Abstract. Curcumin, a phytochemical from rhizomes of the plant *Curcuma longa*, has been reported to exert potential anticancer properties in various cancer types, including acute myeloid leukemia (AML). However, the underlying mechanism remains poorly understood. The present study demonstrated that curcumin had a stronger cytotoxic activity against AML cells compared with three other types of phytochemicals (epigallocatechin gallate, genistein and resveratrol). Protein phosphorylation profiling using an antibody array identified that curcumin treatment increased the phosphorylation levels of 14 proteins and decreased those of four proteins. A protein-protein interaction network was constructed using the STRING database, in which AKT was identified as a hub protein with the highest connectivity (PRAS40, 4E-BP1, P70S6K, RAF-1 and p27). Western blotting results indicated that curcumin dose-dependently suppressed the phosphorylation of AKT, PRAS40, 4E-BP1, P70S6K, RAF-1 and p27 in AML cell lines (ML-2 and OCI-AML5). It was also demonstrated that curcumin regulated the cell cycle- and apoptosis-related proteins (cyclin D1, p21, Bcl2, cleaved-caspase-3 and cleaved-PARP), leading to cell cycle arrest and apoptosis in both ML-2 and OCI-AML5 cells. These effects of curcumin were enhanced by the AKT inhibitor afuresertib but were suppressed by the AKT activator SC-79, indicating that curcumin functions via AKT. In the AML xenograft mouse model, curcumin and afuresertib synergistically suppressed the engraftment, proliferation and survival of AML cells. Collectively, the present study demonstrated that curcumin exerted anti-AML roles by inactivating AKT and these findings may aid in the treatment of AML.

Introduction

Acute myeloid leukemia (AML) is a hematological cancer type that is characterized by the clonal expansion and differentiation arrest of myeloid progenitor cells (1). The standard treatment for AML is induction chemotherapy, which is based on a backbone of cytarabine plus anthracycline treatment (2). However, the survival time for most patients who receive conventional therapy is short. Especially, the median survival of patients aged ≥65 years is only 6 months (3). The 5-year survival rate of patients with AML has not significantly increased despite significant advances in targeted therapy and immunotherapy over recent years (4). Therefore, there is an urgent requirement for the further identification of novel agents and therapeutics for AML.

Phytochemicals, which are natural compounds from plants, have been recognized as vital resources for novel drugs (5). For example, curcumin (6), epigallocatechin gallate (EGCG) (7), genistein (8) and resveratrol (9) have been reported to possess anti-AML properties. Curcumin is the main polyphenol component extracted from rhizomes of the plant *Curcuma longa*, and its therapeutic benefit has been demonstrated in various cancer types, including AML (10). However, the underlying mechanism is complex and remains poorly understood, as curcumin has multiple targets and is involved in various signaling pathways (11). Previous studies have reported that curcumin can exert its antitumor effects by acting as an inhibitor of kinases, such as protein kinase B (AKT/PKB) in head and neck cancer cells (12), JAK1 in retinoblastoma cells (13) and p38MAPK in endothelial cells (14). In the present study, protein phosphorylation profiling using an antibody array demonstrated...
that curcumin treatment increased the phosphorylation levels of 14 proteins but decreased those of four proteins. Among the 18 proteins, AKT/PKB was found to be the main target of curcumin. Moreover, it was identified that curcumin promoted cell cycle arrest and apoptosis of AML cells by inactivating AKT.

Materials and methods

Chemicals and antibodies. Curcumin, genistein, epigallocatechin gallate (EGCG), resveratrol and decitabine were purchased from Target Molecule Corp. Afuresertib (GSK2110183) and SC79 were purchased from Selleck Chemicals. Antibodies against phosphorylated (p)-P70S6 kinase (P70S6K; T421/S424; cat. no. AP0540), p-AKT1(S473; cat. no. AP0140), total AKT1 (cat. no. A11016), poly(ADP-ribose) polymerase (PARP; cat. no. A11010), ACTB (cat. no. AF0198) and caspase 3 (cat. no. A2156) were obtained from ABclonal Biotech Co., Ltd. Antibodies against p-RAF-1 (S301; cat. no. AF0047), p-proline-rich Akt substrate, 40 kDa (PRAS40; T246; cat. no. AF2387), p-p27/Kip1 (T198; cat. no. AF3325), p-eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1; T36; cat. no. AF3431), β-tubulin (cat. no. AF7011) and Ki67 (cat. no. AF0198) were obtained from Affinity Biosciences. PE-conjugated (clone HI30; cat. no. 560975; BD Bioscience) and unconjugated mouse anti-human CD45 antibody (clone HI30; cat. no. 555480; BD Bioscience) were used for flow cytometry and immunohistochemistry (IHC), respectively. A FITC TUNEL cell apoptosis detection kit was purchased from Wuhan Servicebio Technology Co., Ltd.

Cell lines and culture. AML cell lines (HL-60, ML-2, MOLM-13, OCI-AML3, OCI-AML5 and U937) were obtained from the American Type Culture collection, and were cultured according to the manufacturer's instructions. All cell lines were mycoplasma-free and were authenticated by Yubo Biological Technology Co., Ltd. using short tandem repeat analysis.

Cytotoxicity assay. Cells were cultured in a 96-well plate until the cell confluence reached ~70%, and then cells were treated with different concentrations (0, 5, 10, 20, 40 and 80 µM) of curcumin, genistein, EGCG, resveratrol or decitabine. After 48 h, cell viability was determined using a MTT assay as described previously (15). Based on the results of the MTT assay, the half maximal inhibitory concentration (IC₅₀) of each chemical was calculated.

Cell cycle and apoptosis analyses. As reviewed by Kouheipkar et al (16), in vitro examination of the efficacy of curcumin against AML cells was conducted using 10-50 µM curcumin to treat cells for 24-48 h. In the present study, cells were treated with 25 µM curcumin for 24 h. After treatment, cell cycle and apoptosis were analyzed using a PI staining kit [Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.] and an Annexin V-FITC/PI staining kit (Invitrogen; Thermo Fisher Scientific, Inc.), respectively, according to the manufacturer's instructions. After staining, cells were analyzed using a flow cytometer (CytoFlex; Beckman Coulter, Inc.).

Phosphorylation profiling. A human phosphorylation pathway profiling array (cat. no. AAH-PPP-1-4) was purchased from RayBiotech, Inc., which can detect 55 phosphorylated proteins in five signaling pathways: MAPK, AKT, JAK/STAT, NF-κB and TGF-β. ML-2 cells were cultured in a 10-cm dish until cells reached 90% confluence, and then cells were treated with or without curcumin (25 µM) for 6 h. After treatment, the cells were harvested and lysed using the cell lysis buffer with a protease inhibitor cocktail and a phosphatase inhibitor cocktail. Phosphorylation array analysis was performed according to the manufacturer's protocol. The array was sequentially incubated with the sample and horseradish peroxidase-conjugated antibodies (provided within the kit), and then scanned with ImageQuant LAS4000 Scanner (Cytiva). In total, two biological replicates were performed, and the average expression levels were compared between the treatment and control samples.

Western bloting. Cells were cultured in a 12-well plate until the cell confluence reached ~90%, and then cells were treated with the indicated chemicals. Cell lysates were prepared using the cell lysis buffer with a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Cell Signaling Technology). After quantification of the protein concentration, cell lysates containing equal amounts of total protein were denatured and separated on 10-12% SDS-PAGE. Following separation, the proteins were blotted onto PVDF membranes and blocked. After sequentially incubated with primary antibodies and appropriate secondary antibodies, the membranes were exposed to Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.) and were imaged using a gel imaging system (Tanon 4600SF; Tanon Science and Technology Co., Ltd.).

Xenograft mouse models of AML. A total of 20 male NOD/SCID mice (age, 5-6 weeks; average weight, 23 mg) were purchased from Hunan Slaccas Jingda Laboratory Animal Co. Ltd., and housed in groups of 5 per cage with water and food ad libitum, in a specific-pathogen-free room with filtered air and controlled light/dark cycle (12/12 h), temperature (24±2˚C) and relative humidity (45-65%). All mice were pretreated with an intraperitoneal injection of 20 mg/kg busulfan (APEXBio Technology LLC) 24 h before inoculation, and were then injected intravenously with 1x10⁶ ML2 cells. At 15 days after inoculation, the mice were randomly divided into four groups (5 mice per group), and then treated with vehicle, curcumin (2 mg/mouse), afuresertib (1 mg/mouse) or curcumin (2 mg/mouse) + afuresertib (1 mg/mouse) via oral gavage every other day for 16 days. Curcumin and afuresertib were dissolved with 5% DMSO + 10% PEG300 + 5% Tween-80. The humane endpoints were defined by body weight loss of 20%. All mice were euthanized by asphyxiation (CO₂ displacement rate was ~20% vol/min) 4 days after the last treatment, and the death was verified by respiratory arrest and cardiac arrest for >10 min. The experiments were performed from July 10 to August 12. The spleens were fixed in 10% formalin and processed for hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC) analysis and TUNEL assay, as described previously (17,18). Bone marrow (obtained from tibias and femurs) was crushed...
in PBS and created into single cell suspensions for flow cytometry analysis.

Statistical analysis. RStudio (https://rstudio.com) was used for statistical analysis. ANOVA and Tukey’s post hoc test were performed to evaluate the significance of difference between samples, adjust P<0.05 was considered as the level of significance.

Results

Screening for anti-AML phytochemicals. The four phytochemicals (curcumin, EGCG, genistein and resveratrol) have been reported to function as epigenetic modulating agents in cancer (19), while the DNA methyltransferase inhibitor decitabine is an FDA-approved chemical for the treatment for AML. Thus, the present study compared the cytotoxicity of these four phytochemicals with decitabine in six AML cell lines (HL-60, ML-2, MOLM-13, OCI-AML3, OCI-AML5 and U937). Cell viabilities at 48 h after exposure to various concentrations of drugs were determined using MTT assays, and IC_{50} values were calculated. Compared with decitabine, curcumin had a similar or lower IC_{50} in the AML cell lines (Table I). Moreover, curcumin had the strongest cytotoxicity against AML cells (except for OCI-AML3) among the four phytochemicals, and ML-2 cells were the most sensitive to curcumin. Therefore, curcumin was selected for further study of its function and mechanism in AML.

Phosphorylation array analysis indicates AKT is the key target of curcumin. It has been reported that curcumin can affect protein phosphorylation (12-14). Thus, the influence of curcumin treatment on protein phosphorylation was examined using a human phosphorylation pathway profiling array, which can detect 55 phosphorylated proteins in five signaling pathways: MAPK, AKT, JAK/STAT, NF-κB and TGF-β. The results demonstrated that the phosphorylation levels of 14 proteins were downregulated (fold change ≤0.83), while those of four proteins were upregulated (fold change ≥1.2) after treatment with curcumin for 6 h (Fig. 1A; Table SII). Functional annotation analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database identified that the five most enriched pathway were the ‘PD-L1/L1 pathway’, ‘AML’, ‘ErbB signaling pathway’, ‘EGFR tyrosine kinase inhibitor (TKI) resistance’ and ‘B cell receptor signaling pathway’ (Fig. 1B; Table SII).

To confirm the result of the phosphorylation array analysis, western blotting was conducted to detect the influence of curcumin (CCM) on the phosphorylation status of AKT and its interacting proteins in ML-2 cells. Curcumin suppressed the phosphorylation of AKT1, PRAS40, 4E-BP1, P70S6K, RAF-1 interacting proteins in ML-2 cells. Curcumin suppressed the phosphorylation of AKT (CCM), an AKT activator (SC-79) or afuresertib led to cell cycle arrest in the G1 phase, while treatment with the AKT inhibitor afuresertib augmented its cytotoxicity (Fig. 2A). SC-79 reversed the antitumor effects of curcumin, while afuresertib augmented its cytotoxicity on both OCI-AML5 and ML-2 cells. Flow cytometry results demonstrated that treatment with curcumin or the AKT inhibitor afuresertib led to cell cycle arrest in the G1 phase, while treatment with the AKT activator SC-79 promoted cell division (Fig. 2B and C). Moreover, AKT activation by SC-79 rescued the curcumin-induced cell cycle arrest, while AKT inhibition by afuresertib enhanced this effect (Fig. 2B and C). These results suggested that curcumin suppressed AML cell arrest in the G1 phase by inactivating AKT.

Curcumin promotes AML cell arrest in the G1 phase by inactivating AKT. To determine whether the cytotoxicity of curcumin is dependent on AKT activity, AML cells were treated with curcumin (CCM), an AKT activator (SC-79) or an AKT inhibitor (afuresertib) alone (AFU) or in combination (CCM+SC79/CCM+AFU). Curcumin suppressed the phosphorylation of AKT1, PRAS40, 4E-BP1, P70S6K, RAF-1 and p27 in a dose-dependent manner (Fig. 1D).
However, SC‑79 produced the opposite results. Thus, the effects of curcumin were enhanced by afuresertib but attenuated by SC‑79.

**Curcumin promotes AML cell apoptosis by inactivating AKT.** Annexin V and PI labeling followed by flow cytometry were used to detect apoptotic cells. The results demonstrated that both curcumin and afuresertib promoted apoptosis, while SC‑79 suppressed apoptosis. Moreover, curcumin‑induced apoptosis was stimulated by afuresertib, but diminished by SC‑79 (Fig. 3A and B).

To identify the proteins involved in curcumin‑induced apoptosis, the expression of three apoptosis‑related proteins, including Bcl‑2, caspase‑3 and PARP, were examined. The results indicated that curcumin treatment decreased the antiapoptotic Bcl‑2 protein expression but increased the cleavage of caspase‑3 (C‑Casp3) and PARP (C‑PARP) (Fig. 3C and D). Furthermore, the influence of curcumin on these three proteins could be enhanced by afuresertib, but was abrogated by SC‑79.

The above results suggested that curcumin promoted AML cell arrest and apoptosis by inactivating AKT. However, IC50 values of curcumin were very weakly correlated with the levels of phosphorylated AKT in AML cell lines (Fig. S1). Thus suggested that curcumin also exerted antitumor roles via other pathways, besides the AKT pathway.

**Curcumin and afuresertib synergistically reduce the leukemia burden in an AML xenograft mouse model.** Next, the in vivo efficacy of curcumin and afuresertib for the treatment of
AML was evaluated. NOD/SCID mice were intravenously injected with 1x10⁶ ML-2 cells. Drug treatment began 15 days after injection and continued every other day for 16 days. After treatment, peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were isolated and evaluated for human hematopoietic (hCD45) chimerism via flow cytometry (Fig. 4). Compared with the control group (VEH), the mice treated with curcumin (CCM) or afuresertib (AFU) either alone or in combination (CCM+AFU) had fewer human CD45+ cells in the bone marrow and peripheral blood. Moreover, combination drug therapy was more effective than single drug therapy in reducing the chimerism of hCD45 (Fig. 4). These results indicated that curcumin and afuresertib synergistically suppressed the engraftment of AML cells.

The mice treated with curcumin and afuresertib either alone or in combination had a smaller and lighter spleen compared with the control mice (Fig. 5A and B). Thus, it was suggested that treatment with curcumin or afuresertib could decrease splenomegaly in AML mice. IHC using an anti-hCD45 antibody demonstrated that, compared with control mice, the mice treated with curcumin or afuresertib had decreased dissemination of AML cells in the spleen, and the combinational use of curcumin and afuresertib was more effective compared with the use of a single drug (Fig. 5C).

Subsequently, Ki-67 staining and TUNEL assay were conducted to evaluate cell proliferation and apoptosis, respectively. The results (Fig. 5C) demonstrated that treatment with curcumin or afuresertib significantly increased apoptosis but decreased AKT phosphorylation and the cell proliferation rate in spleen, while treatment with both drugs had stronger effects compared with treatment with a single drug. These findings suggested that treatment with curcumin or afuresertib suppressed the engraftment, proliferation and survival of AML cells, and that combination therapy had increased efficacy compared with monotherapy.

![Figure 2. Curcumin (CCM) suppresses AML cell proliferation by inactivating AKT. OCI-AML5 and ML-2 cells were treated with CCM (25 µM), SC-79 (10 µM) or AFU (10 µM) alone or in combination for 24 h. After treatment, MTT assay, flow cytometry and western blotting were performed. (A) Cell viability results from triplicate experiments. (B) Percentage of cells in the G1 phase. Data are presented as the mean ± SD of triplicate experiments. (C) Representative images of flow cytometry. (D and E) Expression levels of cell cycle-related proteins were detected via western blotting in OCI-AML5 and ML-2 cells. *P≤0.05 and **P≤0.01 vs. DMSO; ##P≤0.01 vs. CCM. AFU, afuresertib; CCM, curcumin; AML, acute myeloid leukemia.](image-url)
Discussion

The present study compared the cytotoxicity of four phytochemicals (curcumin, EGCG, genistein and resveratrol) and identified that curcumin had the strongest anti-acute myeloid leukemia (AML) efficacy. It has been reported that curcumin has multiple targets and exerts its role via different molecular mechanism in various cancer types (11). Recently, several studies have revealed that curcumin can inhibit the phosphorylation of certain kinases, such as AKT (12), JAK1 (13) and p38MAPK (14). To identifying the targets of curcumin in AML, the present study performed a phosphorylation antibody array to detect the influence of curcumin on 55 phosphorylated proteins in five signaling pathways (MAPK, AKT, JAK/STAT, NF-κB and TGF-β). The present results suggested that curcumin decreased the phosphorylation levels of 14 proteins but increased the phosphorylation levels of four proteins. Then, a protein-protein interaction (PPI) network of these 18 proteins was conducted, in which AKT was a hub, indicating that AKT was a main target of curcumin.
Protein kinase B (AKT/PKB) is frequently overactivated in AML, and its phosphorylation is an independent poor prognostic factor of overall survival in adult de novo AML (23). AKT is a serine threonine kinase that contains three isoforms: AKT1, AKT2 and AKT3. It has been reported to serve roles in various cellular pathways, including proliferation, apoptosis and angiogenesis. Cyclin D1, which regulates the G1/S check point of the cell cycle, has been reported to be upregulated by the AKT/glycogen synthase kinase 3β axis (24). However, p21, a negative regulator of the cell cycle G1/S transition, is negatively regulated by AKT (25). AKT also promotes leukemia T cells by enhancing the transcription of Bcl-2 (26). The present results suggested that curcumin treatment increased AKT phosphorylation and p21 expression but decreased the expression levels of cyclin D1 and Bcl-2 in AML cells. Moreover, the effects of curcumin on the expression levels of p21, cyclin D1 and Bcl-2 were enhanced by the AKT inhibitor but were suppressed by the AKT activator. Therefore, it was indicated that curcumin may function via AKT. However, the sensitivities to curcumin of AML cell lines were not significantly correlated with their levels of AKT phosphorylation, suggesting that curcumin still functioned via other pathways, besides the AKT pathway.

In conclusion, the present study demonstrated that curcumin decreased the survival and proliferation of AML cells in vitro, as well as AML cell proliferation in hematopoietic tissue and

Figure 4. Curcumin (CCM) and afuresertib (AFU) synergistically inhibit engraftment of AML cells in PB and BM of mice. NOD/SCID mice were pretreated with intraperitoneal injection of 20 mg/kg busulfan 24 h before inoculation, and were then injected intravenously with 1x10^6 ML-2 cells. At 15 days after inoculation, the mice were randomly divided into four groups (5 mice per group), and were treated with VEH, CCM, AFU or CCM+AFU via oral gavage every other day for 16 days. PBMCs and BMMCs were isolated and evaluated for human hematopoietic (hCD45) chimerism via flow cytometry. (A and B) Representative images from flow cytometry. (C and D) Data are presented as the mean ± SD of three mice. **P≤0.01 vs. VEH; ***P≤0.01 vs. CCM. PB, peripheral blood; BM, bone marrow; VEH, vehicle; PBMCs, peripheral blood mononuclear cells; BMMCs, bone marrow mononuclear cells; AFU, afuresertib; CCM, curcumin; AML, acute myeloid leukemia.
ZHOU et al.: CURCUMIN SUPPRESSES AML CELL PROLIFERATION VIA AKT

Mechanistically, curcumin treatment suppressed AKT activation, leading to cell cycle arrest and apoptosis.

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Availability of data and materials

All data generated and/or analyzed during the study are available from the corresponding author on reasonable request.

Authors' contributions

CZ and XD conceived and designed the study. HZ, YN and GZ performed the experiments. HZ, YN, CZ and XD analyzed dissemination into non-hematopoietic tissues. Mechanistically, curcumin treatment suppressed AKT activation, leading to cell cycle arrest and apoptosis.

Figure 5. Curcumin (CCM) and afuresertib (AFU) synergistically inhibit engraftment, proliferation and survival of AML cells in the spleens of mice. NOD/SCID mice were pretreated with intraperitoneal injection of 20 mg/kg busulfan 24 h before inoculation and were then injected intravenously with 1x10^6 ML-2 cells. At 15 days after inoculation, the mice were randomly divided into four groups (5 mice per group), and were treated with VEH, CCM, AFU or CCM+AFU via oral gavage every other day for 16 days. (A) Gross appearance of spleen. Scale bar, 1 cm. (B) Average weight of spleen. (C) H&E, IHC and TUNEL assays of the spleen. Scale bar, 50 µm. *P≤0.05 and **P≤0.01 vs. VEH; †P≤0.05 vs. CCM. VEH, vehicle; AFU, afuresertib; CCM, curcumin; AML, acute myeloid leukemia; H&E, hematoxylin and eosin; IHC, immunohistochemistry.
and interpreted the data. HZ, CZ and XD wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Ethics Committee of Hunan Normal University and performed according to institutional animal care guidelines (no. 2018-037).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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