCR-ABL, phospho-BCR-ABL and phospho-Stat 5. Based on these results, we conclude that PAB-induced BCR-ABL downregulation in CML cells is at least partially dependent on caspase pathways.

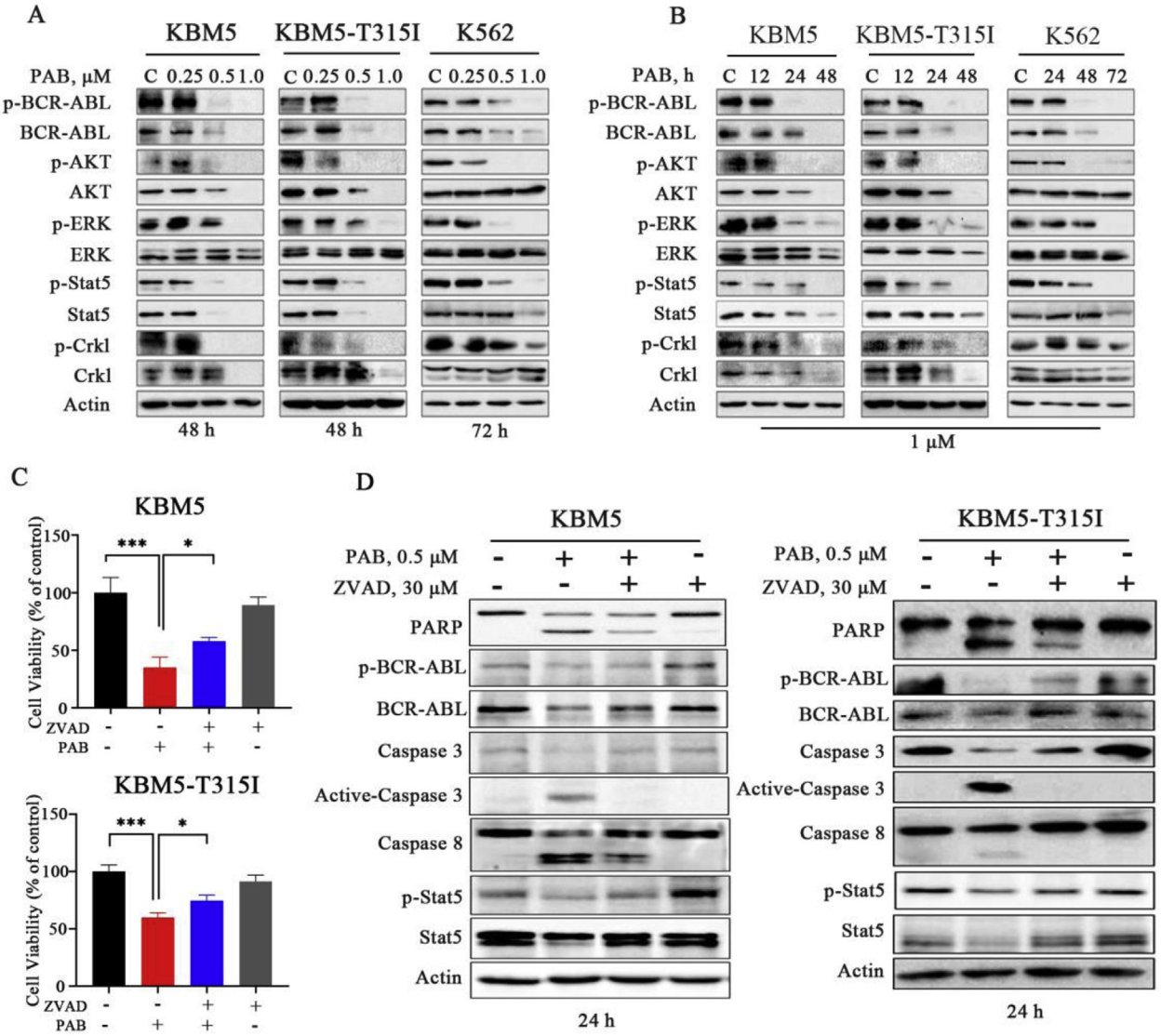

Fig. 5. PAB-induced BCR-ABL downregulation was caspase-dependent. (A) (B) KBM5, KBM5-T315I and K562 cells were cultured with the indicated doses of PAB for 48 h or 72 h, and then c-abl, phospho-c-abl (Y245), Stat 5, phospho-Stat 5A/B (Y694, Y699), ERK1/2, phospho-ERK1/2 (T202/Y204), AKT, phospho-AKT, Crkl, and phospho-Crkl (Y207) were detected. Actin was used as a loading control. (C) Cell viability were evaluated with or without 30 μM ZVAD and 1 μM PAB after 24 h in KBM5 and KBM5-T315I cells. Mean ± S.D. (n = 3). *P < 0.05, ***P < 0.001. (D) KBM5-T315I and KBM5 cells were treated with PAB for 24 h in the presence or absence of 30 μM pan-caspase inhibitor ZVAD. Cell lysates were immunoblotted to analyse the expression of PARP, c-abl, phospho-c-abl (Y245), caspase3, active caspase3, caspase 8, phospho-Stat 5A/B (Y694, Y699). Actin was used as a loading control.

3.6. PAB inhibits the growth of IM-sensitive and IM-resistant CML xenografts

Due to the substantial effects of PAB treatment observed *in vitro*, we established a nude mouse xenograft model subcutaneously inoculated with KBM5 or KBM5-T315I cells. The mice were divided into the vehicle, imatinib (50 mg/kg/day), PAB (50 mg/kg/day), and PAB plus imatinib group when the tumours reached an approximate size of 50 mm^3. Compared to vehicle, PAB significantly reduced tumour volume (Fig. 6A) and tumour weight (Fig. 6B), while the body weights of the mice were unchanged (Fig. 6C). These results indicate that PAB significantly suppressed the growth of IM-sensitive and IM-resistant CML xenografts with low toxicity. No significant synergistic effects of PAB and imatinib were observed. BCR-ABL and BCR-ABL downstream pathway proteins were decreased in the tumours treated with PAB, while the levels of the mitosis regulator p53 and phosphor-cdc2 were increased (Fig. 6D). Additionally, immunohistochemical analysis revealed that BCR-ABL and Ki67 (a proliferation indicator) were downregulated (Fig. 6E). In conclusion, these data demonstrate the potent in vivo antitumour activity of PAB in CML cells, including CML cells expressing the BCR-ABL-T315I mutation.

4. Discussion

Chronic myeloid leukaemia accounts for 15–20% of all cases of leukaemia in adults (Sawyers, 1999). Imatinib is considered an important strategy for targeting BCR-ABL through precision medicine; however, several BCR-ABL-dependent and -independent mechanisms of IM resistance have arisen. Hence, second- and third-generation TKIs such as dasatinib, bosutinib, nilotinib, and ponatinib have been clinically approved to override this resistances (de Klerk et al., 2018). Unfortunately, several of these drugs, except ponatinib, are insensitive to the obstinate T315I mutant (Tan et al., 2019). Therefore, it is imperative to identify more novel BCR-ABL inhibitors that can disrupt the BCR-ABL signalling pathways and overcome IM resistance. Many natural products have been demonstrated outstanding anticancer effects

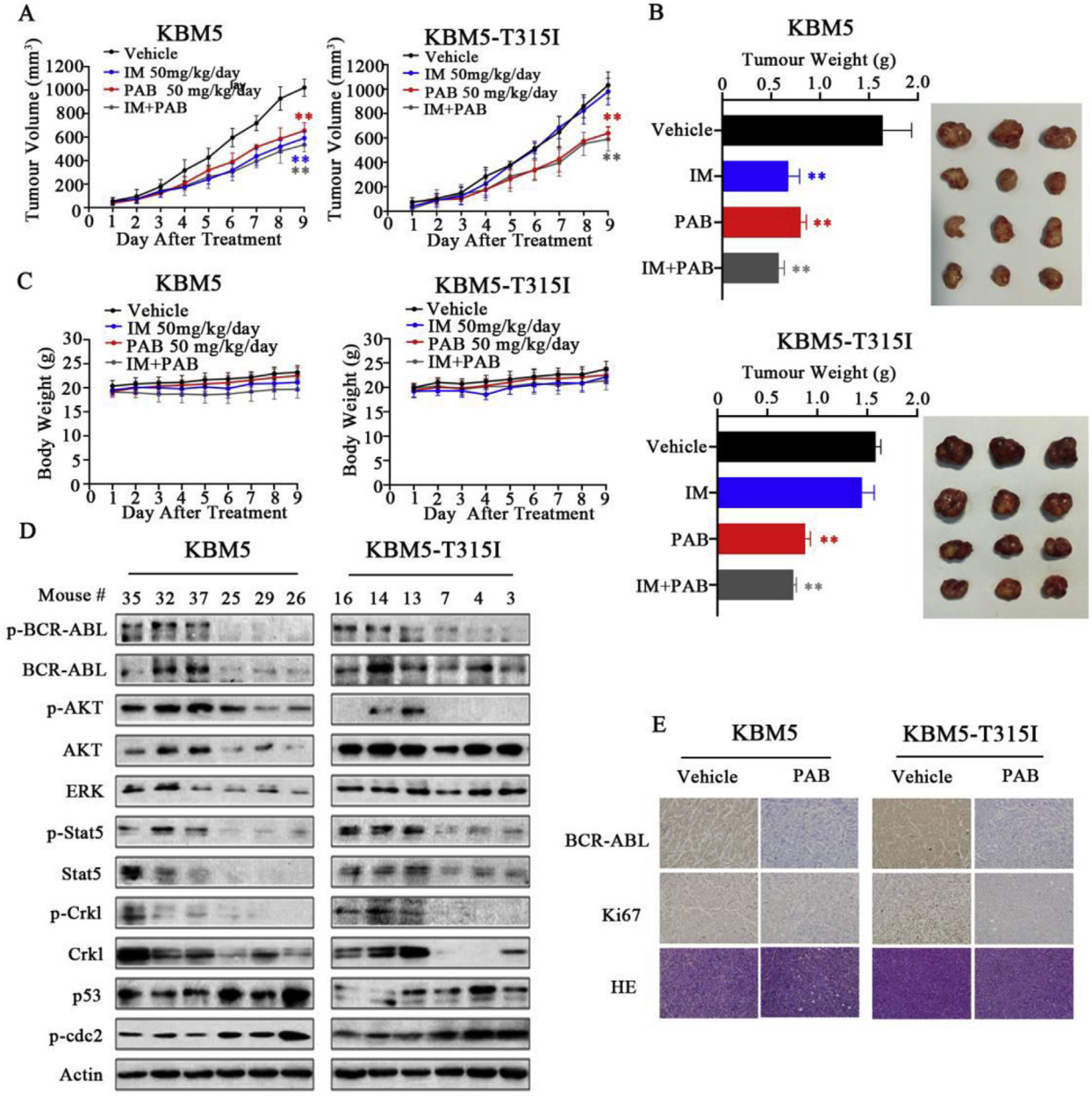

Fig. 6. PAB inhibits growth in both KBM5 and KBM5-T315I CML cell xenografts. (A) Nude mice bearing KBM5 and KBM5-T315I CML cells were treated with vehicle, imatinib (50 mg/kg/d, i.g.), PAB (50 mg/kg/d, i.p.), or the combination of PAB and imatinib for 9 days. Tumour volume was recorded every day. Mean ± S.D. (n = 6). **P < 0.01. (B) Tumour tissues were weighed and imaged after the mice were killed. Mean ± S.D. (n = 6). **P < 0.01. (C) The body weight was recorded every day. (D) Changes in BCR-ABL, BCR-ABL downstream pathways and mitotic arrest-related proteins were assessed by Western blotting. Mice #35, #32, and #37 represent vehicle-treated KBM5 xenografts; mice #25, #29, and #26 represent PAB (50 mg/kg/d)-treated KBM5 xenografts; mice #16, #14, and #13 represent vehicle-treated KBM5-T315I xenografts; and mice #7, #4, and #3 represent PAB (50 mg/kg/d)-treated KBM5-T315I xenografts. Actin was used as a loading control. (E) Immunohistochemical analysis of BCR-ABL and Ki67. Tumour morphology was visualised by staining with haematoxylin and eosin.

with few adverse effects. Numerous research groups are devoted to exploring effective and nontoxic active components from traditional Chinese medicines. We previously reported that gambogic acid and shikonin elicit anti-tumour effects through proteasome inhibition (Shi et al., 2015; Yang et al., 2009). Recently, several reports suggested that PAB, an effective component isolated from the root and trunk bark of *Cortex pseudolaricis*, is a potential anti-inflammatory and immunosuppressive agent, and PAB has been explored for treatment or prevention of various cancers. In the current study, we explored the anticancer effects and mechanisms of PAB in IM-sensitive and -resistant CML cells.

It has been shown that PAB can target microtubules by inducing their depolymerisation, resulting in mitotic cell cycle arrest (Chiu et al., 2010). Our results demonstrated that PAB perturbed cell cycle progression and impeded mitosis evidence by the upregulation of cyclin B1 and phospho-histone 3 in IM-sensitive and -resistant CML cell lines. Interestingly, we found that PAB arrested K562 cells in S phase for a short period of time and then induced G_2/M phase arrest. Ikejima, T et al. reported that G_1 or S phase arrest can reverse PAB-induced apoptosis in HeLa cells (Yao et al., 2014). This PAB-induced transient S phase arrest in K562 cells might serve as a self-protection mechanism, which could explain why K562 cells were less sensitive than KBM5 cells.

Consistent with other reports, our studies showed that PAB

treatment caused the blockade of cell cycle progression in M phase, which may have been responsible for or contributed to its ability to inhibit proliferation and overcome IM resistance in CML cells. In both normal and cancer cells, senescence and cell death occur spontaneously during and after mitotic arrest. Yao GD et al. reported that senescence rather than apoptosis can be induced when the cell cycle is permanently arrested after culture with PAB and that lower glucose might elevate PAB-induced apoptosis in lung cancer cells (Yao et al., 2017). PAB-induced cell death is diverse, related to cancer, and involves apoptosis, ferroptosis and autophagy (Yu et al., 2013; Wang et al., 2018). Recent studies have also shown that PAB can induce apoptosis in various cancers models, including colorectal, breast, melanoma, lung, prostate, etc. Multiple important pathways are involved in PAB-induced apoptosis, including AKT/mTOR, NF-κB, caspase, Bcl-2/Bax and p38 (Wang et al., 2017; Yu et al., 2014; Qi et al., 2012). Our present study demonstrated that PAB-induced apoptosis is associated with caspase activation and the downregulation of anti-apoptotic Bcl-2 family proteins in CML cells. PAB activated caspase 8 and triggered the loss of the mitochondrial membrane potential, resulting in the release of cytochrome C, which subsequently activated caspase 9. Our results suggest that PAB induced G_2/M arrest first, which occurred after less than 1 h of treatment and gradually peaked at 12 h. Subsequently, the apoptosis indicator PARP was slightly cleaved at 12 h. All of these results illustrate that PAB first induced M phase arrest and then caspase-dependent mitochondrial apoptosis was followed.

Previously it was reported that PAB can overcome multidrug resistance in gastric cancer via decreasing the Cox-2-PKC-α-P-gp/MDR1 signalling pathway (Yu et al., 2015; Sun and Li, 2014). Additionally, the ability of PAB to overcome multidrug resistance has been observed in colorectal cancer and liver cancer (Wen et al., 2016a). We found in the current study that PAB overrode imatinib resistance in CML cells and that the molecular mechanism involved downregulation of BCR-ABL protein expression through caspase-dependent cleavage and the suppression of its downstream cascades. This conclusion was supported by the finding that the general caspase inhibitor z-VAD-fmk abolished PAB-induced BCR-ABL downregulation and PARP cleavage (Fig. 5).

In conclusion, we have shown that PAB possesses potent cytotoxic effects in both IM-sensitive and IM-resistant CML cells. PAB inhibits the viability and proliferation of CML cells as well as primary mononuclear cells from CML patients. The underlying mechanisms include mitotic arrest and caspase activation. Mitotic arrest induces proliferation inhibition and cell death in association with BCR-ABL cleavage through the activation of caspases and induction of mitochondrial apoptosis. Moreover, the in vivo results demonstrate that PAB displays potent anti-CML activity and inhibits the growth of IM-sensitive and IM-resistant xenograft tumours without being toxic to mice. We suggest that PAB could be used as a potential treatment for CML patients, especially as an agent to sensitize IM-resistant CML.

Author contributions

X.S. and J.L. designed the experiments and analysed the data. L. J., C.W., Q.H. and Y.S. performed most of the experiments. J.W., X.L. and S.R. provided administrative, technical and material support. X.S. and J.L. wrote the manuscript, and Q.P.D. revised the manuscript. All authors reviewed the manuscript.

Funding

Partially supported by NSFC (81670154/H0812, 81470355/H1616 and 81100378/H0812), projects (201707010352, 1201410214, 2014A030313492, 201528042) from the Foundation of Guangzhou Science and Technology Innovation Committee, the Bureau of Education of Guangzhou Municipality, GD-NSF and Guangdong Special Support Scheme (to X.S.), the National Natural Science Foundation of China (81773213), the National Funds for Developing Local Colleges and Universities (B16056001), the Natural Science Foundation Research Team of Guangdong Province (2018B030312001), the Science and Technology Program of Guangzhou (201604020001), the Project of Department of Education of Guangdong Province (2016KTSCX119), and the Research Team of Department of Education of Guangdong Province (2017KCXTD027). This research received no external funding.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2020.173064.

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