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# The combination of PRL-3 inhibitor with sorafenib synergistically promotes AML apoptosis

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Phosphatase of regenerating liver-3 (PRL-3) is recognized as a novel independent crucial driver for AML progression. Thus, the specific inhibitor of PRL-3 would be a potential therapeutic agent to AML in clinics, but there are not enough preclinical applications reported yet. Here we evaluated the cytotoxicity of PRL-3 inhibitor, BR-1, against AML cells ML-1 and MOLM-13. Meanwhile, the effect of BR-1 on the biological characteristics of AML cells and the underlying mechanism was investigated along with the combination of BR-1 and sorafenib on the AML cell viability. Our results show that BR-1 promotes apoptosis by inactivation of the JAK/STAT5 and PI3K/AKT pathways, while inhibits cell proliferation through arresting cell cycle in the S phase. In addition, a combination of BR-1 with sorafenib can further improve the therapeutic effect on AML. Thus, our results demonstrated that BR-1 would be a novel and potent therapeutic agent to AML, and its combination with other anti-AML drugs would be a promising strategy for AML therapy.

*Key words: PRL-3 inhibitor, sorafenib, acute myeloid leukemia, apoptosis, therapy*

Acute myeloid leukemia (AML) is a heterogeneous disease. With the development of tumor genetics and molecular biology, diverse molecular characteristics of oncogenes have been disclosed along with applicable prognostic and therapeutic value [1]. However, there are still many patients with undetected molecular abnormalities, indicating the complexity and heterogeneity of cancer cases. Thus, identification of new prognostic genes and therapeutic targets would be of help to the individualized clinical treatment of cancer, including AML patients.

Phosphatase of regenerating liver-3 (PRL-3), also known as PTP4A3, belongs to the protein tyrosine phosphatase (PTP) family [2]. In this family, there are about 107 members that modulate the phosphorylation and dephosphorylation of many biologically significant molecules with numerous kinases, which all participate in signaling pathways and play roles in development and tumor progression [3]. We previously showed that PRL-3 is highly expressed in AML patients and affects the cell cycle, cell proliferation, and apoptosis in AML cell lines, demonstrating PRL-3 as an independent prognostic marker for AML [4]. Thus, we suspect that usage of PRL-3 inhibitors would be a potential therapeutics for AML.

Rhodamine- and 1,3-thiazolidine-2,4-dione-based compounds are composed of a very important group of heterocyclic compounds in drug development [5]. The rhodamine derivatives have been characterized by abilities to inhibit cancer cell migration and the enzymatic activity of PRL-3 [5]. But there are few clinical studies on their use in cancer therapy and no mention of their application on AML.

FMS-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) is a common gene mutation in AML and patients with this mutation are not sensitive to chemotherapy [6, 7]. Impressively, one of the tyrosine kinase inhibitors, sorafenib can provide an effective treatment to AML patients with this mutation [8]. However, the overall poor efficacy of sorafenib limits its large-scale application on AML patients [9, 10]. Therefore, overcome of drug resistance and improvement of curative efficacy are urgent needs in clinics. Evidence shows that the combination of sorafenib and other anti-tumor drugs can play a synergistic anti-tumor effect to improve therapy efficacy [11–13].

Thus, in this study, we tentatively combined the inhibitors of both PRL-3 and FLT3-ITD to characterize their synergetically therapeutical effects as well as the underlying mechanisms on AML progression. Our results here demon-

strate that the PRL-3 inhibitor, BR-1 alone can exert an efficient anti-AML effect. Moreover, a combination of BR-1 with sorafenib can further improve the cytotoxicity against AML cells, suggesting that targeting PRL-3 would be a new therapeutic strategy for AML treatment, and the combined inhibition of both PRL-3 and FLT3-ITD would be a potential strategy for AML therapy.

## Patients and methods

**Patients.** According to the NCCN Clinical Practice Guidelines in Oncology for myeloid neoplasms and acute leukemia in 2019 [14], the newly diagnosed AML at the First Affiliated Hospital of Sun Yat-sen University from September 2020 to December 2020 were classified and sampled. Each patient underwent a bone marrow puncture at the time of diagnosis with informed consent. Prior to this study, all patients (or their parents or guardians) have given their written informed consent and the study protocol was approved by the institute's committee on human research. All procedures were strictly followed to the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Leukemia cells were extracted from the bone marrow of each patient for culture, using lymphocyte separation fluid and erythrocyte lysate. Bone marrows from 10 patients were eventually succeeded to be steadily cultured for the later experiments. The main clinical features of these 10 patients are listed in Table 1.

**Cell lines and reagents.** Human leukemia lines, ML-1, MOLM-13, and HL-60 (without MLL fusion protein) were obtained from the American Type Culture Collection (ATCC) and grown in the RPMI medium (Hyclone) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin. The ML-1-PRL3 cells were established in our lab previously [4]. BR-1 and sorafenib were purchased from GLPBIO. All drugs were dissolved at a 10 mM concentration in dimethyl sulfoxide (DMSO) and stored in small aliquots at –20 °C until further use.

**Cell proliferation, cell cycle, and apoptosis assays.** The CCK-8 cell proliferation kit (Dojindo Laboratories, Kumamoto, Japan) was used to examine the growth rate of cells. Flow cytometry was carried out for cell cycle analysis with a flow cytometer (Beckman, Danvers, MA) upon propidium iodide staining. Cell apoptosis was also analyzed by flow cytometry using the VIFC/PI Apoptosis Detection kit (4A Biotech Co., Ltd, China). All experiments were carried out in triplicate each time, and at least 3 independent experiments were repeated.

**Trypan blue assays.** AML cells were seeded into the 96-well plates with a density of $5\times10^3$ cells/well. After 24 h of drug treatment, cells were fully mixed with 0.4% trypan blue with a ratio of 10:1 (V/V) for another 3 min, followed by the observation and photographing under a microscope immediately.

**Soft agar assay for colony formation.** For soft agar plate preparation, 2 ml agar mixture (1% agar, 2× RPMI with 20% FBS, and 2× antibiotics) was plated onto each culture Petridish with 35 mm diameter and set aside for 5 min to solidified. For cell seeding on the soft agar plate, 0.35% agarose (W/V) was prepared with RPMI with 10% FBS and 1× antibiotics. Until the temperature of this solution is in a range of 37 °C to 40 °C; 5,000 ML-1 cells were mixed with this agar solution and plated on top of each prepared soft agar plate with a final 10 μM BR-1 or with the equal volume of DMSO as a control. Plates were incubated at 37 °C in a humidified incubator for 7 to 21 days. During this culturing process, cells were feed 1–2 times with 500 μl RPMI per week. After colonies could be observed on plates, the colonies were stained with 0.5 ml 0.5% crystal violet (W/V) for about 20 min and the colonies were counted and photographed under a microscope.

**Western blots.** PRL-3 antibody and western blot analysis were described previously [4]. The blots were detected by ECL

**Table 1. Main characteristics of AML patients.**

| Patients | Gender | Age (years) | WBC ($\times10^9$/l) | Hb (g/l) | PLT ($\times10^9$/l) | Blasts in bone marrow (%) | FLT3-ITD mutation | NCCN Prognostic stratification |
|---|---|---|---|---|---|---|---|---|
| #1 | Male | 32 | 23.98 | 64 | 39 | 79.4 | Negative | Favorable |
| #2 | Male | 44 | 39.12 | 56 | 27 | 54 | Negative | Intermediate |
| #3 | Female | 26 | 191.95 | 77 | 93 | 84.7 | Negative | Intermediate |
| #4 | Female | 59 | 53.39 | 103 | 61 | 76.4 | Positive | Poor |
| #5 | Female | 51 | 3.92 | 53 | 74 | 52.4 | Negative | Intermediate |
| #6 | Male | 60 | 77.19 | 66 | 22 | 93 | Positive | Poor |
| #7 | Female | 50 | 1.91 | 65 | 92 | 75.5 | Negative | Intermediate |
| #8 | Female | 58 | 8.03 | 128 | 35 | 79 | Negative | Favorable |
| #9 | Female | 27 | 4.32 | 117 | 923 | 70 | Negative | Intermediate |
| #10 | Male | 26 | 6.32 | 61 | 76 | 75 | Negative | Intermediate |

(Clarity Western ECL Substrate, Cat. #170-5060; BioRad) and visualized with ChemiDoc (MP, Serial #731BR00765; BioRad).

**Tumor models in immunodeficient mice.** Four-week-old nude mice were purchased from the Experimental Animal Center of Sun Yat-sen University and randomly divided into two groups, of which, one group was used for subcutaneous injection and the other for intravenous injection. For subcutaneous injection, each mouse was injected subcutaneously with $1\times10^6$ ML-1 cells. When tumor volume reached 8 $mm^3$, half of the mice were randomly selected and injected with BR-1 at 15 mg/kg dose intraperitoneally once every three days, for 4 times in total, while the other half was injected with the same volume of corn oil as a control group. Mice were sacrificed on the 13$^{th}$ day after the first injection, and the tumor size and weight were measured and compared between the two groups. For intravenous injection, all mice were injected with $1\times10^6$ ML-1 cells into the caudal vein. After 14 days, mice were also randomly assigned into two groups (n=5/group) and then treated with corn oil and BR-1 (15 mg/kg), respectively once every three days, 3 times in total. For another 10 days from the first drug administration, mice were sacrificed and the dissected spleen and liver were analyzed as above. This animal experiment was strictly performed following the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-Sen University.

**Statistical analysis of clinical samples.** The SPSS 25.0 software (SPSS, Chicago, IL) was adopted to perform the statistical analysis. Comparisons between two groups were analyzed using Student's t-test, while Bonferroni test was used for comparisons between three or more groups. All experiments were carried out in triplicate each time. Differences were considered to be statistically significant at $p<0.05$.

## Results

**BR-1 enhances AML cell apoptosis.** To investigate whether BR-1 could induce apoptosis of AML cells, we treated ML-1, MOLM-13, and HL-60 cells with BR-1 at a concentration gradient (0 μM, 5 μM, 10 μM, 20 μM). Immunoblotting results revealed that with BR-1 concentration increase, AML cells were accompanied by the increased activation of apoptosis-related signaling, including the cleaved amounts of caspase-7 and PARP as well as the activated p-H2A.X (Figures 1A–1C). Flow cytometry analysis also confirmed the indicated apoptosis induced by BR-1 (Figures 1D–1F, $p<0.0001$, respectively). Given that JAK/STAT5 and PI3K/AKT pathways are associated with the anti-apoptosis effect in tumors [15–17], we sought to clarify whether PRL-3 inhibitor BR-1 could suppress these two pathways to induce the apoptosis of tumors. Our results showed that BR-1 can effectively suppress the phosphorylation of STAT5 and AKT. As expected, the expression of PI3K was decreased along with BR-1 treatment (Figures 1G–1I).

**BR-1 arrests AML cell cycle and inhibits AML cell proliferation.** We then explored the role of BR-1 in AML cell proliferation. The CCK-8 assay indicated that BR-1 significantly repressed ML-1, MOLM-13, and HL-60 cells' proliferation, respectively (Figures 2A–2C, $p<0.0001$). Soft agar colony formation assay showed that BR-1 treatment led to fewer and smaller colonies of ML-1cells, compared to the DMSO control group (Figures 2D–2F, $p=0.0024$), which confirmed the inhibition of BR-1 to AML cell proliferation. Next, flow cytometry analysis further verified the inhibitory effect of BR-1 on AML cell cycle progression, while demonstrated that BR-1 could arrest cell cycle progression by inducing cell cycle arrest at the S phase in ML-1, MOLM-13, and HL-60 cells a dose-dependent manner (0 μM, 5 μM, 10 μM, 20 μM, Figures 2G–2I). Moreover, we also determined the expression of cell cycle-related proteins of the S phase and showed that BR-1 obviously attenuated the expressions of CDK2 and CDC25A (Figures 2J–2L), further validating the inhibitory effect of BR-1 on AML cell cycle progression and proliferation.

**BR-1 renders more efficacy in cytotoxicity in PRL-3 highly expressing cells.** Considering BR-1 was characterized as an inhibitor of PRL-3 [5], and PRL-3 is also elevated in AML patients [4], we treated the parental ML-1 cells with the PRL-3-overexpressing ML-1 cells with BR-1 (25 μM) respectively. In the PRL-3-overexpressing cells, more apoptosis was observed than that of the wild-type parental cells upon the BR-1 treatment (Figure 3A). Accordingly, BR-1 also exerted obvious cell proliferation inhibition of PRL-3 forced expression cells (Figure 3B). The statistical analysis further proved that BR-1 presented a more inhibitory effect on PRL-3 highly expressing cells than PRL-3 low cells (Figure 3C, $p<0.0001$), indicating the promising application on AML therapy.

**Combination of BR-1 with sorafenib renders synergetic cytotoxicity on AML cells.** To tentatively promote the cytotoxicity efficacy of BR-1, we used and compared BR-1 with a tyrosine kinase inhibitor, sorafenib as well as their combination to examine their therapeutical potential in both ML-1 and MOLM-13 cells. The CCK-8 assay and flow cytometry both demonstrated that the combination of BR-1 with sorafenib could significantly inhibit AML cell proliferation, compared to both the individual BR-1 or sorafenib, respectively (Figures 4A, 4B; $p<0.0001$). As sorafenib generally blocks cells in the G1 phase, a combination of sorafenib with BR-1 majorly enhanced the G1 phase-arrest of AML cells, compared to the singly treated cells (Figures 4C, 4D). Likewise, the supplemental addition of sorafenib efficiently prompted the apoptotic cell ratio, compared to each individual agent (Figures 4E–4H, $p<0.0001$). As MOLM-13 cells are FLT3-ITD-positive cells, sorafenib showed more cytotoxicity to these cells than ML-1 cells that are FLT3-negative cells. However, the combination of BR-1 was capable to enhance the apoptotic effect of sorafenib (Figures 4E–4H). We also checked the expression status of FLT3 and found that BR-1 could downregulate FLT3 (Figure 4I), suggesting the mutual

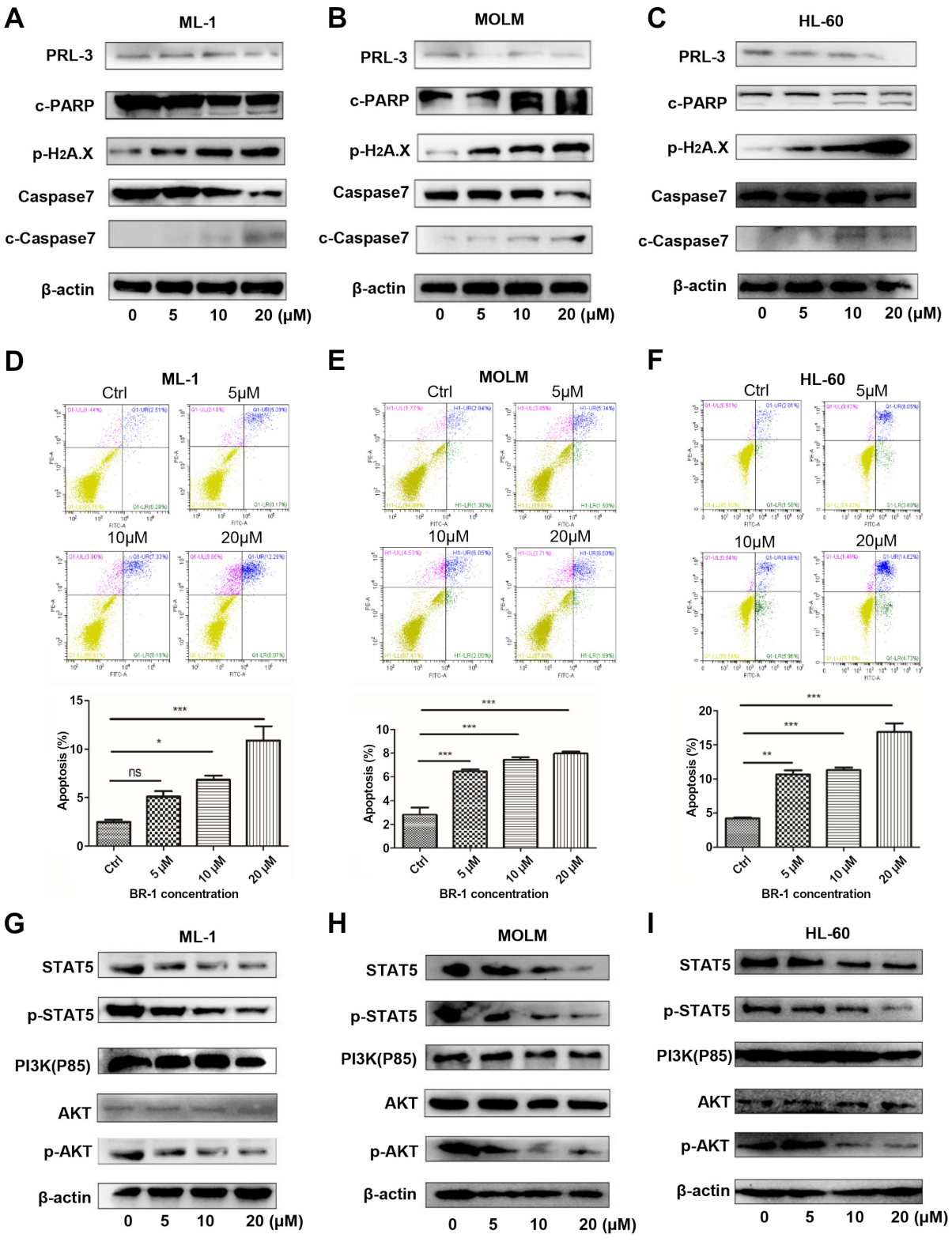


**Figure 1. BR-1 enhances AML cell apoptosis. A–C) Expression of apoptosis-related proteins with various concentrations of BR-1 treatment in ML-1 (A), MOLM-13 (B), and HL-60 (C) cells. D–F) Flow cytometry analysis and cell ratio of apoptosis by VIFC/PI staining in ML-1 (D), MOLM-13 (E), and HL-60 (F) cells with indicated concentrations of BR-1 treatment. G–I) Expression of STAT5, PI3K, and AKT with various concentrations of BR-1 treatment in ML-1 (G), MOLM-13 (H), and HL-60 (I) cells.**

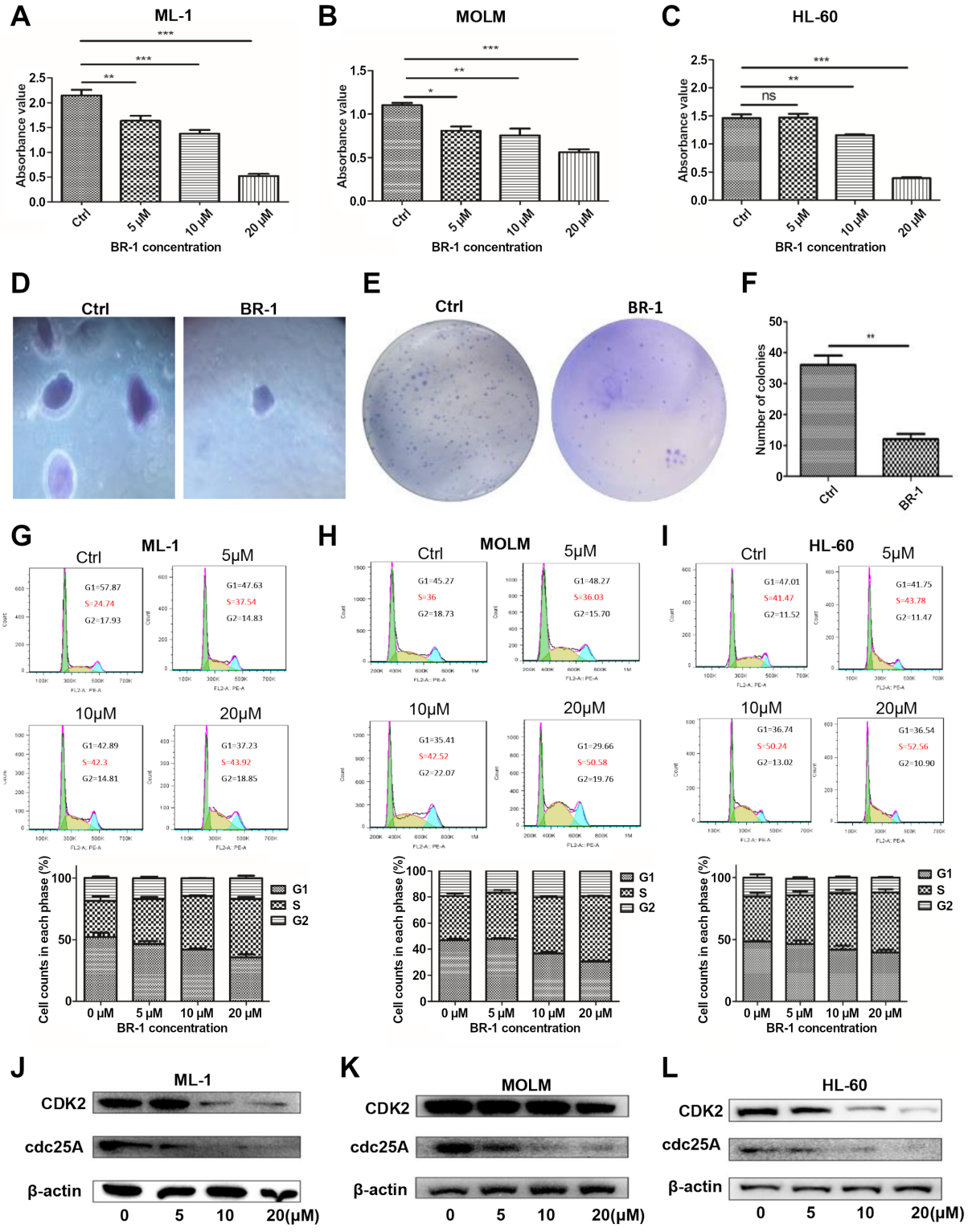


**Figure 2. BR-1 arrests AML cell cycle and inhibits AML cell proliferation. A–C) Cell viability analyses of ML-1 (A), MOLM-13 (B), and HL-60 (C) cells with different concentrations of BR-1 treatment. D) The soft agar colony formation assay was evaluated using a microscope (40×) after crystal violet staining. E) The soft agar colony formation assay was observed by the naked eye after crystal violet staining. F) Quantification of colony numbers of the control group and the BR-1 group. G–I) Effect of BR-1 on cell cycle progression of ML-1 (G), MOLM-13 (H), and HL-60 (I) cells with indicated concentrations of BR-1 treatment. J–L) Expression of the cell cycle regulatory molecules in the S phase in ML-1 (J), MOLM-13 (K), and HL-60 (L) with shown concentrations of BR-1 treatment.**

**Figure 3. BR-1 renders more efficacy of cytotoxicity in PRL-3 highly expressing cells. A) A comparison of the expression of apoptosis-related proteins between ML-1-GFP and ML-1-PRL-3 cells upon BR-1 treatment. B) Cell viability analyses of ML-1-GFP and ML-1-PRL-3 cells upon BR-1 treatment. C) A comparison of the variation between ML-1-GFP and ML-1-PRL-3 cells upon BR-1 treatment.**

regulation between PRL-3 and FLT3 expressions as reported [18]. Overall, our results revealed the evident synergetic effect of BR-1 and sorafenib.

**BR-1 represses AML progression *in vitro* and *in vivo*.** Eventually, to confirm the therapeutic potential of BR-1 on AML, we performed xenograft experiments in nude mice. The experimental design is shown in Figure 5A. Results showed that, in the subcutaneous inoculation of AML cell groups, BR-1 efficiently inhibited tumor formation of AML cells, as the tumor size were smaller, compared with the corn oil control group, although there was no statistical significance, which might be due to the short tumor formation time after cell inoculation (Figure 5B, p=0.5594). However, the tumor weight of the mice after treating with BR-1 was lower than that of the corn oil control group (Figure 5C, p=0.0144). In the intravenous injection groups, the treatment was not harmful to mice, as there was no significant weight loss observed, compared to the control group (Figure 5D). Results show that the parental ML-1 cells could invade into other mouse tissues and caused the typically enlarged spleens (Figure 5E, p=0.0067) and livers (Figure 5F, p=0.0117), respectively. In contrast, BR-1 significantly abrogated the cell invasiveness and maintained relatively normal spleen and liver sizes, compared to those of control. Interestingly, we also noted that mice with leukemia could develop hind limb paralysis, but BR-1 can effectively delay this.

To further validate the cytotoxicity of BR-1 on patient-derived primary AML cells, we extracted and isolated AML cells from patients in our hospital. A total of 10 patients (4 males and 6 females) with a median age of 47 years (range, 26–60 years) were enrolled in the study. According to the NCCN prognostic stratification, 2 patients were classified as favorable-risk, 6 as intermediate-risk, and 2 as poor-risk. Meanwhile, 2 patients contained FLT3-ITD mutation. The main clinical features of those 10 patients are listed in Table 1.

Upon treatment of these cells with BR-1, cells showed evident proliferation inhibition by the CCK-8 analysis (Figure 5G), while presented an obvious apoptosis-promoting effect from BR-1 (Figure 5H). The combination of BR-1 with sorafenib showed a remarkable inhibitory effect of cell proliferation with the CCK-8 assay (Figure 5I) and cytotoxic effect on induction of cell death visualized with trypan blue staining (Figure 5J) on the two patients with FLT3-ITD mutation. Taken together, our *in vitro* AML primary cells and *in vivo* xenograft results evidently manifested that BR-1 is a potential agent for AML therapy.

## Discussion

It is reported that the malfunction of PTP family members leads to various diseases including cancer, neurological disorder, and diabetes [19]. Among them, PRL-3 is known to play roles in cancer progression by involvement in invasion and metastasis of cancer cells and tumor angiogenesis [2]. Increasing evidence further suggests that PRL-3 is an important driver of cancer metastasis [20–22]. For instance, PRL-3 reinforces the PI3K/Akt activation and consequently promotes the epithelial-mesenchymal transition of cancer cells [23]. Knockdown of PRL-3 conversely reduces tumor size and inhibits metastasis as well as the invasion and growth of cancer cells [24, 25]. Our previous study also showed that PRL-3 is an independent poor prognostic factor for AML [4], thus the PRL3 inhibitor would be a promising therapeutic agent for leukemia. Rhodamine- and 1, 3-thiazolidine-2, 4-dione-based compounds have been shown to possess antibacterial, antifungal, antiviral, antimalarial, insecticidal, herbicidal, antitumor, anti-inflammatory, and cardiotonic activities [26]. Min et al. identified that one rhodamine-based compound, BR-1, can be used as a PRL-3 inhibitor, which strongly inhibits the migration and invasion of PRL-3

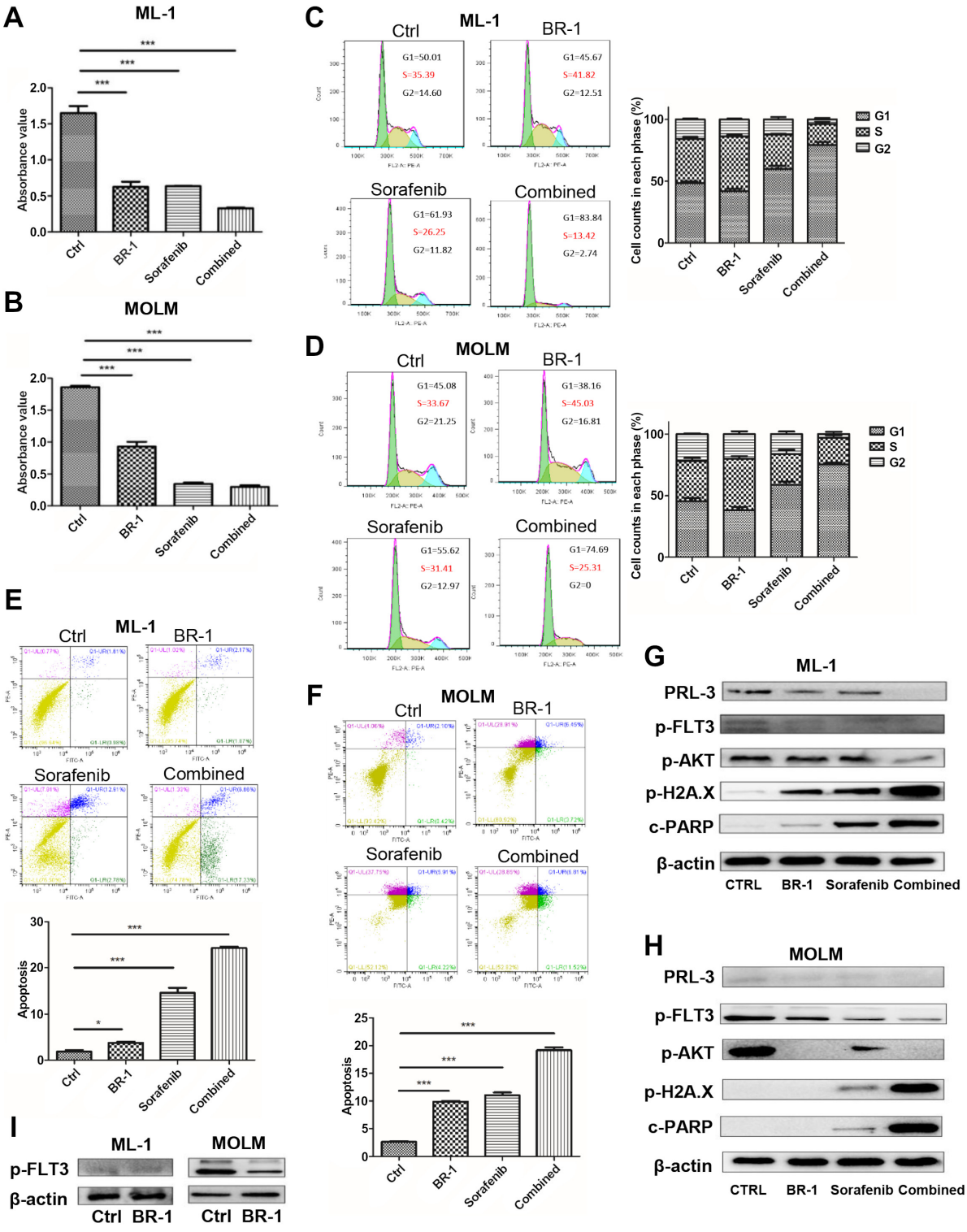


**Figure 4. Combination of BR-1 with sorafenib renders synergetic cytotoxicity on AML cells. A, B) Cell viability analyses of ML-1 (A) and MOLM-13 (B) cells with treatments of BR-1 (10 μM), sorafenib (1 μM), and their combination. C, D) Effect on cell cycle progression of ML-1 (C) and MOLM-13 (D) cells with treatments of BR-1 (10 μM), sorafenib (1 μM), and their combination. E, F) Flow cytometry analysis and cell ratio of apoptosis by VIFC/PI staining in ML-1 (E) and MOLM-13 (F) cells with treatments of BR-1 (10 μM), sorafenib (1 μM), and their combination. G, H) Expression of apoptosis-related proteins and AKT in ML-1 (G) and MOLM-13 (H) cells with treatments of BR-1 (10 μM), sorafenib (1 μM), and their combination. I) Expression of FLT3 after treating with BR-1 (10 μM) in ML-1 and MOLM-13 cells.**

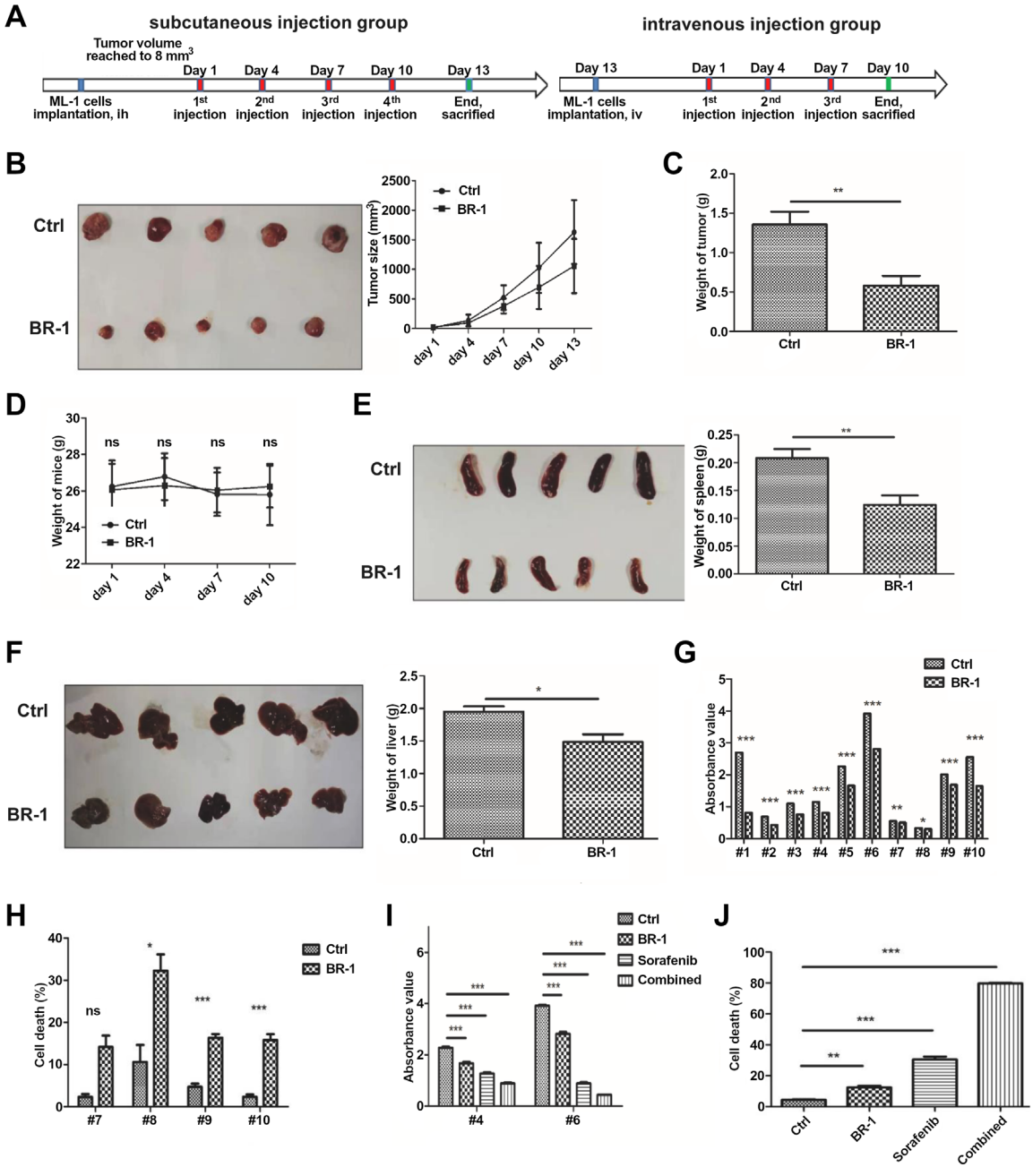


**Figure 5. BR-1 represses AML progression *in vitro* and *in vivo*. A) Schematic outline of the xenograft experiments. B) Subcutaneous tumor formation with treatments of corn oil or BR-1 (15 mg/kg). C) Comparison of the tumor weight between treatments of corn oil and BR-1 (15 mg/kg). D) Comparison of the mice weight between treatments of corn oil and BR-1 (15 mg/kg). E) Comparison of the spleen's weight between treatments of corn oil and BR-1 (15 mg/kg). F) Comparison of the liver's weight between treatments of corn oil and BR-1 (15 mg/kg). G) Cell viability analyses of primary AML cells upon BR-1 treatment (10 μM). H) Cell death ratio of primary AML cells upon BR-1 treatment (10 μM) by trypan blue staining assay. I) Cell viability analyses of primary AML cells with treatments of BR-1 (10 μM), sorafenib (1 μM), and their combination. J) Cell death ratio of primary AML cells with treatments of BR-1 (10 μM), sorafenib (1 μM), and their combination by trypan blue staining assay.**

overexpressing colon cancer cells through the dephosphorylation of known PRL-3 substrates, including ezrin and cytokeratin 8 [5]. However, there are few reports on this BR-1 on leukemia therapy.

To fulfill our suspicion, we tentatively examined the cytotoxicity of BR-1 on AML cells and our experiments clearly demonstrate that BR-1 is an effective anti-tumor agent against AML cells. Mechanistically, it is known that

PRL-3 activates both JAK/STAT5 and PI3K/AKT pathways to confer an anti-apoptotic effect [15–17]. Our results here verify the real effect of BR-1 on AML by promoting apoptosis, while effectively represses both JAK/STAT5 and PI3K/AKT pathways, validating the specific inhibition of PRL-3 and its downstream oncogenic signaling by BR-1.

In addition, we disclose that BR-1 can specifically arrest AML cell cycle in the S phase, while promotes cell apoptosis. Studies have confirmed that cdc25A was one of the intra-S phase checkpoints. The degradation of cdc25A could inhibit the activity of cyclin A/E-CDK2 and blocks the recruitment of CDC45 at the DNA replication origins, so as to inhibit the duplication of DNA [27, 28]. Our results show that BR-1 can effectively downregulate this checkpoint molecule thus indicating that BR-1 would be a useful reagent for cell cycle study, though further exploration should be performed.

Zhou et al. showed that PRL-3 is involved in FLT3-ITD signaling and can be a therapeutic target for AML [18]. PRL-3 is also a downstream effector of FLT3 signaling [29] and ectopic expression of PRL-3 conferred therapeutic resistance to FLT3 inhibitor in AML [18]. Therefore, here we validated the synergetic effect of BR-1 and sorafenib, a tyrosine kinase inhibitor that can treat the patients with FLT3 mutation, and showed that a combination of BR-1 with sorafenib would be a promising therapeutic strategy to AML patients, as our *in vitro* therapy in AML cell lines and the patient-derived primary AML cells obviously demonstrate the impressive therapy effect, which is better than either one used alone. Mechanistically, we suspect that BR-1 inhibits the PRL-3 activity, thus further sensitize AML cells to sorafenib, as blockade of PRL-3 by BR-1 can effectively reduce FLT3 phosphorylation.

Taken together, our results show that BR-1 would be a novel and efficient anti-AML agent, which should be carried out on the pre-clinical investigation. Moreover, a combination of BR-1 with sorafenib would be a promising strategy for AML therapy.

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