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PBA2, a novel inhibitor of the β-catenin/CBP pathway, eradicates chronic myeloid leukemia including BCR-ABL T315I mutation

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Abstract

Background BCR-ABL is a constitutively active tyrosine kinase that stimulates multiple downstream signaling pathways to promote the survival and proliferation of chronic myeloid leukemia (CML) cells. The clinical application of specific BCR-ABL tyrosine kinase inhibitors (TKIs) has led to significantly improved prognosis and overall survival in CML patients compared to previous treatment regimens. However, direct targeting of BCR-ABL does not eradicate CML cells expressing T315I-mutated BCR-ABL. Our previous study revealed that inhibiting CREB binding protein (CBP) is efficacious in activating β-catenin/p300 signaling, promoting cell differentiation and inducing p53/p21-dependent senescence regardless of BCR-ABL mutation status. We hypothesize that the specific inhibition of CBP may represent a novel strategy to promote β-catenin/p300-mediated differentiation and suppress cancer cell proliferation for treating CML patients.

Methods The anticancer efficacy of PBA2, a novel CBP inhibitor, in CML cells expressing wild-type or T315I-mutated BCR-ABL was investigated in vitro and in vivo. Cell differentiation was determined by the nitroblue tetrazolium (NBT) reduction assay. The extent of cellular senescence was assessed by senescence-associated β-galactosidase (SA-β-Gal) activity. Cytotoxicity was measured by MTS assay. RNA interference was performed to evaluate the cell proliferation effects of CBP knockdown. The interaction of β-catenin and CBP/p300 was examined by co-immunoprecipitation assay.

Results PBA2 exhibited significantly higher anticancer effects than imatinib in CML cells harboring either wild-type or T315I-mutated BCR-ABL both in vitro and in vivo. Mechanistically, PBA2 reduced CBP expression and promoted β-catenin-p300 interaction to induce cell differentiation and senescence.

Conclusion Our data supported the rational treatment of CML by inhibiting the β-catenin/CBP pathway regardless of BCR-ABL mutation status.

Keywords CBP, p300, Cell differentiation, Cell senescence, Chronic myeloid leukemia, T315I mutation

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Introduction

Chronic myeloid leukemia (CML) is a clonal malignant disorder of pluripotent hematopoietic stem cells (HSCs). It is characterized by the presence of Philadelphia chromosome (Ph) translocation t(9;22)(q34;q11) encoding the BCR-ABL fusion gene, which constitutively activates multiple downstream signaling pathways, resulting in survival and proliferation of cancer cells [1, 2]. Although most CML patients attain a durable complete cytogenetic response with imatinib, the first-line BCR-ABL tyrosine kinase inhibitor (TKI), minimal residual disease persists in almost all patients, which confers disease recurrence upon treatment cessation. Significantly, imatinib discontinuation resulting from resistance or intolerance occurs in up to 30% of patients within the first five years of therapy [3, 4]. The most frequent drug resistance mechanism is kinase domain mutations, notably the T315I mutation, which reduces or completely ablates drug efficacy [5–8]. With the availability of second-generation BCR-ABL inhibitors, such as nilotinib and dasatinib, better treatment response could be achieved in most CML patients harboring various BCR-ABL mutations. However, BCR-ABL T315I kinase domain mutation confers high-level resistance to all three drugs (imatinib, nilotinib, and dasatinib). Despite the significant antileukemic potency of ponatinib, a third-generation BCR-ABL TKI against T315I mutation, its side effects and long-term tolerability limited its clinical use. Worse still, ponatinib was temporarily suspended in 2013 due to the occurrence of severe cardiovascular thrombotic events. However, considering the potent antileukemic activity of ponatinib, the combination of low-dose ponatinib with other anticancer agents working on different molecular pathways has been recommended for CML treatment [9–12]. Therefore, to discover more promising therapeutic options, it is important to explore BCR-ABL-independent targets for treating CML patients with various BCR-ABL mutations, including T315I.

It is well established that HSCs have multipotency and self-renewal capacities [13, 14]. HSCs undergo continuous differentiation to produce multiple lineages of different blood cells, while properly maintaining the HSC pool size throughout life by precisely balancing differentiation and self-renewal [15]. The Wnt pathway has been implicated in self-renewal and differentiation of HSCs. It is regulated by β-catenin, which recruits CREB binding protein (CBP) or E1A binding protein p300 (p300) and binds to T cell factor (TCF)/lymphocyte enhancer factor-1 (LEF) in the nucleus to activate transcription of target genes in mammalian cells [16]. The dichotomous behavior of CBP and p300, recruited by β-catenin in self-renewal and differentiation, has been reported in HSCs, respectively [15, 17, 18]. Aberrations in the Wnt/β-catenin pathway are known to play critical roles in the carcinogenesis of CML. Accumulating evidence revealed that activation of the Wnt pathway promotes cancer cell proliferation and survival, and confers resistance to the TKIs. Our previous study revealed that β-catenin recruited significantly more CBP than p300 in CML cells. Inhibition of CBP could enhance the binding of β-catenin to p300, thereby promoting cell differentiation and p53/p21-dependent senescence in CML cells harboring either wild-type or T315I-mutated BCR-ABL both in vitro and in vivo [19]. It follows that the specific inhibition of CBP may represent a novel treatment strategy to promote β-catenin/p300-mediated differentiation and to suppress cancer cell proliferation for the treatment of CML cells, including those expressing T315I-mutated BCR-ABL.

Materials and methods

Reagents

PBA2 was synthesized by PharmaBlock Sciences (Nanjing), Inc, and its purity is more than 99% (Fig. 1a). PBA2 was dissolved in DMSO to prepare a stock solution (10 mM) and it was diluted with the cell culture medium to the desired concentration in different experiments. Imatinib mesylate was obtained from Selleck Chemicals (Shanghai) (Fig. 1b). MTS was purchased from MACKLIN (Shanghai) and dissolved in PBS, followed by filtration with a 0.22 μm filter to produce the sterile MTS solution.

Cell culture

The human CML cell line K562 was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and the murine 32D myeloid cells stably expressing either wild-type (32D-WT) or T315I-mutated BCR-ABL (32D-T315I) was a kind gift provided by Professor Pan JX (Sun Yat-sen University Ophthalmic Hospital, Guangzhou). BCR- ABL expression in these cells was routinely confirmed by Western blot. HEK293T cell line was purchased from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% FBS, 100U/ml penicillin, and 100U/ml streptomycin. CML cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100U/ml penicillin, and 100U/ml streptomycin in the humidified incubator containing 5% CO_2 at 37 °C. Additionally, the 32D-WT and 32D-T315I cells were supplemented with 0.5 ng/ml WNT3a to imitate the cellular feature of activated Wnt signaling in CML cells.

NBT assay

After incubation with PBA2 or imatinib, K562, 32D-WT, and 32D-T315I cells were cultured in 96-well plates for 72 h. The nitroblue tetrazolium (NBT) reduction assay was conducted as previously described [20].

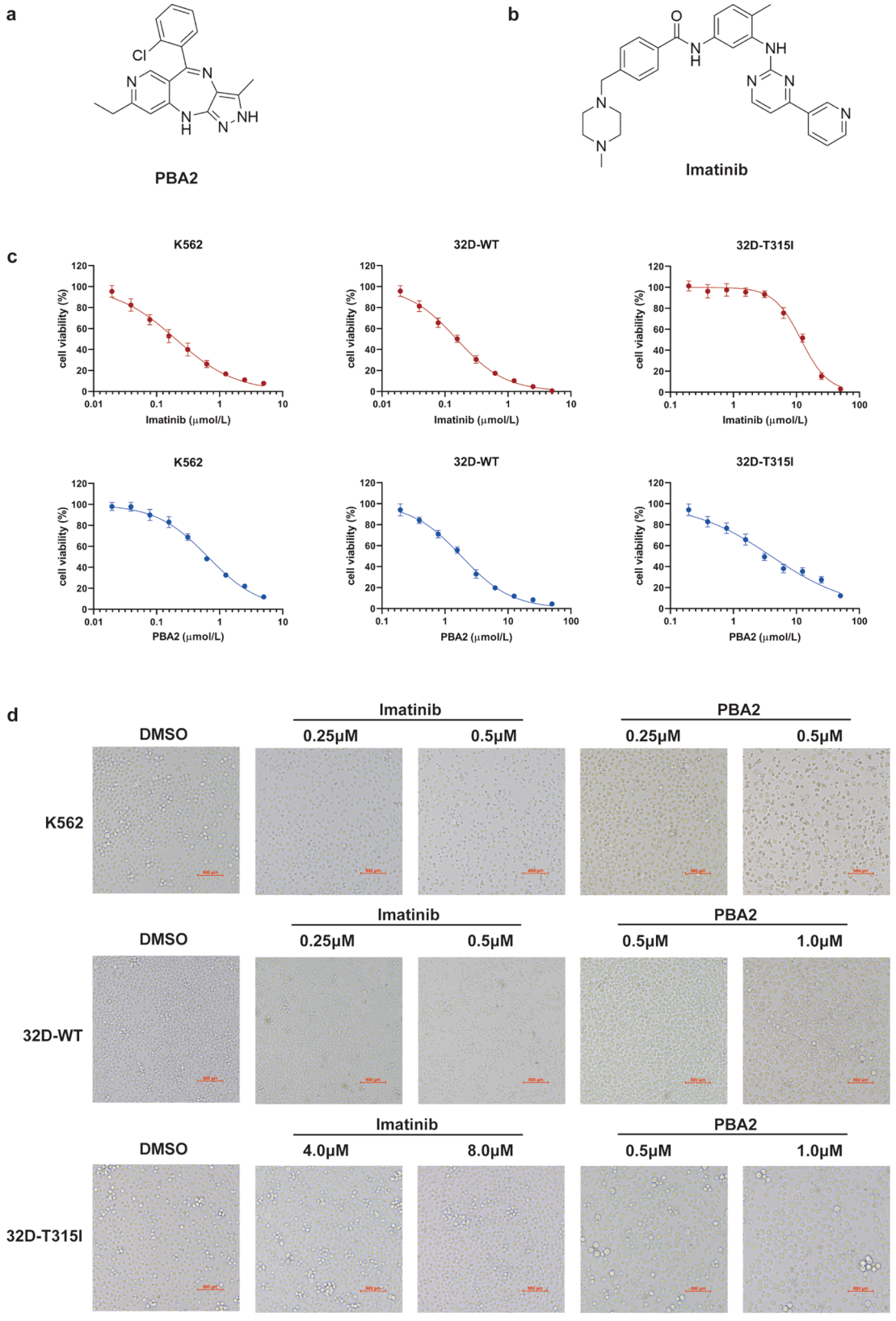

Fig. 1 PBA2 overcomes resistance to imatinib in BAR-ABL T315I-mutated cell. **a-b** Chemical structures of PBA2 and imatinib. **c** Cell viability of K562, 32D-WT and 32D-T315I treated with PBA2 and imatinib using MTS assay. **d** Cell morphology of K562, 32D-WT and 32D-T315I treated with indicated treatments

SA-β-Gal staining

The senescence-associated β-galactosidase (SA-β-Gal) activity was measured by the Senescence β-Galactosidase Staining Kit (Beyotime Biotechnology, China). SA-β-Gal staining was performed according to the protocol of the manufacturer. Senescent cells were identified as blue-stained cells under an Olympus IX71S8F inverted microscope.

Cytotoxicity assay

The cytotoxic effect of PBA2 and imatinib on CML cells expressing wild-type or T315I-mutated BCR-ABL was evaluated by MTS assay. Approximately 10,000 cells were seeded into the round-bottom 96-well plates, followed by treatment with the indicated concentrations of PBA2 or imatinib for 48 h. After that, 20 μl MTS was added into each well and incubated at 37 ℃ for 3–4 h. Finally, the OD values were detected at 490 nm. The cell viability was calculated using the formula: (Experimental group OD-Blank group OD) / (Control group OD-Blank group OD) × 100%.

Cell proliferation assay

To test the effect of CBP knockdown on cell proliferation, 1000 cells expressing shRNA were seeded into the round-bottom 96-well plates. The 20 μl MTS solution was added into each well and OD values were detected at 490 nm every 24 h. The curves of cell proliferation were drawn using GraphPad Prism.

RNAi

The different shRNA sequences were separately inserted into pLKO.1-eGFP-puro plasmid using T4 DNA ligase. These plasmid vectors were co-transfected with psPAX2 and pMD2.G into HEK293T cells for 48 h to produce lentiviral particles using the Neofect DNA transfection agent (NEOFECT, Beijing). The viral supernatants were collected using 0.45 μm filters before transfecting target cells with 10 μg/ml polybrene. After 24 h, the viral supernatants were removed and the fresh complete culture medium was added. Then the transfected cells were subjected to puromycin selection (1–2 μg/ml) to generate cells stably expressing indicated shRNA. The RNA interference efficiency was confirmed by qRT-PCR and Western blot.

The shRNA sequences are as follows:
CREBBP (human).
shRNA1 (5'- GCTATCAGAATAGGTATCATT-3'),
shRNA2 (5'-GCGTTTACATAAACAAGGCAT-3'),
shRNA3 (5'- ATCGCCACGTCCCTTAGTAAC-3'),
Crebbp (mouse).
shRNA1 (5'- CGCGAATGACAACACAGATTT-3'),
shRNA2 (5'- TAACTCTGGCCATAGCTTAAT-3'),
shRNA3 (5'- GAGGATCATTAACGACTATAA-3').

qRT-PCR

The qRT-PCR assay was performed according to the manufacturer's instructions. The total RNA from cells was extracted using the EZB RNA Purification Kit (EZBioscience). Then, the extracted RNA was converted into cDNA through reverse transcription assay using the Color Reverse Transcription Kit (EZBioscience). The obtained cDNA templates were mixed with primers and 2× SYBR Master Mix (EZBioscience). Finally, the PCR amplification reaction was conducted with the ROCHE 480 instrument. Primer sequences are as follows:

CREBBP (F: CACAGAACCAGTTCCCGTCA, R: GGGTTAGGAAGAGCAGCACC)

GAPDH (F: TTCTTTTGCGTCGCCAGCC, R: TCCCGTTCTCAGCCTTGACG)

Crebbp (F: GCCCATTGTGCATCTTCACG, R: GGCTGGTTCATGTAGGGGAG)

Gapdh (F: TGTCAAGCTCATTTCCTGGTATG, R: TTATGGGGGTCTGGGATGGA)

Western blot

The cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with phenylmethanesulfonyl fluoride (PMSF, 1 mM). Equal amounts of the whole cell lysate were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes, blocked with 5% skimmed milk at room temperature for 1 h and incubated with primary antibodies overnight at 4 °C. After the incubation of secondary antibodies, the protein bands were detected using the enhanced chemiluminescence (ECL) HRP substrate and visualized using Kodak X-AR film. Antibodies against phosphorylated BCR-ABL, BCR-ABL, phosphorylated GSK3β, GSK3β, β-catenin, p53, p21, and cyclinD1 were from Cell Signaling Technology (Danvers, USA). CBP, p300, JUN, phosphorylated PI3K, PI3K, phosphorylated AKT, and AKT were purchased from Santa Cruz Biotechnology Inc (Paso Robles, CA, USA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from KangCheng Biotech Inc (Shanghai, China).

Co-immunoprecipitation (IP) assay

Total cell lysates were prepared in the ice-cold IP lysis buffer. The supernatant of cell lysate was precleared by Protein G Plus/Protein A-agarose beads to reduce nonspecific binding. Then, either mouse IgG or anti-β-catenin antibody was added to the supernatant and incubated at 4 °C overnight. The bound proteins were washed three times with lysis buffer and dissociated with the beads via boiling and centrifugation. The immunoprecipitated proteins were then analyzed by standard western blot.

In vivo tumor xenograft models

All mouse experiments were conducted with the permission of the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-Sen University Cancer Center (No. L102012015120F). Female BALB/c nude mice were purchased from Gempharmatech-GD (Guangdong, China). To detect the antitumor effect of PBA2, 1×10^7 cells of K562, 32D-WT, and 32D-T315I cells were subcutaneously implanted into the left flank of BALB/c nude mice separately. Mice bearing CML tumor xenografts (approximately 100 mm^3) were treated once daily by oral gavage with vehicle, imatinib (100 mg/kg) or PBA2 (at indicated concentrations) for 10 consecutive days. Tumor volume was calculated using the formula: $L\times W^2\times0.5$ (L and W indicate the length and width of tumors, respectively).

Mice survival models

32D-WT or T315I cells were injected into the tail vein of BALB/c nude mice. At 72 h after tumor cells injection, mice were treated once daily by oral gavage with vehicle, imatinib (100 mg/kg) or PBA2 (9 mg/kg) for up to 25 days. Moribund animals were sacrificed as per IACUC guidelines. On necropsy, the mice had marked splenomegaly due to tumor cell infiltration.

Statistical analysis

All experiments were repeated at least three times and the data are presented as mean ± SD or mean ± SEM. The data was compared using One-Way ANOVA or unpaired two-tailed Student's *t*-test. Kaplan-Meier survival curves were used to estimate cumulative survival probabilities with a Log-rank test. The GraphPad Prism software was used for statistical analyses. A value of $p<0.05$ was considered statistically significant.

Results

PBA2 overcomes imatinib-resistant CML cells harboring T315I-mutated BCR-ABL

Imatinib is a potent antitumor drug against CML patients harboring BCR-ABL, however, it shows little efficacy in those with BCR-ABL T315I mutation. Our previous screening experiments identified that PBA2 possessed significant antiproliferative effects against BaF3 cells expressing wild-type or T315I-mutated BCR-ABL [21]. The inhibitory effect of PBA2 and imatinib on the cell growth of K562, 32D-WT, and 32D-T315I cells was determined by cytotoxicity assay. As expected, 32D-T315I was resistant to imatinib compared with 32D-WT (IC_{50} WT = 0.2 μM vs. T315I = 12 μM) (Table 1). The cell viability of K562 and 32D-WT cells was significantly inhibited by both imatinib and PBA2. Importantly, PBA2 also showed superior efficacy against 32D-T315I cells than imatinib (IC_{50} PBA2 = 4.0 μM vs. imatinib = 12 μM) (Fig. 1c; Table 1). Besides, a significantly enlarged and flattened cell morphology occurred in CML cells treated with PBA2, consistent with the distinctive features of cellular senescence (Fig. 1d). Nevertheless, this altered cell morphology was not observed in the imatinib group.

Table 1 IC_{50} values of PBA2 and imatinib in CML cell lines

Cell lines	IC_{50} Mean ± SD (μM)	
	PBA2	Imatinib
K562	0.652 ± 0.041	0.201 ± 0.018
32D-WT	1.817 ± 0.100	0.153 ± 0.009
32D-T315I	3.987 ± 0.455	11.860 ± 0.705

IC_{50} values derived from cytotoxicity assays. IC_{50} values represent mean ± SD of at least 3 independent experiments

PBA2 induces cell differentiation and senescence in CML cells

The emergence of enlarged and flattened CML cells following exposure to PBA2 appealed to us to explore whether PBA2 could induce cell senescence. To this end, SA-β-Gal staining assays were performed. The staining results showed a remarkably greater number of SA-β-Gal positive cells (dark blue-stained cells) in the PBA2 group than in the imatinib group and the negative control group in K562, 32D-WT, and 32D-T315I cells, indicating that PBA2 could induce cell senescence in both BCR-ABL wild-type and T315I-mutated CML cells (Fig. 2a-d). In addition, we also detect whether PBA2 could promote cell differentiation. Notably, NBT reduction assays suggested PBA2 exhibited a significant cell differentiation-inducing ability in all three cell lines, while imatinib failed to contribute to cell differentiation (Fig. 2e-g). These observations demonstrated that PBA2 possessed the function to induce cell differentiation and senescence in CML cells.

PBA2 reduces CBP expression and promotes p53/p21-mediated senescence

Next, we explored the possible mechanism underlying PBA2 induced cell differentiation and senescence. According to our previous study [19], the binding of β-catenin to CBP and p300 determined CML cell proliferation and differentiation, respectively, independent of BCR-ABL mutation status. The inhibition of CBP impaired the β-catenin/CBP-mediated cell proliferation and facilitated β-catenin/p300-mediated cell differentiation, which could be used as an effective treatment strategy for CML harboring the imatinib-resistant BCR-ABL-T315I mutant. As PBA2 was found to significantly promote cell differentiation and senescence, the effect of PBA2 on CBP expression was also evaluated.

In the absence of Wnt stimulation, β-catenin is known to be sequentially phosphorylated within the destruction complex consisting of Axin, APC, CK1 and glycogen synthase kinase-3β (GSK-3β). Then phosphorylated

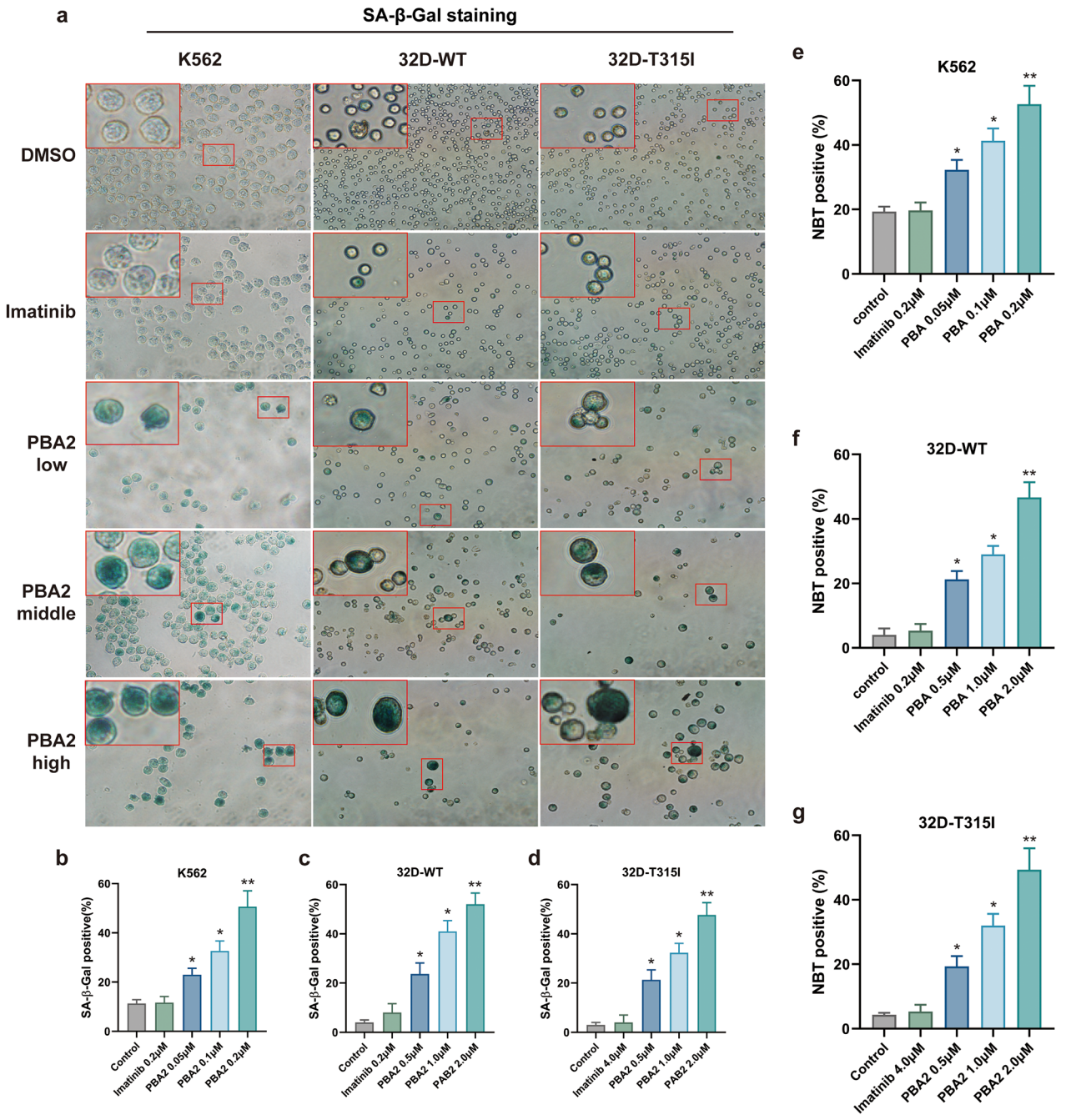

Fig. 2 PBA2 promotes CML terminal differentiation and senescence. **a** The representative images of senescent cells were taken by the Olympus IX71 inverted microscope following treatment with PBA2 and imatinib. **b-d** Senescence status was assessed in K562, 32D-WT, and 32D-T315I cells treated with indicated concentrations of PBA2 and imatinib for 72 h using the β-galactosidase staining assay. Senescent cells were identified as blue-stained cells under an Olympus IX71 inverted microscope. **e-g** K562, 32D-WT, and 32D-T315I cells were incubated with the indicated concentrations of PBA2 and imatinib for 72 h. Differentiation status was determined using the NBT reduction assay. The data represent mean ± SEM derived from three independent experiments. Significant changes are indicated as follows: *, $p < 0.05$; **, $p < 0.01$, compared to the control group (unpaired Student's *t*-test)

β-catenin is recognized and subsequently ubiquitinated by ubiquitin E3 β-transducin repeat-containing protein (β-TrCP). Then, ubiquitinated β-catenin is rapidly degraded by the proteasome [22]. Therefore, inhibition of GSK3β is known to promote the cellular accumulation of β-catenin. PBA2 significantly decreased the expression of GSK3β, while imatinib showed little effect on GSK3β expression (Fig. 3a). Additionally, compared with imatinib, PBA2 markedly reduced the expression of CBP but slightly increased the expression of p300 in

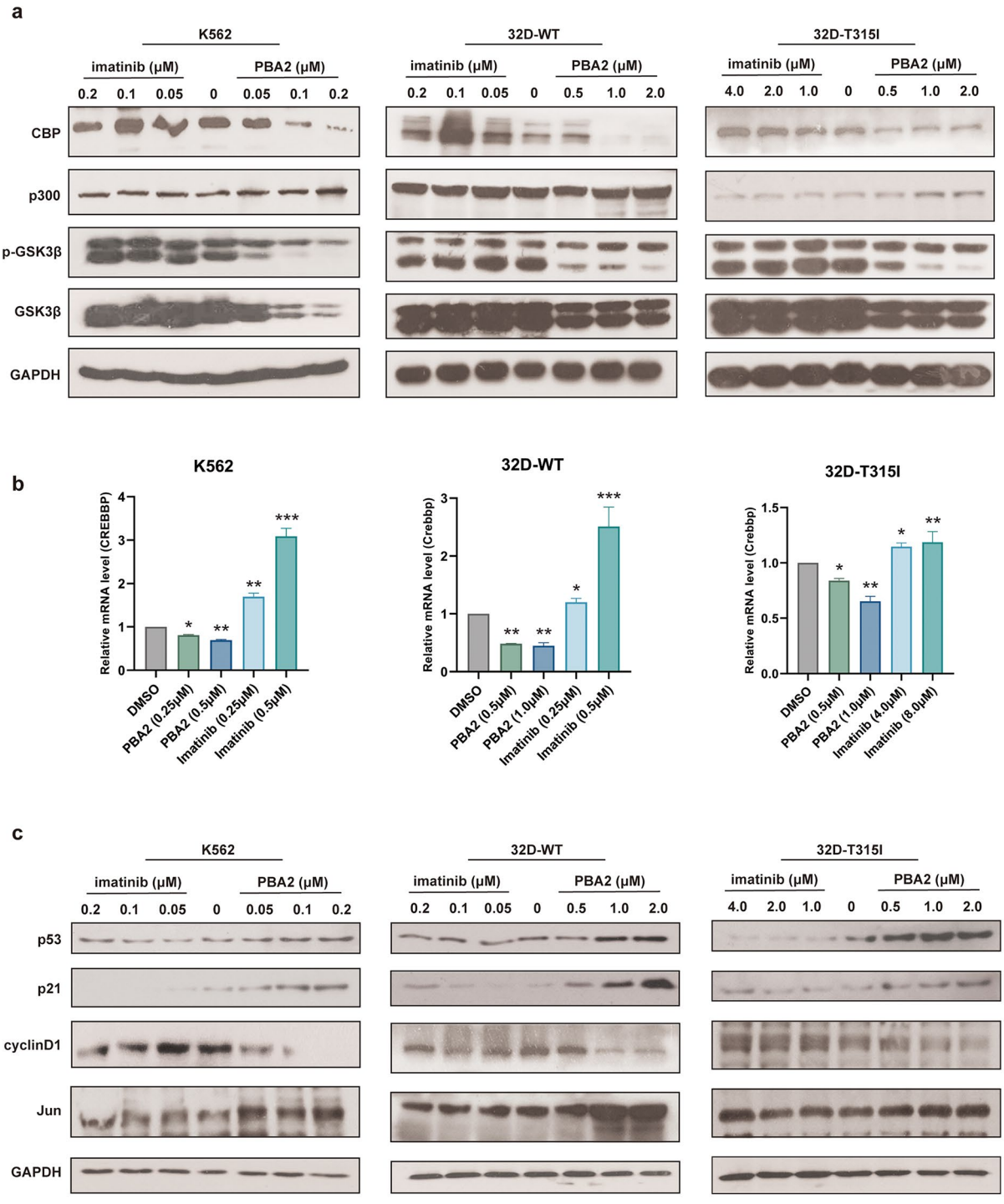

Fig. 3 PBA2 inhibits CBP expression and promotes p53/p21-dependent senescence of BCR-ABL-WT and BCR-ABL-T315I cells. **a** Western blot analysis of the expression of CBP, p300, p-GSK3β and total GSK3β in K562, 32D-WT, and 32D-T315I cells treated with PBA2 or imatinib at the indicated concentrations for 48 h. **b** The mRNA level in CML cells following treatment with PBA2 or imatinib at the indicated concentrations for 48 h by qPCR assay. **c** Western blot analysis of the indicated proteins in K562, 32D-WT, and 32D-T315I cells incubated with imatinib or PBA2 at indicated concentrations for 48 h

K562, 32D-WT and 32D-T315I cells (Fig. 3a). Notably, the qPCR assay demonstrated that PBA2 decreased the mRNA level of CBP in CML cells, which indicated that PBA2 inhibited the expression of CBP through transcriptional regulation (Fig. 3b).

We further investigated the mechanism by which PBA2 induced CML cell senescence. The western blot results of senescence-associated proteins indicated that PBA2 contributed to the accumulation of p53 and p21 in K562, 32D-WT and 32D-T315I (Fig. 3c). In addition, PBA2 was found to significantly decrease the expression of cyclin D1 (a CBP downstream molecule), but remarkably increase the expression of Jun (a p300 downstream protein) (Fig. 3c). These observations suggested PBA2 promoted p53/p21-mediated senescence.

Knockdown of CBP inhibits cell proliferation of CML cells

To evaluate the effect of CBP knockdown on CML cell proliferation, we transfected CML cells with lentiviral vectors carrying shNC, shCREBBP, or shCrebbp plasmids using RNA interference (RNAi) technology. The western blot analysis demonstrated the successful CBP knockdown in K562, 32D-WT, and 32D-T315I (Fig. 4a). In addition, the mRNA level was also significantly decreased in CML cells (Fig. 4b). Then, 1000 CML cells expressing shRNA were seeded into 96-well plates and the OD values were detected every 24 h for consecutive five days. The cell growth curve showed CBP knockdown inhibited cell proliferation in K562, 32D-WT, and 32D-T315I (Fig. 4c). The viable cells of CML cells with CBP knockdown were significantly reduced compared with the control counterparts (Fig. 4d). Overall, CBP inhibition suppressed cell proliferation of CML cells.

PBA2 shows superior antitumor efficacy in CML tumor xenograft models

Given that PBA2 overcame resistance to imatinib in CML cells in vitro, the in vivo antitumor activity of PBA2 was investigated in tumor xenograft models of CML. In K562-derived tumor-bearing mice, tumor growth was significantly inhibited in the PBA2 or imatinib group compared with the vehicle-treated group (Fig. 5a, b). The tumor volume and weight were markedly reduced in the PBA2 or imatinib-treated mice (Fig. 5c, d). A similar significant superior antitumor effect of PBA2 or imatinib over vehicle control was also observed in 32D-WT-bearing mice (Fig. 5e-h). Notably, in the 32D-T315I-driven xenograft model, CML tumor growth was remarkably suppressed by PBA2 (Fig. 5i-j). Compared with the vehicle group, PBA2, but not imatinib, prominently decreased the tumor volume and weight (Fig. 5k-l). During in vivo experiments, no animal deaths or significant body weight loss were observed in all groups, indicating the well-tolerated antitumor profiles of PBA2 and imatinib. These results demonstrated that PBA2 exhibited significant antitumor efficacy in both BCR-ABL wild-type and T315I-driven mouse xenograft models, regardless of BCR-ABL mutation status.

Furthermore, we also studied the effect of PBA2 on survival in mouse models where 32D-WT or 32D-T315I cells were intravenously injected in BALB/c nude mice. In the 32D-WT-driven model, the median survival period of vehicle-treated mice was 35 days and daily oral treatment with PBA2 (9 mg/kg) prolonged the median survival time to 46 days (Fig. 5m). These results were comparable to those receiving daily oral administration of 100 mg/kg imatinib, where the median survival was 43 days (Fig. 5m). However, in the 32D-T315I-driven model, the oral administration of imatinib displayed no appreciable effect on survival time (Fig. 5n). In contrast, PBA2 (9 mg/kg) treatment was found to significantly prolong survival time to 47 days, compared to 35 days in vehicle-treated mice and 27 days in imatinib-treated mice (Fig. 5n).

PBA2 significantly enhances β-catenin/p300 interaction and inhibits BCR-ABL signaling

To further investigate the mechanism of the anti-tumor effect of PBA2, the interaction of β-catenin to CBP versus p300 and BCR-ABL signaling was evaluated. The cytoplasmic and nuclear expression of β-catenin were detected, respectively. We found PBA2 increased β-catenin expression in a concentration-dependent manner in the cytoplasm and nucleus of K562, 32D-WT and 32D-T315I cells, indicating β-catenin was significantly stabilized and its nuclear accumulation was increased by PBA2 (Fig. 6a, b). Despite β-catenin accumulation in the nucleus following PBA2 treatment, the significant inhibitory effect of PBA2 on CBP suggested that PBA2 may result in a switch of β-catenin binding from CBP to p300.

Consistently, the results from the Co-IP assay indicated that PBA2 enhanced the interaction of β-catenin and p300 in CML cells expressing wild-type or T315I mutant BCR-ABL (Fig. 6c). The above findings indicated that PBA2 significantly reduced CBP expression but increased β-catenin level, thereby promoting the interaction of β-catenin/p300 to trigger cell differentiation and senescence in CML cells independent of BCR-ABL mutation status. As shown in Fig. 6d, it is noteworthy that although both imatinib and PBA2 exhibited significant inhibitory effects against wild-type BCR-ABL and its downstream PI3K/AKT pathway, only PBA2 effectively inhibited BCR-ABL-T315I signaling.

Discussion

The long-term maintenance of HSCs homeostasis is regulated by balancing self-renewal and differentiation. Activation of the Wnt signaling pathway contributes to the accumulation of β-catenin in the nucleus and the

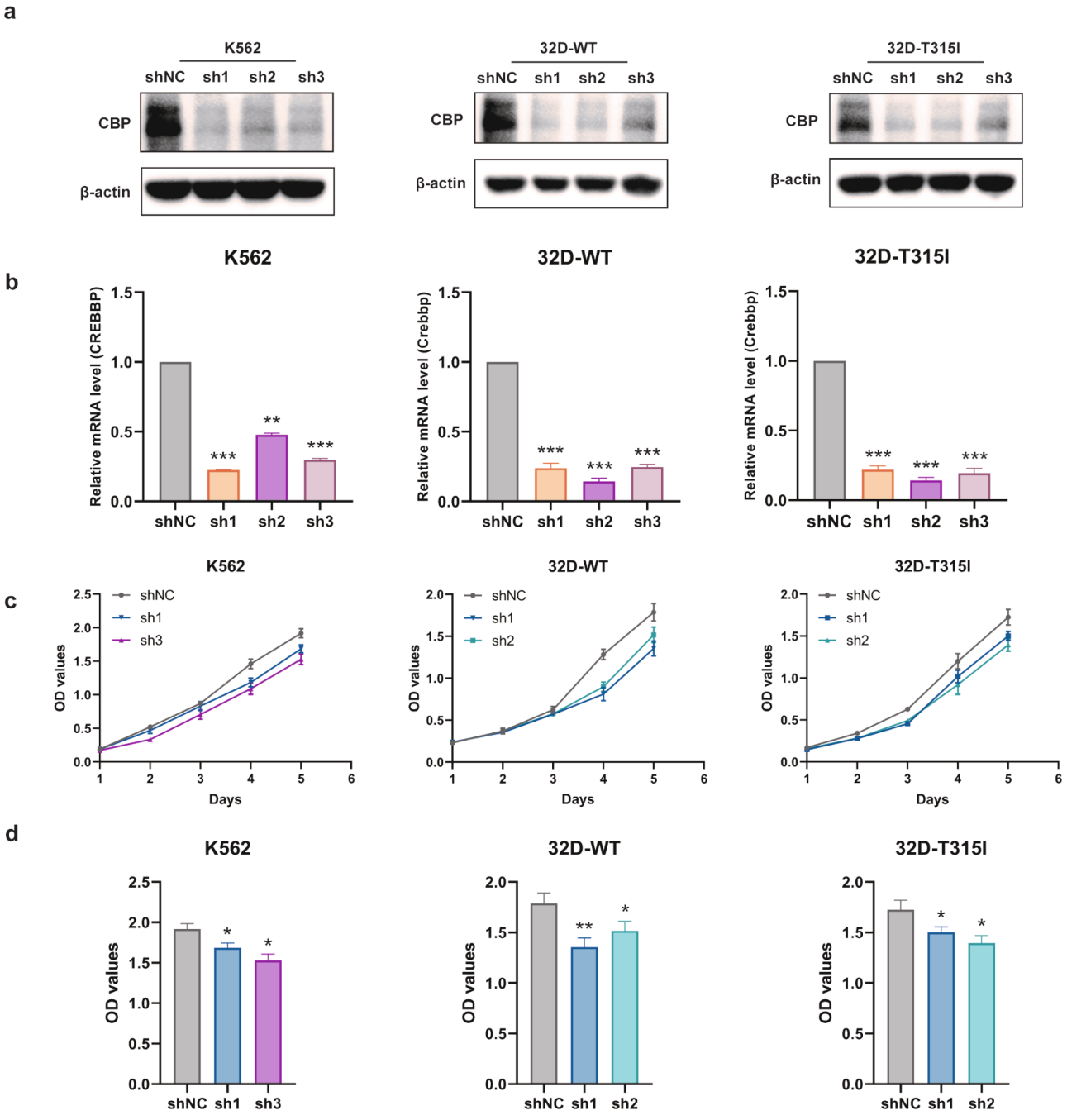

Fig. 4 Knockdown of CBP inhibits cell proliferation of CML cells. **a** Western blot analysis of CBP protein expression in CML cells transfected with lentiviral vector carrying shNC, shCREBBP, or shCrebbp. **b** qPCR assay of CBP mRNA level in CML cells transfected with lentiviral vector carrying shNC, shCREBBP, or shCrebbp. **c** The cell growth curve of CML cells with CBP knockdown and the control counterparts. **d** The OD values of CML cells at the endpoint of experiments

recruitment of transcriptional factors of the TCF/LEF family, and subsequent activation of downstream target genes, which plays a critical role in determining the fate of HSCs towards self-renewal and differentiation [23, 24]. Differentiation-inducing agents including all-*trans* retinoic acid (ATRA), have shown promising therapeutic efficacy and significantly improved survival of hematological malignancies, particularly in acute promyelocytic leukemia (APL) treatment [25]. However, there is generally a lack of effective differentiation therapy in CML. To this end, a dichotomous model has been proposed where specific interaction of β-catenin with either CBP or p300 could regulate self-renewal and differentiation of HSCs, respectively [15, 24]. We have previously reported that

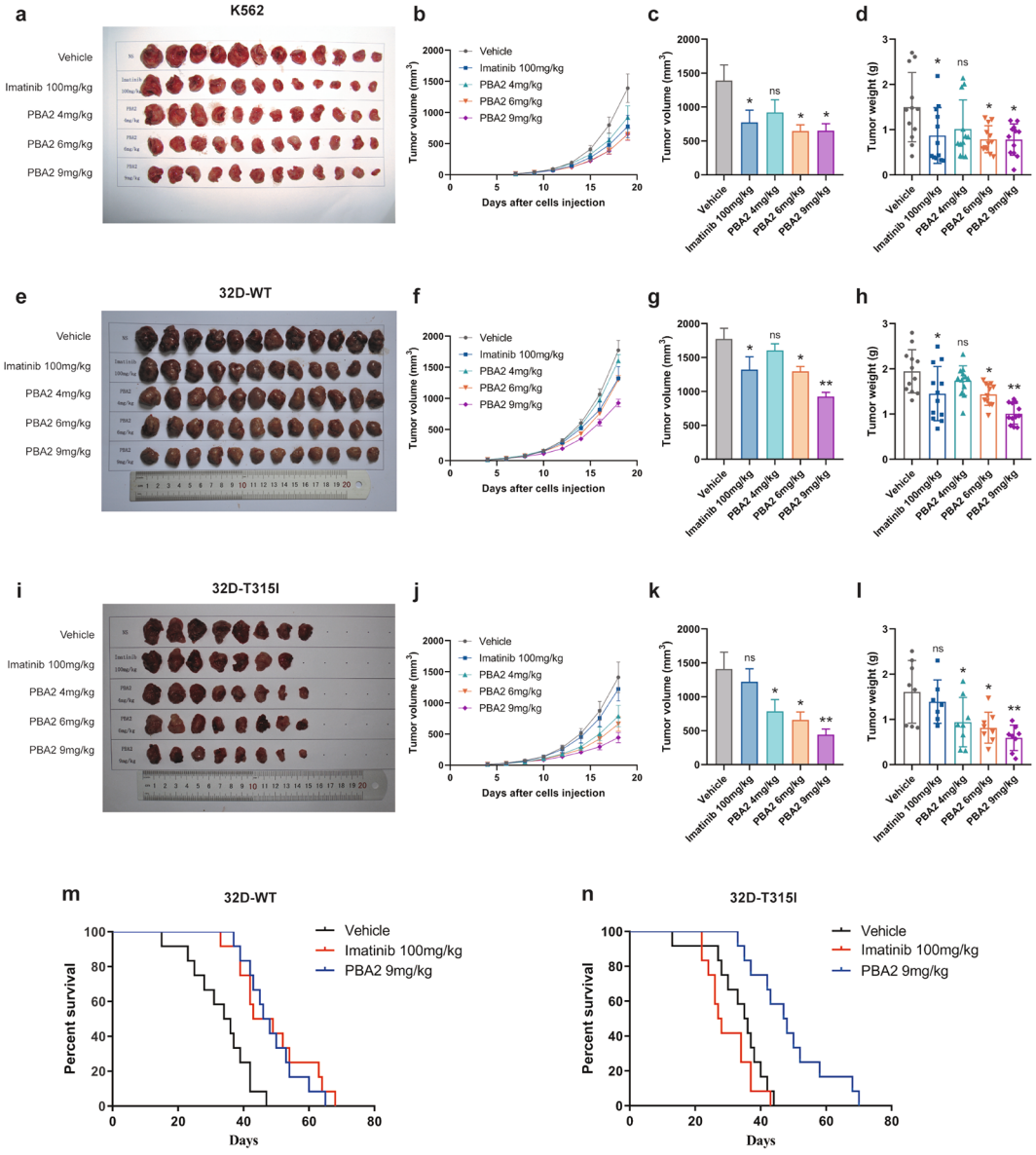

Fig. 5 PBA2 suppresses tumor xenograft growth in mouse models of CML expressing either wild-type BCR-ABL or T315I-mutated BCR-ABL. Tumor-bearing mice were treated once daily by oral gavage with vehicle or the indicated doses of PBA2 and imatinib for 8 or 10 consecutive days. **a**, **e**, **i** Images showing tumor size of BALB/C nude mice with K562, 32D-WT or 32D-T315I derived-tumors after treatment with vehicle, imatinib and PBA2. **b**, **f**, **j** Tumor growth curves were plotted to illustrate the change in tumor volumes over time after tumor implantation. **c**, **g**, **k** The bar graphs showed tumor volumes at the endpoint of the experiment. The data shown represent the mean ± SEM of tumor volumes for each group. **d**, **h**, **l** The bar graphs showed tumor weights at the endpoint of the experiment. The data shown represent the mean ± SD of tumor weight for each group. **m**, **n** 32D cells expressing wild-type or T315I-mutated BCR-ABL were injected into the tail vein of BALB/c nude mice, and the animals were treated once daily by oral gavage with vehicle, imatinib, or PBA2 at the indicated doses. Animal survival rate curves were plotted and the data were analyzed by Kaplan-Meier method. Statistical significance was evaluated to compare the survival of the treatment groups with the vehicle group

β-catenin recruits more p300 when CBP expression is silenced in CMLs expressing wild-type or T315I mutant BCR-ABL [19]. It follows that the suppression of CBP could switch the balance towards β-catenin/p300-mediated cell differentiation, representing an entirely novel approach for the treatment of CML patients and possessing the therapeutic potential to treat those with either wild-type or T315I-mutated BCR-ABL.

The successful clinical application of histone deacetylase inhibitors (HDACi) has increased the interest in the development of histone acetyltransferase inhibitors. The two histone acetyltransferases, CBP and p300, have been studied widely. Notably, CBP inhibitors have been investigated in certain cancer types, including solid tumors and hematological malignancies [26, 27]. C646, a CBP/p300 inhibitor, suppressed cell proliferation and tumor

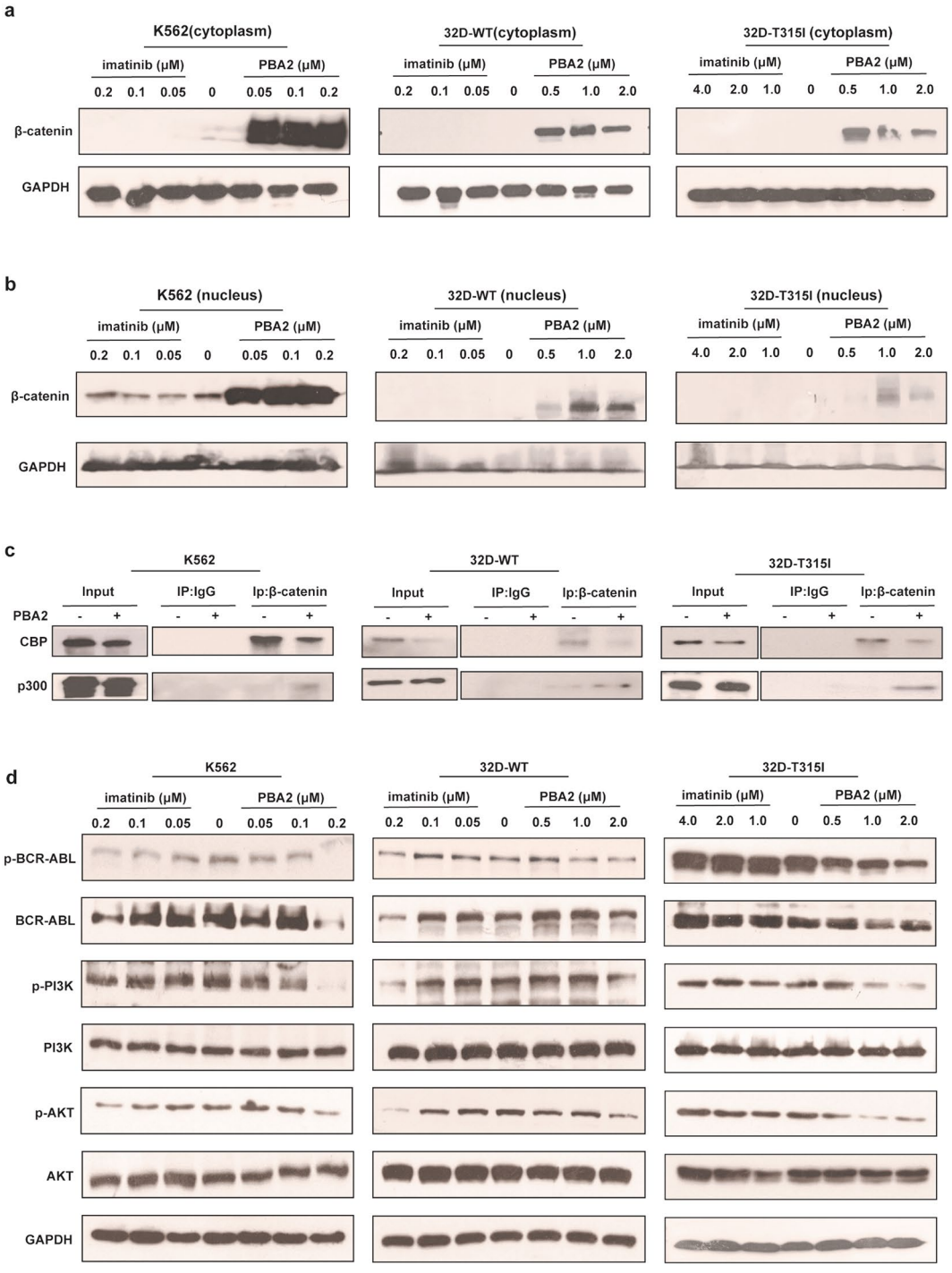

Fig. 6 PBA2 promotes β-catenin/p300 interaction and inhibits BCR-ABL signaling. **a** Western blot analysis of β-catenin in the cytoplasm of K562, 32D-WT and 32D-T315I cells incubated with imatinib or PBA2 at indicated concentrations. **b** Western blot analysis of β-catenin in the nucleus of K562, 32D-WT and 32D-T315I cells incubated with imatinib or PBA2 at indicated concentrations. **c** Co-IP analysis of the interaction between β-catenin and CBP or p300 in K562, 32D-WT and 32D-T315I cells following PBA2 treatment. **d** Western blot analysis of the indicated proteins in K562, 32D-WT and 32D-T315I cells incubated with imatinib or PBA2 at indicated concentrations

growth of glioblastoma in vitro and in vivo, respectively [28]. In addition, C646 exhibited inhibitory effects on cell migration, invasion, and lung metastasis of osteosarcoma [29]. Another CBP/p300 inhibitor B029-2 presented marked antitumor effects in hepatocellular carcinoma (HCC) in vitro and in vivo by disrupting the metabolic reprogramming of HCC cells [30]. The selective β-catenin/CBP inhibitor, ICG001, significantly restrained

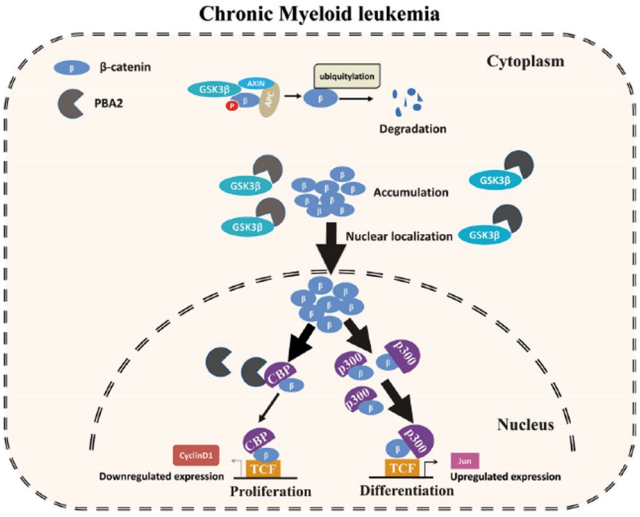

Fig. 7 Proposed mechanism of PBA2 for the treatment of CML. Inhibition of CBP significantly increased the interaction of p300 and β-catenin, which induces cell differentiation and growth inhibition regardless of BCR-ABL mutation status in CML cells

the cell proliferation of HER2-positive breast cancer, which was associated with decreased levels of HER2 [27]. Importantly, ICG001 also contributed to the differentiation of pre-B acute lymphoblastic leukemia (ALL) cells and the decrease of self-renewal capacity in ALL. ICG001 eradicated the drug-resistant primary leukemia cells and significantly prolonged the survival of the mouse model of primary ALL when combined with chemotherapy [31]. These important results suggested that CBP inhibitors possessed promising antitumor efficacy in different types of cancer.

Consistent with these observations, we found that PBA2, a novel potent CBP inhibitor, remarkably suppressed cell proliferation in CML cells harboring either wild-type or the T315I-mutated BCR-ABL, which was correlated with β-catenin/p300-mediated cell differentiation and senescence (Fig. 7). Cellular senescence is considered a potent tumor-suppressive mechanism as it hampers cell division and proliferation. Two well-established tumor suppressive networks, p53/p21 and p16/p-Rb, are known to induce cell senescence and arrest cell proliferation. In this study, the results demonstrated that the increased interaction of β-catenin and p300 was implicated in p53/p21-mediated cell senescence. PBA2 also exhibited potent antitumor effects in mouse models of CML with wild-type or T315I-mutated BCR-ABL. Moreover, PBA2 significantly prolonged the survival of tumor-bearing mice harboring T315I mutation compared with imatinib. These findings demonstrated that PBA2 could achieve remarkable antitumor efficacy in both imatinib-sensitive and -resistant CML in vitro and in vivo, supporting the rational treatment of CML by targeting CBP, and further clinical investigations of the novel compound PBA2 are warranted.

Conclusion

PBA2 exhibited several important and novel biological properties that supported its further clinical development. It is highly effective against CML expressing wild-type or T315I mutant BCR-ABL. The significant antitumor activity is presumably mediated by a novel mechanism related to the induction of cell differentiation and senescence. Importantly, PBA2 was shown to inhibit tumor growth and prolong survival in mouse models of wild-type and T315I-mutated BCR-ABL-driven CML. Numerous BCR-ABL TKIs have been reported to suppress the proliferation of CML cells expressing BCR-ABL, however, the vast majority of them are not efficacious against those harboring BCR-ABL-T315I mutation [6, 32]. We found a novel compound, PBA2, that exhibited highly potent antitumor efficacy in both imatinib-sensitive and -resistant CML in vitro and in vivo. These significant findings support the conclusion that inhibiting CBP is a promising strategy for the treatment of CML, and the safety profile of PBA2 is warranted to investigate in further studies.

Supplementary Information

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Supplementary Material 1

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Author contributions

Liwu Fu designed the research. Ke Yang and Kai Fu conducted the experiments. Hong Zhang, Xiaokun Wang, Caibo Yang, and Fang Wang analyzed data and provided technical assistance. Ke Yang and Kai Fu wrote the paper and prepared the figures. Kenneth K.W. To and Zhe-Sheng Chen

revised the manuscript. All authors contributed to this article and approved the submitted version.

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Data availability
http://www.researchdata.org.cn/ (RDDB2024808155)

Declarations

Ethics approval and consent to participate
All animal experiments were performed with the permission of the Institutional Committee of the Sun Yat-sen University Cancer Center and complied with the protocol approved by the Guangdong Provincial Animal Care and Use Committee and experimental guidelines of the Animal Experimentation Ethics Committee of Sun Yat-sen University Cancer Center (L102012015120F).

Competing interests
The authors declare no competing interests.

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