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The combination of Nutlin-3 and Tanshinone IIA promotes synergistic cytotoxicity in acute leukemic cells expressing wild-type p53 by co-regulating MDM2-P53 and the AKT/mTOR pathway

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ABSTRACT

P53 dysfunction has been associated with various malignant tumors, including acute leukemia. The overexpression of mouse double minute 2 (MDM2) causes the inactivation of p53 in acute leukemia. MDM2 inhibitors that activate p53 and induce apoptosis are currently being developed for potential treatment of acute leukemia. However, MDM2 inhibitors alone have limited efficacy in acute leukemia therapeutics. Combining other drugs to enhance the efficacy of MDM2 inhibitors is the thus considered as a potential treatment scheme. Here, we report that the combination of Nutlin-3 and Tanshinone IIA synergistically induces cytotoxicity, cell cycle arrest, apoptosis, and autophagic cell death, thereby imparting anti-leukemia effect in an acute leukemia cell line with wild-type p53 by effectively activating p53, inhibiting the AKT/mTOR pathway, and activating the RAF/MEK pathway. Using primary samples from acute leukemia patients, we show that the combination of Nutlin-3 plus Tanshinone IIA synergistically induces cytotoxicity by activating p53 and inhibiting the AKT/mTOR pathway. This specific combination of Nutlin-3 and Tanshinone IIA is also effective in preventing the recurrence of refractory leukemia, such as Ph+ ALL with the ABL kinase T315I mutation and AML with the FLT3-ITD mutation. Taken together, the results of this study demonstrate that the Nutlin-3 plus Tanshinone IIA combination exerts synergistic anti-leukemia effects by regulating the p53 and AKT/mTOR pathways, although further investigation is warranted. Small-molecule MDM2 antagonists plus Tanshinone IIA may thus be a promising strategy for the treatment of acute leukemia.

1. Introduction

The past few decades have witnessed advances in the treatment of acute leukemia, including achieving remission in acute leukemia patients using intensive chemotherapy and the use of allogeneic hematopoietic stem cell transplantation as a potential curative therapy for patients with high-risk acute leukemia. However, recurrence and treatment failure continue to occur in most patients. Therefore, the identification of novel therapeutic strategies for refractory/relapsed patients is imperative.

The p53 tumor suppressor is involved in cell cycle arrest, DNA repair, senescence, and apoptosis and plays a key role in maintaining genomic stability and preventing malignant transformation of cells (Levine, 1997). P53 dysfunction has been associated with various malignant tumors, including acute leukemia. Inactivated p53 mutations have been detected in approximately 50% of all human solid tumors (Levine, 1997). Unlike most solid tumors, inactivated p53 mutation only occurs in approximately 10–15% of both myeloid and lymphoid leukemia at diagnosis (Mitani et al., 2007). Although p53 mutations may be less prevalent in hematological malignancies, the loss of p53 function is characteristic of virtually all tumors, even those that retain wild-type p53 utilize alternative mechanisms to hamper its function (Lane, 1992). The overexpression of murine double minute (MDM2) is one such mechanism in acute myeloid (Bueso-Ramos et al., 1993; Faderl et al., 2003) and lymphoid leukemia (Marks et al., 1997; Zhou et al., 2000) that is often associated with chemoresistance and poor

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Table 1
Clinical characteristics of patients.

Patient number	Gender	Age	Sample type	Disease status	Molecular genetics abnormalities
#1	M	85	BM	Newly diagnosed B-ALL	bcr/abl
#2	F	40	BM	Newly diagnosed AML	CEBPA mutation
#3	M	17	PB	Newly diagnosed AML	AML-ETO, TET2, ASXL
#4	M	27	BM	Newly diagnosed B-ALL	bcr/abl
#5	M	40	BM	Newly diagnosed B-ALL	bcr/abl
#6	M	26	BM	Relapsed AML	FLT3-ITD
#7	M	38	PB	Relapsed Ph + ALL after dasatinib treatment	bcr/abl T315I mutation
#8	M	19	PB	Newly diagnosed AML	AML-ETO
#9	M	40	BM	Newly diagnosed B-ALL	bcr/abl
#10	M	34	BM	Newly diagnosed Mixture lineage M/T	Neg
#11	F	37	PB	Relapsed Ph + ALL after imatinib treatment	bcr/abl T315I mutation
#12	M	44	BM	Newly diagnosed B-ALL	bcr/abl
#13	M	52	BM	Relapsed AML	EVI and FLT3-ITD mutation
#14	M	52	PB	CML acute B lymphoblastic transformation	bcr/abl T315I mutation
#15	M	20	PB	Newly diagnosed T-ALL	Neg
#16	M	20	PB	Newly diagnosed AML	CEBPA mutation
#17	M	16	BM	Newly diagnosed T-ALL	Neg
#18	M	31	BM	Newly diagnosed B-ALL	bcr/abl E255K mutation
#19	M	62	BM	Newly diagnosed AML	FLT3-ITD and c-KIT mutation
#20	M	27	BM	Newly diagnosed B-ALL	bcr/abl
#21	M	21	BM	Newly diagnosed AML	AML-ETO
#22	M	59	BM	Relapsed AML	FLT3-ITD mutation
#23	F	16	BM	Newly diagnosed AML	Neg
#24	F	17	PB	Newly diagnosed AML	Neg
#25	M	42	BM	Newly diagnosed B-ALL	Neg
#26	M	48	BM	Newly diagnosed AML	Neg
#27	F	20	BM	Newly diagnosed T-ALL	Neg
#28	F	22	PB	Relapsed Ph + ALL after imatinib treatment	bcr/abl T315I mutation
#29	M	37	PB	Newly diagnosed T-ALL	HOX11 and HOX11L2 mutation
#30	F	51	BM	Newly diagnosed B-ALL	bcr/abl
#31	F	49	BM	Newly diagnosed AML	NMPI mutation

prognosis. MDM2 is an E3 ubiquitin ligase of p53 and controls p53 half-life via ubiquitin-dependent degradation. Nutlin-3, a selective small-molecule inhibitor of the p53-MDM2 interaction, binds MDM2 at the p53 binding pocket and releases p53 from the negative control, leading to effective stabilization of p53 and activation of the p53 pathway (Vassilev et al., 2004). It has been demonstrated that treatment with Nutlin-3 results in the upregulation of p53 protein and subsequent induction of cell cycle arrest and apoptosis in a variety of tumor cells (Vassilev, 2007). Recent studies have also demonstrated that Nutlin-3 induces cytotoxic cell death in most $TP53^{wild\text{-}type}$ primary hematologic malignancies, including acute myeloid leukemia (Kojima et al., 2005; McCormack et al., 2012) and acute lymphoblastic leukemia (Kaindl et al., 2014; Trino et al., 2016). The phytochemical compound 1,6,6-Trimethyl-6,7,8,9-tetrahydro-phenanthro [1,2-b] furan-10,11-dione (Tanshinone IIA) has been isolated from the Chinese medicinal herb *Salvia miltiorrhiza*. The molecular formula of Tanshinone IIA is $C_{19}H_{18}O_3$ and its molecular mass is 294.344420 g/mol. Tanshinone IIA has been reported to exert diverse biological properties, including anti-inflammatory, anti-oxidative, and anti-angiogenic activities (Jang et al., 2003; Tsai et al., 2011). Importantly, anti-tumor activities have been demonstrated in various tumors, including leukemia (Chiu et al., 2013; Ding et al., 2017; Munagala et al., 2015; Shan et al., 2009; Su and Chiu, 2016; Sung et al., 1999; Wang et al., 2008; Ye et al., 2017; Yu et al., 2014). Tanshinone IIA has been shown to activate the p53 pathway (Zhang et al., 2009).

In our previous study, we found that in the Philadelphia chromosome-positive acute lymphoblastic leukemia cell line SUP-B15, imatinib combined with Nutlin-3 plus Tanshinone IIA synergistically inhibits cell proliferation and induces apoptosis by activating the p53 pathway (Guo et al., 2017). However, the underlying mechanism of the Nutlin-3 plus Tanshinone IIA combination in imparting an anti-leukemic effect and whether similar results could be obtained in other leukemia cell lines or leukemia primary cells remain unclear. Thus, the present study aimed to investigate the effect and elucidate the mechanism of Nutlin-3 plus Tanshinone IIA in acute leukemia cell lines and primary leukemia cells obtained from patients.

2. Materials and methods

2.1. Antibodies and chemicals

Nutlin-3 was purchased from Cayman Chemical. Tanshinone IIA was obtained from Shanghai Shi Feng Biological Co., Ltd. A 10-mM stock solution of Nutlin-3 in DMSO and a 20-mM stock solution of Tanshinone IIA in DMSO were prepared and stored at −20 °C. The p-AKT (Ser473, #4060), p-mTOR (Ser2448, #5536), p-4EBP1(Thr37/46, #2855), p-P70S6 (Thr389, #9234), p-cRAF (Ser338, #9427), p-MEK1/2 (Ser217/221, #9154), p-ERK1/2 (Thr202/Tyr204, #4370), PTEN (#9188), AKT (#4691), mTOR (#2983), 4EBP1(#9644), P70S6 (#2708), MEK1/2 (#8727), BCL-2 (#4223), LC3 (#2775), and SQSTM1/p62 (#39749) antibodies were obtained from Cell Signaling Technologies. The p53(sc-126), MDM2 (sc-965), and p-MDM2 (Ser166, sc-293105) antibodies were purchased from Santa Cruz Biotechnology. The p21(K0081-3)antibody was purchased from Medical & Biological Laboratories Co., Ltd. The BAX (14-6997) antibody was purchased from Affymetrix eBioscience. The MCL1 (TA500990S) antibody was purchased from ORIGENE. The Annexin V-FITC apoptosis detection kit was obtained from KeyGen Biotech. Co., Ltd. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Sigma. The 3-Methyladenine (3-MA) was obtained from Cayman Chemical. IMDM, RPMI 1640 medium, penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from Hyclo Company.

2.2. Cell lines and patient specimens

Acute leukemia cell lines SUP-B15, NALM-6, HL-60, and MV4-11 were obtained from ATCC. SUP-B15 was cultured in IMDM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-

streptomycin, and 2 mM L-glutamine. NALM-6, HL-60, and MV4-11 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2 mM L-glutamine.

Bone marrow (BM) or peripheral blood (PB) samples were obtained from 17 acute lymphoid leukemia (ALL) patients, 13 acute myeloid leukemia (AML) patients, and 1 mixed-phenotype acute leukemia (MPAL, T/myeloid) patient at the Department of Hematology in the West China Hospital of Sichuan University, upon written informed consent, according to the Declaration of Helsinki. Patient specimen collection was approved by Biomedical Ethics Committee of West China Hospital of Sichuan University. Patient characteristics are shown in Table 1. The mononuclear cells were isolated by density centrifugation (Ficoll-Hypaque).

2.3. Cell viability assay and median effect analysis

Cell viability/proliferation was measured using the MTT assay. Cells were incubated for the indicated time at 37 °C in a 96-well plate with different drugs in a final volume of 100 µL. Control cells were treated with 0.1% dimethyl sulfoxide (DMSO). Thereafter, 20 µL of MTT at a concentration of 5 mg/mL in PBS was added to each well, and the cells were incubated for an additional 4 h at 37 °C. Then, 100 µL of a SDS-isobutanol-HCl solution was added, and the cells were incubated overnight at room temperature. Following incubation, the optical density (OD) was measured using Quant MQX200 Microplate Spectrophotometer (Biotek) at a wavelength of 570 nm. Appropriate controls lacking cells were included to determine background absorbance. Cellular viability was calculated as the percentage of viable cells relative to the control using the following formula: $OD_{Experimentsamples} - OD_{Blank}/(OD_{Control} - OD_{Blank}) \times 100\%$. All of the experiments were conducted in triplicate.

To characterize drug interactions, cell viability was analyzed using the median effect method of Chou and Talalay. The combination index (CI) values at fixed Nutlin-3:Tanshinone IIA concentration ratios were calculated using the commercially available software CalcuSyn 2.1 (Biosoft, Cambridge, UK). CI values < 1.0 indicate synergism, CI values = 1.0 depict an additive effect, and CI values > 1.0 show antagonism.

2.4. Apoptosis analysis by flow cytometry

Induction of apoptosis was quantified by Annexin V-FITC/propidium iodide (PI) staining followed by analysis using a Cytomics FC500 flow cytometer equipped with CXP software as previously described (Guo et al., 2015). For each analysis, 10,000 events were recorded.

2.5. Cell cycle analysis by flow cytometry

Cell cycle progression following drug treatment was measured by flow cytometry using PI DNA staining. The cells were plated at a density of 1×10^6 cells/6-cm dish in complete medium for 24 h. Following treatment, the cells were collected and fixed in ice-cold 70% ethanol overnight at -20° C. The cells were centrifuged and washed with PBS, then the cell pellets were treated with 1 µg/mL PI solution containing 100 µg/mL RNase at 37° C for 30 min. Subsequently, the samples were analyzed using a Cytomics FC500 flow cytometer. A minimum of 10,000 cells from each sample were analyzed in terms of DNA content, and the percentage of cells in each cell cycle phase was quantified.

2.6. Western blot analysis

For Western blot analysis, the cells were lysed, and equal amounts of protein from each sample were separated in dodecyl sulfate, sodium salt (SDS)-polyacrylamide gels and then blotted onto nitrocellulose filters as previously described (Guo et al., 2012). The membrane was incubated with a primary antibody (1:1000 vol/vol diluted in Tris Buffered saline + 0.1% Tween + 5% bovine serum albumin) overnight at 4° C, washed, and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000 vol/vol diluted in TBST + 5% bovine serum albumin) for 1 h at room temperature. Protein signals were detected using enhanced chemiluminescence (ECL) detection systems and film imaging (Bio-Rad Laboratories Inc.), following the manufacturer's instructions.

2.7. Quantitative real-time PCR analysis

Quantitative PCR was performed on a CFX96™ Real-Time PCR system using the manufacturer's protocol. RNA was prepared using RNAzol®RT. For mRNA quantification, reverse transcription was performed using the Thermo Scientific RevertAid First STRAND cDNA Synthesis kit. Expression of genes was assessed using a KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (KAPABIOSYSTEMS) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control. The following primers were used:

P53: 5′-GTGCGTGTTTGTGCCTGTCCT-3′ (Forward);
P53: 5′-CAGTGCTCGCTTAGTGCTCCCT-3′ (Reverse);
MDM2: 5′-GGCAGGGGAGAGTGATACAGA-3′ (Forward);
MDM2: 5′-GAAGCCAATTCTCACGAAGGG-3′ (Reverse);
BAX: 5′-CCTTTTGCTTCAGGGTTTCA-3′ (Forward);
BAX: 5′-TCCATGTTACTGTCCAGTTCGT-3′ (Reverse);
P21: 5′-TGTCCGTCAGAACCCATGC-3′ (Forward); and
P21: 5′-AAAGTCGAAGTTCCATCGCTC-3′ (Reverse).

2.8. Immunofluorescence of the p53 protein

The p53 protein was localized using immunofluorescence. Cells were grown in six-well plates and treated with the indicated drugs for 24 h. After incubation, the cells were collected in Eppendorf tubes. Then, the cells were washed twice with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.25% Triton X-100 diluted in PBS for 10 min. The cells were blocked with PBST containing 1% bovine serum albumin for 30 min at room temperature, followed by incubation overnight at 4 °C with mouse monoclonal anti-p53 antibody (1:100 vol/vol diluted in PBST + 1% bovine serum albumin). After washing, the cells were incubated with Alexa Fluor® donkey anti-mouse IgG secondary antibody (1:1000 diluted in PBST + 1% bovine serum albumin) (Life Technologies) at room temperature for 1 h. After washing, the nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) in glycerin jelly, and then the cells were dropped onto glass slide and coverslipped. Images were obtained using a Zeiss AxioImager Z2 microscope with ZEN lite software.

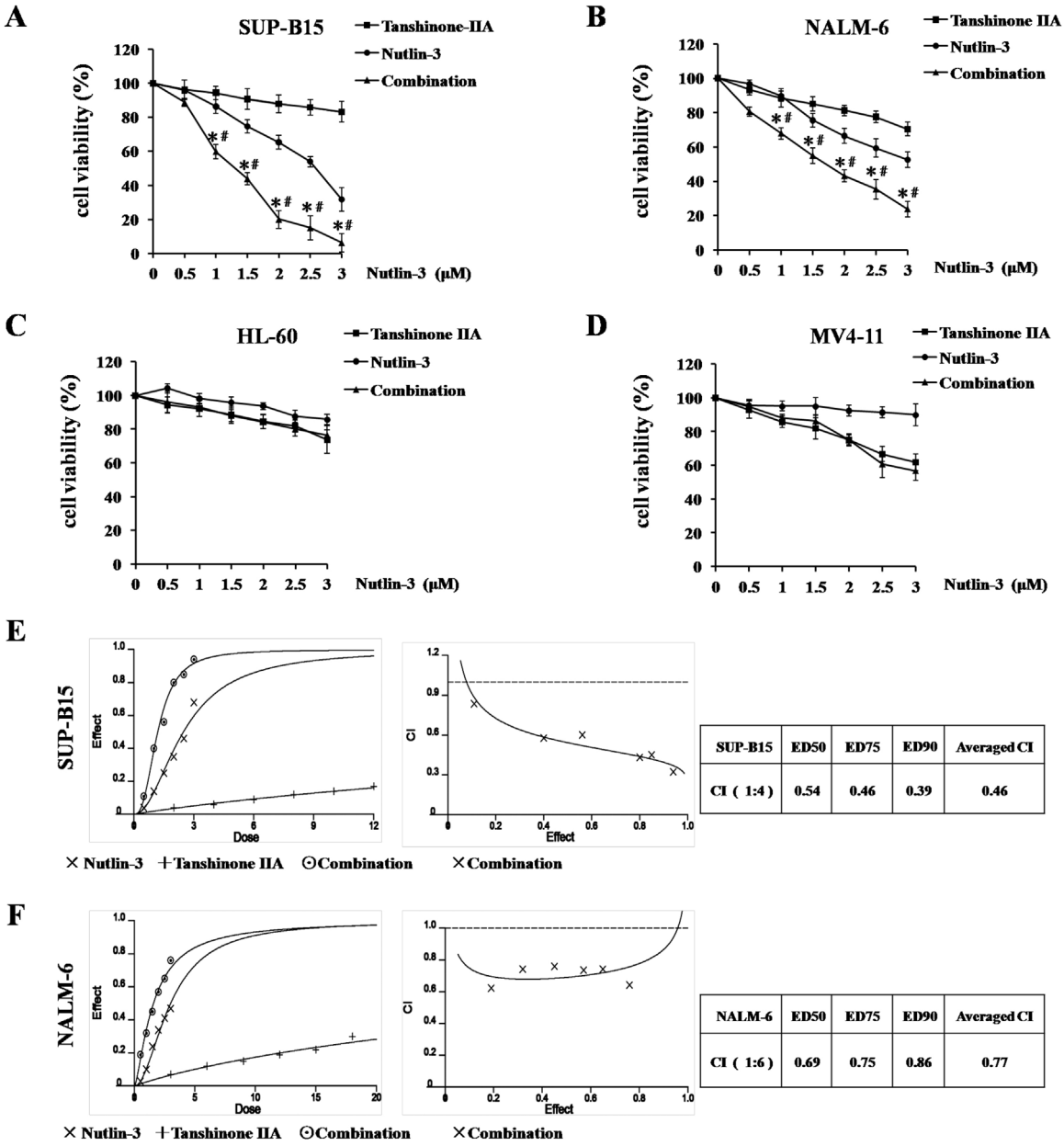
2.9. Statistical analysis

All of the data were expressed as the mean ± standard deviation unless otherwise indicated. Statistical analysis was performed by one-way ANOVA with Bonferroni's corrected *t* test for post hoc pair-wise comparisons.

3. Results

3.1. Nutlin-3 plus Tanshinone IIA induces synergistic cytotoxicity in leukemic cells expressing wild-type p53

Cell viability was analyzed using the MTT assay. Nutlin-3 alone had dose-dependent cytotoxicity on both SUP-B15 ($p53^{wild\text{-}type}$) and NALM-6 ($p53^{wild\text{-}type}$) cells, with the IC_{50} (50% inhibitory concentration) value at 72 h treatment was 1.96 ± 0.32 µM and 2.19 ± 0.45 µM, respectively. Tanshinone IIA alone was also cytotoxic to SUP-B15 and NALM-6 cells, with IC_{50} value at 72 h treatment was 8.80 ± 1.34 µM (about 4



SUP-B15	ED50	ED75	ED90	Averaged CI
CI (1:4)	0.54	0.46	0.39	0.46

NALM-6	ED50	ED75	ED90	Averaged CI
CI (1:6)	0.69	0.75	0.86	0.77

Fig. 1. Nutlin-3 plus Tanshinone IIA synergistically imparts cytotoxicity effects in acute leukemia cell lines expressing wild-type p53. SUP-B15, NALM-6, HL-60, and MV4-11 cells were treated with serial concentrations of nutlin-3 (0.5, 1, 1.5, 2, 2.5, and 3 μM), Tanshinone IIA (2, 4, 6, 8, 10, and 12 μM for SUP-B15 and 3, 6, 9, 12, 15, 18 μM for NALM-6, HL-60, and MV4-11) alone or in combination at a constant nutlin-3:Tanshinone IIA ratio 1:4 for SUP-B15 and 1:6 for NALM-6, HL-60 and MV4-11cells for 48 h using 0.1% DMSO treatment as vehicle control. (A–D) Cell viability was determined using the MTT assay, and the data were expressed as the means ± SD of three independent experiments. The significance of the differences was determined with a one-way ANOVA with Bonferroni post-test (*$p < 0.05$ vs. Nutlin-3 alone; #$p < 0.05$ *vs.* Tanshinone IIA alone). (E and F) The dose–effect plots to determine drug efficacy in the SUP-B15 and NALM-6 cells are shown; the decrease in cell viability, labeled as "effect" on the *Y*-axis, was determined in assays using triplicates, and the combination index for cytotoxic effects was calculated and plotted on a graph using Calcusyn 2.1 software. The dashed line indicates a combination index of 1.

times of the value of Nutlin-3 treatment) and 12.05 ± 2.72 μM (about 6 times of the value of Nutlin-3 treatment), respectively. The cytotoxicity of Nutlin-3 against HL-60 ($p53^{deleted}$) and MV4-11 ($p53^{mutated}$) was weak, and Nutlin-3 greater than 20 μM cannot inhibit the proliferation of these cells. Tanshinone IIA has a weak cytotoxic effect on HL-60 and MV4-11 cells, with IC_{50} values greater than 50 μM. The combined treatment was performed at fixed ratios relative to their respective IC_{50} concentrations. Since Nutlin-3 had no dose-effect relationship in HL-60 and MV4-11 cells, we selected the drug concentration and ratio with reference to NALM-6 cells. The combined treatment can significantly accelerate the production of cytotoxic effects, so we reduced the drug treatment time to 48 hours. The SUP-B15 ($p53^{wild\text{-}type}$), NALM-6 ($p53^{wild\text{-}type}$), HL-60 ($p53^{deleted}$), and MV4-11 ($p53^{mutated}$) cells were exposed to serial concentrations of Nutlin-3 (0.5–3 μM), Tanshinone IIA (2–18 μM), used either alone or in combination at a constant Nutlin-3:Tanshinone IIA ratio (1:4 for SUP-B15 and 1:6 for the other cells). Cell viability was analyzed using the MTT assay at 48 h after treatment. Fig. 1A and B shows that the SUP-B15 and NALM-6 leukemia cells with wild-type p53 exhibiting moderate cytotoxic effects after treatment with 3 μM Nutlin-3. The cell viability of the SUP-B15 and NALM-6 cells was 31.79 ± 6.92% and 52.66 ± 4.52%, respectively. Relatively mild cytotoxic effects were observed in the SUP-B15 and NALM-6 cells after treatment with 12 μM and 18 μM Tanshinone IIA, with cell viabilities of 83.24 ± 1.14% and

70.42 ± 3.98%, respectively. Tanshinone IIA significantly enhanced the Nutlin-3-induced cytotoxic effects on SUP-B15 and NALM-6, as indicated by a decrease in cell viability to 6.25 ± 5.38% and 23.76 ± 4.62%, respectively. However, in the $p53^{deleted}$ HL-60 (Fig. 1C) and $p53^{mutated}$ MV4-11 cells (Fig. 1D), Nutlin-3 had weaker cytotoxic effects, whereas Tanshinone IIA still imparted some cytotoxic effect, although the combination of Nutlin-3 and Tanshinone IIA did not enhance these cytotoxic effects. To evaluate whether the combined cytotoxic effect of Nutlin-3 plus Tanshinone IIA was synergistic, the data were analyzed using the method of Chou and Talalay, and combination index (CI) values were calculated using the software Calcusyn 2.1. Fig. 1E and F shows that Nutlin-3 plus Tanshinone IIA synergistically promotes cytotoxicity in the SUP-B15 and NALM-6 cells, with CI < 1 (CI values at ED50, ED75, and ED90 were 054, 0.46, and 0.39 in SUP-B15 cells and 0.69, 0.75, and 0.86 in NALM-6 cells, respectively). These results demonstrate that the combination of Nutlin-3 plus Tanshinone IIA exerts synergistic cytotoxicity only in the wild-type p53 cells, and thus the subsequent experiments were conducted using the p53 wild-type cell lines.

3.2. Nutlin-3 plus Tanshinone IIA synergistically induces cell cycle arrest and apoptosis in leukemic cells expressing wild-type p53

Based on the cell viability data, we next investigated the effects of Nutlin-3 plus Tanshinone IIA on cell cycle progression and apoptosis modulation in SUP-B15 and NALM-6 cells. The SUP-B15 and NALM-6 cells were treated with Nutlin-3 and Tanshinone IIA alone or in combination for 24 h, and then cell cycle was analyzed by flow cytometry using PI DNA staining, and apoptosis was analyzed by flow cytometry using Annexin V-FITC/PI staining. Fig. 2A and B shows that in both SUP-B15 and NALM-6 cells, Nutlin-3 alone induced a G_2/M phase arrest and Tanshinone IIA alone induced a S phase arrest, whereas the combination of Nutlin-3 plus Tanshinone IIA simultaneously induced the arrest of the G2/M and S phases. Fig. 3A and B shows that the Nutlin-3-induced apoptotic rates of the SUP-B15 and NAML-6 cells were 19.96 ± 2.45% and 18.36 ± 2.87% and the Tanshinone IIA-induced apoptosis rates of the SUP-B15 and NAML-6 cells were 11.56 ± 1.76% and 14.26 ± 1.99%, respectively, whereas the combination of Nutlin-3 plus Tanshinone IIA caused a significant increase in apoptotic rate of up to 45.04 ± 5.74% and 36.56 ± 2.01%, respectively.

3.3. Nutlin-3 plus Tanshinone IIA induces autophagic cell death in leukemic cells expressing wild-type p53

The results of the cell viability assay and apoptosis analysis demonstrated that the cytotoxic effect induced by Nutlin-3 plus Tanshinone IIA was greater than their effect on apoptosis induction. The decrease in cell viability induced by exposure to Nutlin-3 plus Tanshinone IIA for 24 h was about 60% in the SUP-B15 cells and 49% in the NALM-6 cells, whereas the apoptotic rate was about 45% and 36%, respectively. These results indicate that the cytotoxic effects induced by the combination of Nutlin-3 plus Tanshinone IIA were not entirely caused by apoptosis. It has been reported that Nutlin-3 or Tanshinone IIA can also exert antitumor effects by inducing autophagic cell death. Thus, we hypothesized that the combination of Nutlin-3 plus Tanshinone IIA also exerts synergistic cytotoxicity by inducing autophagic cell death in leukemic cells expressing wild-type p53. To confirm our hypothesis, we first examined the effect of autophagy inhibitor 3-methyladenine (3-MA) on the cytotoxic effects induced by the Nutlin-3 plus Tanshinone IIA combination. Fig. 4A and B shows that pretreatment with 5 mM 3-MA reduced the cytotoxicity induced by Nutlin-3 plus Tanshinone IIA in SUP-B15 and NALM-6 cells, and cell viability increased from 40.37 ± 4.72% and 51.35 ± 5.01% to 65.94 ± 6.13% and 69.12 ± 4.89%, respectively, indicating that autophagy is indeed involved in Nutlin-3 plus Tanshinone IIA-mediated cytotoxicity. The conversion of LC3-I to LC3-II and the reduction in the autophagic substrate SQSTM1/p62 are specific hallmarks of autophagy, and so to further determine whether Nutlin-3 plus Tanshinone IIA induces autophagy, we next detected the expression of LC3-I, LC3-II, and SQSTM1/p62 by Western blotting and then calculated the ratio of LC3-II/LC3-I by densitometric analyses. Fig. 4C and D shows that in both SUP-B15 and NALM-6 cells, Nutlin-3 alone had a less severe effect on the LC3-II/LC3-I ratio and SQSTM1/p62 level, the LC3-II/LC3-I ratio of the SUP-B15 and NALM-6 cells after Nutlin-3 treatment was 1.8 and 0.8, whereas this was 2.1 and 0.7 in the controls, respectively. Tanshinone IIA alone induced a slight increase in LC3-II/LC3-I ratio and a decrease in SQSTM1/p62 levels, and the LC3-II/LC3-I ratio of the SUP-B15 and NALM-6 cells after Tanshinone IIA treatment was 4.6 and 1.2, and the SQSTM1/p62 level decreased from 1 to 0.8 and 0.7, respectively. The extent of increase in the LC3-II/LC3-I ratio and reduction in SQSTM1/p62 level induced by Nutlin-3 plus Tanshinone IIA was greater than that of Tanshinone IIA alone, the LC3-II/LC3-I ratio of the SUP-B15 and NALM-6 cells after Nutlin-3 plus Tanshinone IIA treatment increased to 9.8 and 2.4, respectively, and SQSTM1/p62 levels in the two cell lines decreased to 0.3. The increase of the LC3-II/LC3-I ratio and the reduction in the SQSTM1/p62 levels was diminished by 3-MA. These results suggest that autophagy is indeed involved in the cytotoxicity mediated by the Nutlin-3 plus Tanshinone IIA combination.

3.4. Nutlin-3 plus Tanshinone IIA synergistically activates the p53 pathway in leukemic cells expressing wild-type p53

Nutlin-3 is a small-molecule inhibitor of p53-MDM2 interactions, and according to the literature, Tanshinone IIA also activates the p53 pathway. Therefore, to elucidate the synergistic anti-leukemia effect of Nutlin-3 and Tanshinone IIA, we first investigated their effects on the p53 pathway by examining the mRNA and protein levels of *p53* and its main transcription targets, namely, *MDM2*, *BAX*, and *p21*, the location of p53 in the cells, as well as their downstream effects on apoptosis.

We detected the effects of drugs on the mRNA expression of *p53* and its target genes using quantitative real-time PCR analysis. Fig. 5A shows that in the SUP-B15 cells, Nutlin-3 alone did not upregulate the expression of p53, and instead slightly inhibited its expression (0.95 *vs*. control), whereas Nutlin-3 upregulated the *p53* target genes of *MDM2*, *BAX*, and *p21* (2.33, 2.08, 2.98 *vs.* the controls, respectively). Unlike Nutlin-3, Tanshinone IIA induced the upregulation of the p53 gene and its target gene *BAX*, as well as *p21* expression (1.65, 1.20, 2.60 *vs*. control, respectively), whereas Tanshinone IIA did not significantly upregulate *MDM2* expression (1.11 *vs*. control). Using Nutlin-3 combined with Tanshinone IIA, *BAX* and *p21* upregulation was observed (2.71 and 5.39 *vs.* control, respectively), and their effects on *p53* and *MDM2* slightly decreased compared to that using single drugs. In the NALM-6 cells (Fig. 5B), Nutlin-3 slightly upregulated p53 expression (1.08 *vs*. control) and moderately upregulated *MDM2*, *BAX*, and *p21* gene expression (2.39, 1.73, and 1.74 *vs*. control, respectively). Tanshinone IIA upregulated *p53* and *p21* moderately (1.40 and 1.62 *vs*. control, respectively), slightly upregulated *BAX* (1.15 *vs.* control), and had less effect on *MDM2* expression (0.99 *vs.* control). Nutlin-3 plus Tanshinone IIA significantly upregulated *BAX* and *p21* (5.18 and 4.34 *vs*. control, respectively), moderately upregulated p53 (1.76 *vs*. control), and inhibited the Nutlin-3-mediated upregulation of the *MDM2* gene (1.19 *vs*. 2.39).

We detected the effects of drugs on the protein expression of p53 and its target genes and their downstream effects on apoptosis using Western blot analysis. Fig. 5C (left panel) shows that in the SUP-B15 cells, Nutlin-3 significantly upregulated p53, MDM2, p-MDM2, BAX, and p21, indicating the activation of p53 pathway, which in turn leads to a significant upregulation of the apoptotic effector cleaved PARP. Tanshinone IIA moderately upregulated p53 and p21 and slightly upregulated BAX, leading to a moderate increase in cleaved PARP levels. Unlike Nutlin-3, Tanshinone IIA did not upregulate MDM2 and p-MDM2, and instead slightly inhibited their expression. Nutlin-3 plus

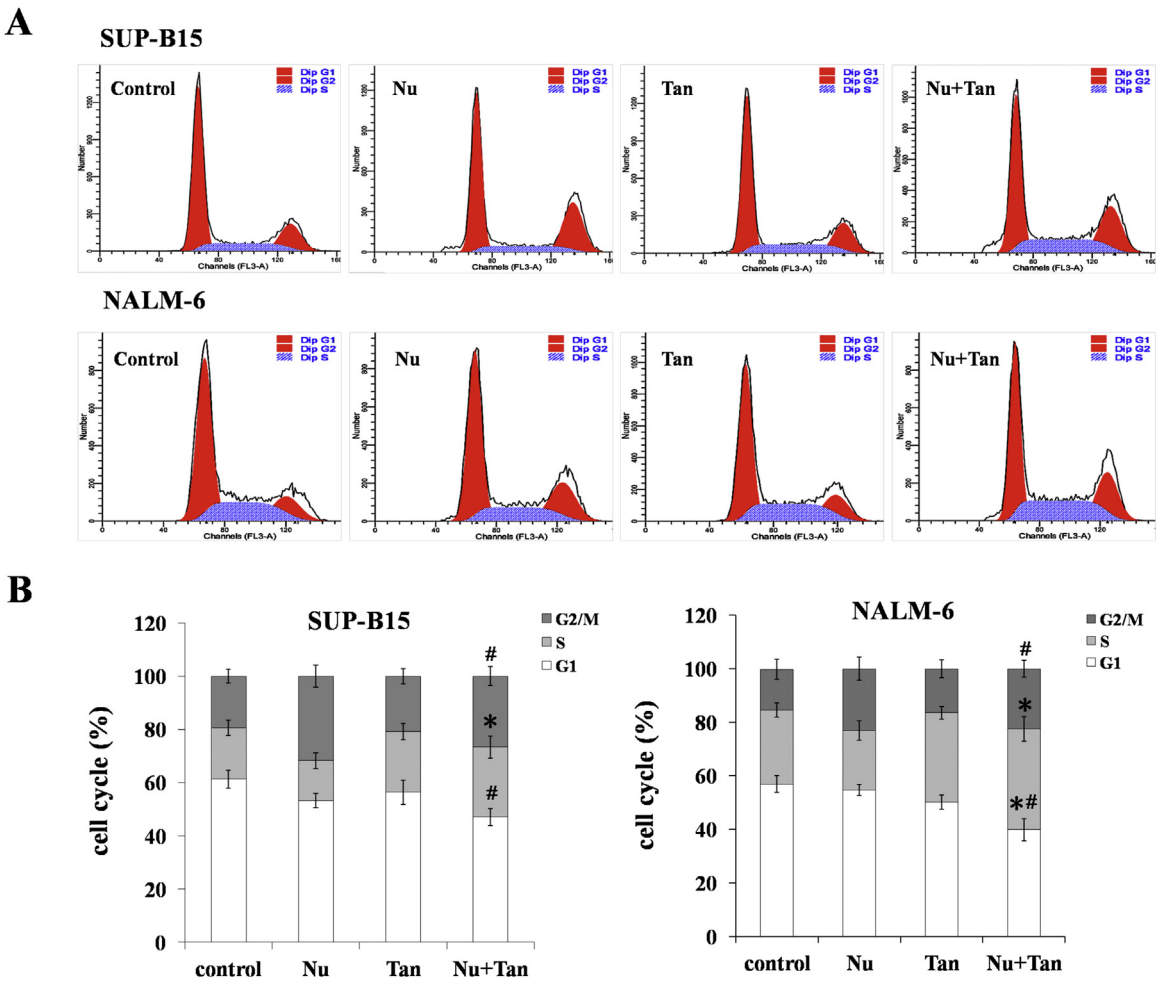


Fig. 2. The combination of Nutlin-3 plus Tanshinone IIA induces cell cycle arrest in SUP-B15 and NALM-6 cells. SUP-B15 cells were treated with 0.1% DMSO (control), 2.5 μM nutlin-3 (Nu), 10 μM Tanshinone-IIA (Tan) alone, or in combination for 24 h. NALM-6 cells were treated with 0.1% DMSO (control), 2.5 μM nutlin-3 (Nu), 15 μM Tanshinone-IIA (Tan) alone or in combination for 24 h. At the end of the treatment period, the cells were collected and fixed, and cell cycle was analyzed by flow cytometry using PI staining. (A) One representative flow cytometry result of cell cycle is presented. (B) Cell cycle distribution percentage is shown. The data were the means of three independent experiments. The significance of the differences was determined with a one-way ANOVA with Bonferroni post-test (*$p < 0.05$ vs. Nutlin-3 alone; #$p < 0.05$ *vs.* Tanshinone IIA alone).

Tanshinone IIA retained the Nutlin-3-induced upregulation effects on P53, BAX, and p21, but weakened those on MDM2 and p-MDM2, and enhanced induction of apoptosis, as evidenced by a significant increase in the levels of cleaved PARP. In the NALM-6 cells (Fig. 5C, right panel), Nutlin-3 significantly upregulated p53, MDM2, and p-MDM2 and moderately upregulated BAX and p21, leading to a moderate increase in cleaved PARP levels. Tanshinone IIA induced weaker upregulation of p53, BAX, p21, and cleaved PARP than Nutlin-3 and slightly inhibited MDM2 and p-MDM2 expression. When Nutlin-3 was combined with Tanshinone IIA, Nutlin-3-induced upregulation of p53 was retained, the upregulatory effects on BAX and p21 were markedly enhanced, and the Nutlin-3-induced upregulation effects on MDM2 and p-MDM2 were weakened, and all these significantly enhanced the apoptosis induction effect, as evidenced by a significant increase in cleaved PARP levels.

The activity of p53 is not only dependent on the concentration of the p53 protein, but more importantly, depends on its localization in the cell. The p53 can only be transcribed in the nucleus. Thus, we next examined the effects of drugs on p53 localization using immunofluorescence. Fig. 5D shows that in both SUP-B15 and NALM-6 cells, although Nutlin-3 alone upregulated p53 protein expression, the p53 protein could not be detected in the nucleus. Tanshinone IIA had a weak effect on upregulating the p53 protein; however, Tanshinone IIA combined with Nutlin-3 not only more effectively upregulated p53 expression than Nutlin-3 alone, but resulted in the detection of p53 in the nucleus, thus enabling p53 function.

3.5. Nutlin-3 plus Tanshinone IIA synergistically regulates the AKT/mTOR and RAF/MEK signaling pathways.

In addition to the dysregulation of the p53 pathway, tumor cells often express constitutively active growth-signaling pathways such as the AKT/mTOR and RAF/MEK/ERK pathways. These signaling pathways play vital roles in tumorigenesis, proliferation, apoptosis, and autophagy. Therefore, we examined the effect of the Nutlin-3 plus Tanshinone IIA combination on the AKT/mTOR and RAF/MEK pathways. Fig. 6A shows that in the SUP-B15 and NALM-6 cells, Nutlin-3 alone had few effects on PETN and few inhibitory effects on the activation of the AKT/mTOR pathway, Tanshinone IIA upregulated PTEN and inhibited the AKT/mTOR pathway, the Nutlin-3 plus Tanshinone IIA combination had stronger inhibitory effects on AKT/mTOR activation than Tanshinone IIA alone by effectively upregulating PTEN. Fig. 6B shows that in both SUP-B15 and NALM-6 cells, Nutlin-3 had no effect on the activation of RAF/MEK, Tanshinone IIA activated the RAF/MEK pathway, and the Nutlin-3 plus Tanshinone IIA combination retained the activation effect of Tanshinone IIA on the RAF/MEK pathway.

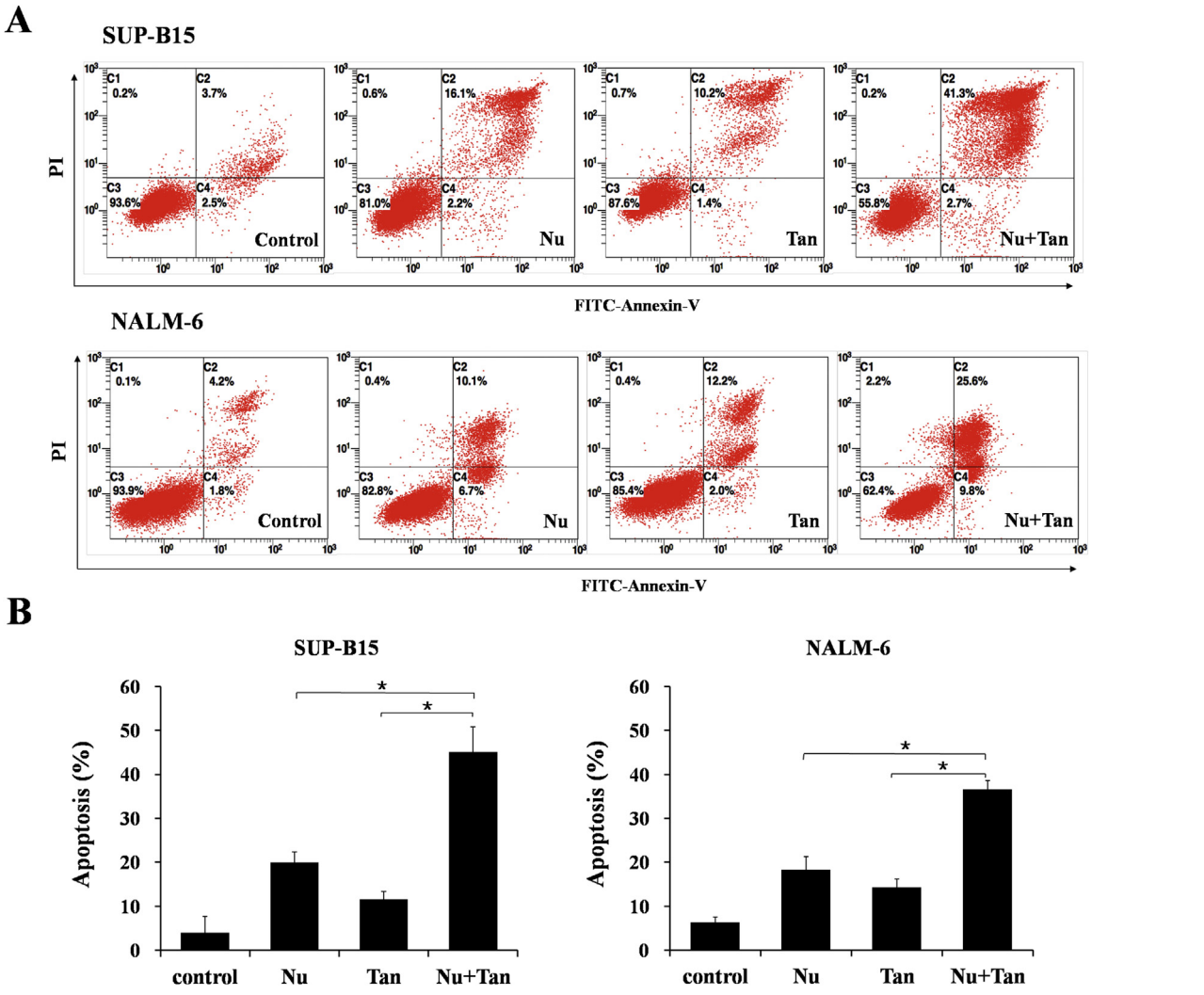


Fig. 3. The combination of Nutlin-3 plus Tanshinone IIA exerts stronger apoptotic effects on the SUP-B15 and NALM-6 cells. The SUP-B15 cells were treated with 0.1% DMSO (control), 2.5 μM nutlin-3 (Nu), 10 μM Tanshinone-IIA (Tan) alone, or in combination for 24 h. NALM-6 cells were treated with 0.1% DMSO (control), 2.5 μM nutlin-3 (Nu), 15 μM Tanshinone-IIA (Tan) alone, or in combination for 24 h. At the end of the treatment period, the cells were collected, and the percentage of apoptotic cells was examined by flow cytometry using the Annexin V-FITC/PI apoptosis detection kit. (A) One representative flow cytometry result of apoptosis is presented. (B) The percentage of apoptosis is shown. The data are presented as the mean ± SD of three independent experiments. The significance of the differences was determined using one-way ANOVA with Bonferroni post-test (* represents significant difference, $p < 0.05$).

3.6. Nutlin-3 plus Tanshinone IIA synergistically regulates anti-apoptosis protein and cell cycle regulatory protein expression

Our previous flow cytometry data has demonstrated that the Nutlin-3 plus Tanshinone IIA combination synergistically induces apoptosis and cell cycle arrest. Next, we assessed its effect on apoptosis and cell cycle regulatory proteins using Western blotting. We had examined the effect of this combination on the pro-apoptotic protein BAX in the previous experiment. Here, we examined their effects on the expression of anti-apoptotic proteins MCL1 and BCL-2. Fig. 6C shows that in SUP-B15 cells, Nutlin-3 and Tanshinone IIA alone did not impart any significant inhibitory effects on the anti-apoptosis protein MCL1 and BCL-2. However, the Nutlin-3 plus Tanshinone IIA combination down-regulated their expression. In NALM-6 cells, Nutlin-3 also had no inhibitory effect on MCL1 and BCL-2 expression, Tanshinone IIA had some inhibitory effect on their expression, and the Nutlin-3 plus Tanshinone IIA combination enhanced the inhibitory effect on their expression. In terms of the cell cycle regulatory proteins cyclin D1 and CDK4, in both SUP-B15 and NALM-6 cells, the effect of Nutlin-3 alone was weak, Tanshinone IIA imparted some inhibitory effect on their expression, and the Nutlin-3 plus Tanshinone IIA combination enhanced the inhibitory effect on cyclin D1 and CDK4 expression.

3.7. Nutlin-3 plus Tanshinone IIA exhibits cytotoxicity effects in primary leukemia cells by activating the p53 pathway and inhibiting the AKT/mTOR pathway

The characteristics of acute leukemia patients cannot be fully represented by cell lines. Therefore, we investigated the cytotoxic effects and mechanisms of the Nutlin-3 plus Tanshinone IIA combination on primary leukemia cells. The characteristics of 31 acute leukemia patients are shown in Table 1, which include 17 cases of acute lymphoblastic leukemia, 13 cases of acute myeloid leukemia, and 1 case of mixed-phenotype acute leukemia. Due to the limited number of cells that were collected from patients, we did not investigate the concentration gradient effect of drugs in primary leukemia cells. However, using cell lines, we selected 2.5 μM and 15 μM as the working concentrations of Nutlin-3 and Tanshinone IIA. First, we detected the effects of different drugs on cell viability using the MTT method. Fig. 7A shows that the Nutlin-3 plus Tanshinone IIA combination effectively induced cytotoxic effects in primary leukemia cells, with a decrease in cell viability of > 25% in 13 of the 17 (76.5%) acute lymphoblastic

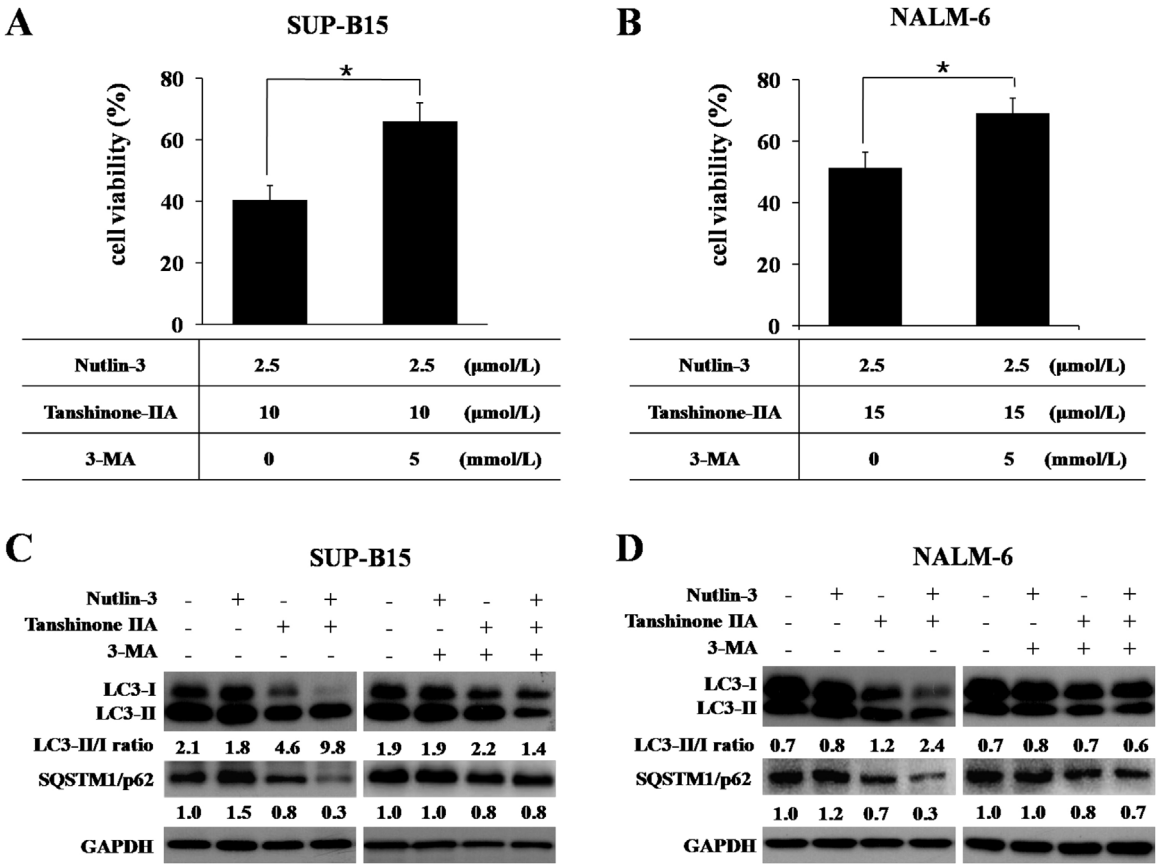


Fig. 4. The combination of Nutlin-3 plus Tanshinone IIA induces autophagic cell death in SUP-B15 and NALM-6 cells. SUP-B15 (A) and NALM-6 (B) cells were incubated with Nutlin-3 plus Tanshinone IIA for 24 h or pretreated with 5 mM 3-MA for 1 h, then incubated with Nutlin-3 plus Tanshinone IIA for 24 h. At the end of incubation, cell viability was determined using the MTT method. The data are expressed as the mean ± SD of three independent experiments. The significance of the differences was determined using one-way ANOVA with Bonferroni post-test (* represents significant difference, $p < 0.05$). SUP-B15 (C) and NALM-6 (D) cells were treated with Nutlin-3, Tanshinone IIA alone or in combination for 24 h, or SUP-B15 and NALM-6 cells were pretreated with 5 mM 3-MA for 1 h, then treated with Nutlin-3, Tanshinone IIA alone or in combination for 24 h. At the end of the incubation, total protein samples were extracted, LC3I, LC3II, and SQSTM1/p62 levels were examined by Western blotting with the indicated antibodies. One representative Western blot of two independent experiments is presented. The numbers labeled below each blot represent relative intensity of the bands. GAPDH was used as reference.

leukemia patients and all of the acute myeloid leukemia patients, and a decrease in cell viability by > 50% in 8 of the 17 (47.1%) cases of acute lymphoblastic leukemia and 10 of the 13 (76.9%) acute myeloid leukemia cases. Furthermore, the Nutlin-3 plus Tanshinone IIA combination induced higher cytotoxic effects than any single drug in 9 of the 17 (52.9%) acute lymphoblastic leukemia and 9 of the 13 (69.2%) acute myeloid leukemia cases, and the decrease in cell viability was about 20% higher than that using a single drug. These findings indicate that the Nutlin-3 plus Tanshinone IIA combination imparts a cytotoxic effect in both acute lymphoblastic leukemia and acute myeloid leukemia, and its cytotoxic effect is more prominent in acute myeloid leukemia. Treatment of relapsed Ph+ ALL patients carrying the ABL kinase T315I mutation and relapsed AML patients harboring the FLT3-ITD mutation are generally very difficult, and most of these patients die after disease progression without allogeneic hematopoietic stem cell transplantation. The Nutlin-3 plus Tanshinone IIA combination effectively imparted cytotoxic effects in three relapsed Ph+ ALL patients with T315I (#7, #11, and #28) and three relapsed AML with FLT3-ITD mutation (#6, #13, and #22), with > 50% decrease in cell viability, which is higher than any single agent.

Next, to explore the mechanism of drug actions in primary leukemia cells, we detected the effects of different drugs on activating the p53 and AKT/mTOR pathways using Western blotting in the samples with adequate cells. Fig. 7B shows the effect of the Nutlin-3 plus Tanshinone IIA combination on the p53 and AKT/mTOR pathways in eight primary leukemia cells, including two newly diagnosed Ph+ ALL (#1 and #5), one relapsed Ph + ALL with ABL kinase T315I mutation (#7), two newly diagnosed AML (#3 and #26), one relapsed AML with FLT3-ITD mutation (#22), and two newly diagnosed T-ALL (#15 and #27). The results were similar to those observed in the cell lines. Nutlin-3 alone induced p53 pathway activation in all eight primary leukemia cells, as well as the upregulation of MDM2 and p-MDM2 in seven of the eight cell lines except for one sample (#22). Tanshinone II alone had minimal effects on p53 activation, when combined with Nutlin-3, Tanshinone IIA not only had no effect on Nutlin-3-mediated p53 activation, but also enhanced p53 activation in four cases (#7, #22, #26, and #27). In six of eight cases, Tanshinone IIA inhibited the phosphorylation of MDM2 (#1, #3, #7, #15, #22, and #26), and when combined with Nutlin-3, Tanshinone IIA inhibited Nutlin-3-mediated upregulation of p-MDM2 in five cases (#1, #3, #7, #15, and #26). Nutlin-3 alone inhibited AKT/mTOR pathway activation in three cases (#1, #5, and #7), Tanshinone IIA alone imparted a mildly inhibitory effect on AKT/mTOR activation in six cases; however, the Nutlin-3 plus Tanshinone IIA combination exerted a significant synergistic inhibitory effect on AKT/mTOR activation in seven cases (#1, #3, #5, #15, #22, #26, and #27). The above data indicate that similar to the cell lines, the Nutlin-3 plus Tanshinone IIA combination also exerts an anti-leukemic effect by synergistically regulating the MDM2-P53 and AKT/mTOR pathways.

4. Discussion

Our present study shows that the Nutlin-3 plus Tanshinone IIA

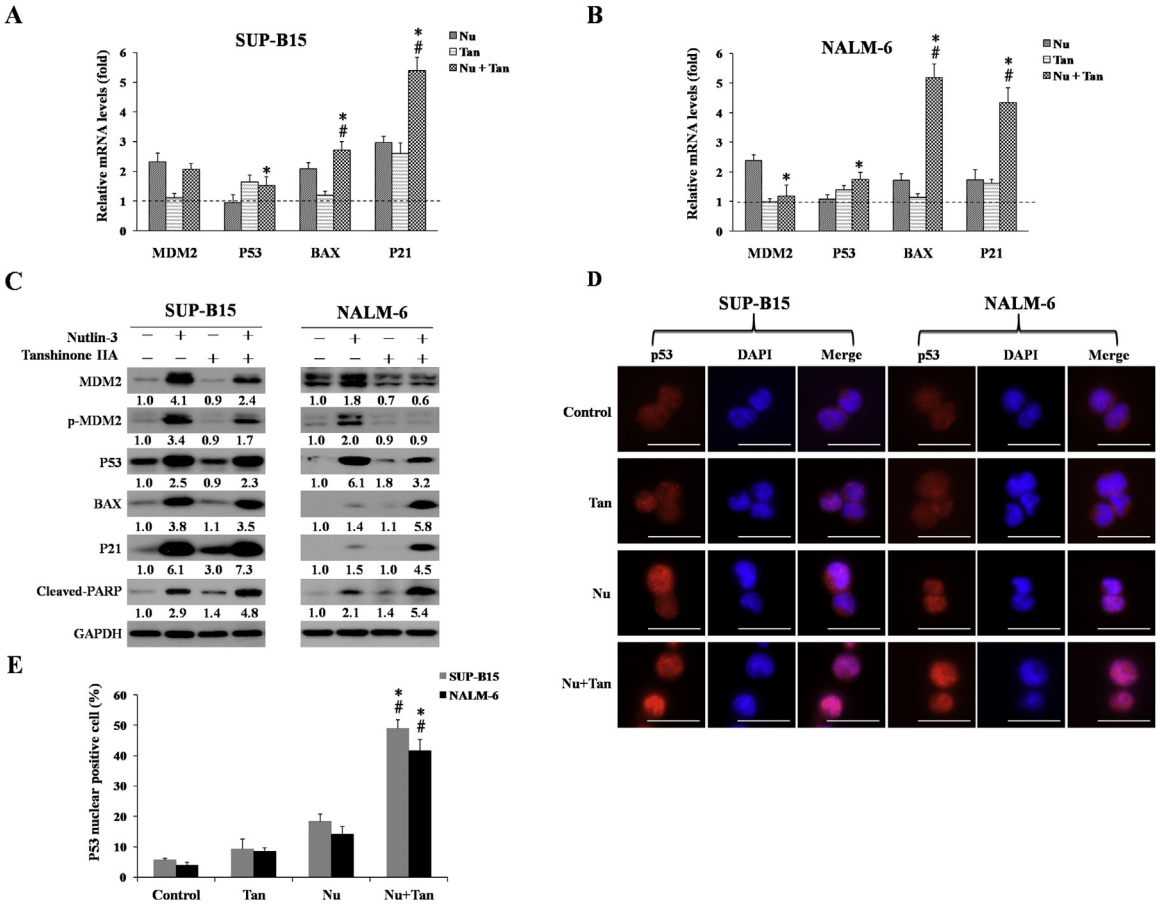


Fig. 5. The combination of Nutlin-3 plus Tanshinone IIA synergistically reactivates the p53 pathway in SUP-B15 and NALM-6 cells. SUP-B15 cells were treated with 0.1% DMSO (control), 2.5 μM nutlin-3 (Nu), 10 μM Tanshinone IIA (Tan) alone or in combination for 24 h. The NALM-6 cells were treated with 0.1% DMSO (control), 2.5 μM nutlin-3 (Nu), 15 μM Tanshinone IIA (Tan) alone or in combination for 24 h. At the end of the treatments, the cells were collected for following experiment. A and B The relative mRNA quantification of p53 and its target genes (MDM2, BAX, and p21) was performed by real-time RT-PCR as described in the Materials and Methods. The data are presented as the mean ± SD of three different experiments, with respect to the control set to 1 (dashed line). The significance of the differences was determined using one-way ANOVA with Bonferroni post-test (*$p < 0.05$ *vs.* nutlin-3 alone; #$p < 0.05$ *vs.* Tanshinone IIA alone). (C) Total proteins lysates were subjected to Western blot analysis for MDM2, p-MDM2, p53, BAX, p21, and apoptotic effector molecule cleaved PARP using the corresponding antibodies. One representative Western blot of two independent experiments is presented. The numbers labeled below each blot represent relative intensity of the bands. GAPDH was used as reference. (D) Cells were stained for p53 (red) and nuclei were counterstained with DAPI (blue). Localization of p53 in the cells is indicated in the merged image. Scale bar = 20 μm. (E) Cells with p53 nuclear positive were quantified by counting a total of 100 cells (magnification 400×) for each treatment. The data are presented as the mean ± SD of 5 independent fields. The significance of the differences was determined with a one-way ANOVA with Bonferroni post-test (*$p < 0.05$ vs. Nutlin-3 alone; #$p < 0.05$ *vs.* Tanshinone IIA alone).

combination synergistically induces cytotoxicity, cell cycle arrest, apoptosis, and autophagic cell death, indicating a synergistic anti-leukemia effect in acute leukemia cell lines with wild-type p53. Further mechanistic studies indicate that Nutlin-3 plus Tanshinone IIA exert their synergistic anti-leukemia effects by effectively activating p53, synergistically inhibiting the AKT/mTOR pathway and activating the RAF/MEK pathway. In primary samples from acute leukemia patients, the Nutlin-3 plus Tanshinone IIA combination synergistically induced cytotoxicity by activating p53 and inhibiting the AKT/mTOR pathway in most samples, including clinical recurrence and refractory leukemia, such as Ph+ ALL with ABL kinase T315I mutation and AML with FLT3-ITD mutation. These results demonstrate that the Nutlin-3 plus Tanshinone IIA combination might be a promising strategy for the treatment of patients with acute leukemia.

Nutlin-3 activates p53 by blocking the interaction between p53 and MDM2, thus inducing cell cycle arrest and apoptosis of tumor cells. Tanshinone IIA is an important component of *Salvia miltiorrhiza*, a Chinese medicine with antitumor activity, and it has the effects of inhibiting pro-growth signal pathway and activating p53 in tumor cells. In our study, as reported in the literature, Nutlin-3 activates the p53 pathway in leukemia cells with wild-type p53 and Tanshinone IIA alone had a certain inhibitory effect on AKT activation, whereas its effect on p53 activation was weak. However, when Nutlin-3 was combined with Tanshinone IIA, these imparted a significant synergistic regulatory effect on p53 and the AKT pathway. We inferred that the co-regulation of p53 and AKT pathways and the crosstalk between p53 and AKT were the most important mechanisms for the synergistic anti-leukemic effect of the Nutlin-3 plus Tanshinone IIA combination. We think that the Nutlin-3 plus Tanshinone IIA combination co-regulates p53 and AKT as follows. First, the Nutlin-3 plus Tanshinone IIA combination impairs the negative feedback loop of the p53 pathway, namely, the MDM2-P53 loop. MDM2 is not only the negative regulatory protein of p53, but also the target protein of p53, thus forming an autoregulatory feedback loop in which p53 positively regulates *mdm2* expression and Mdm2 negatively regulates p53 (Moll and Petrenko, 2003; Wu et al., 1993). Nutlin-3 activates p53 by blocking the interaction between MDM2 and p53, and in turn the activated p53 transcriptionally upregulates MDM2 expression, limiting the level and duration of p53 activation, thus resulting in insufficient P53 activation. Nutlin-3-mediated upregulation of MDM2 restricts the depth and duration of p53 activation as well as

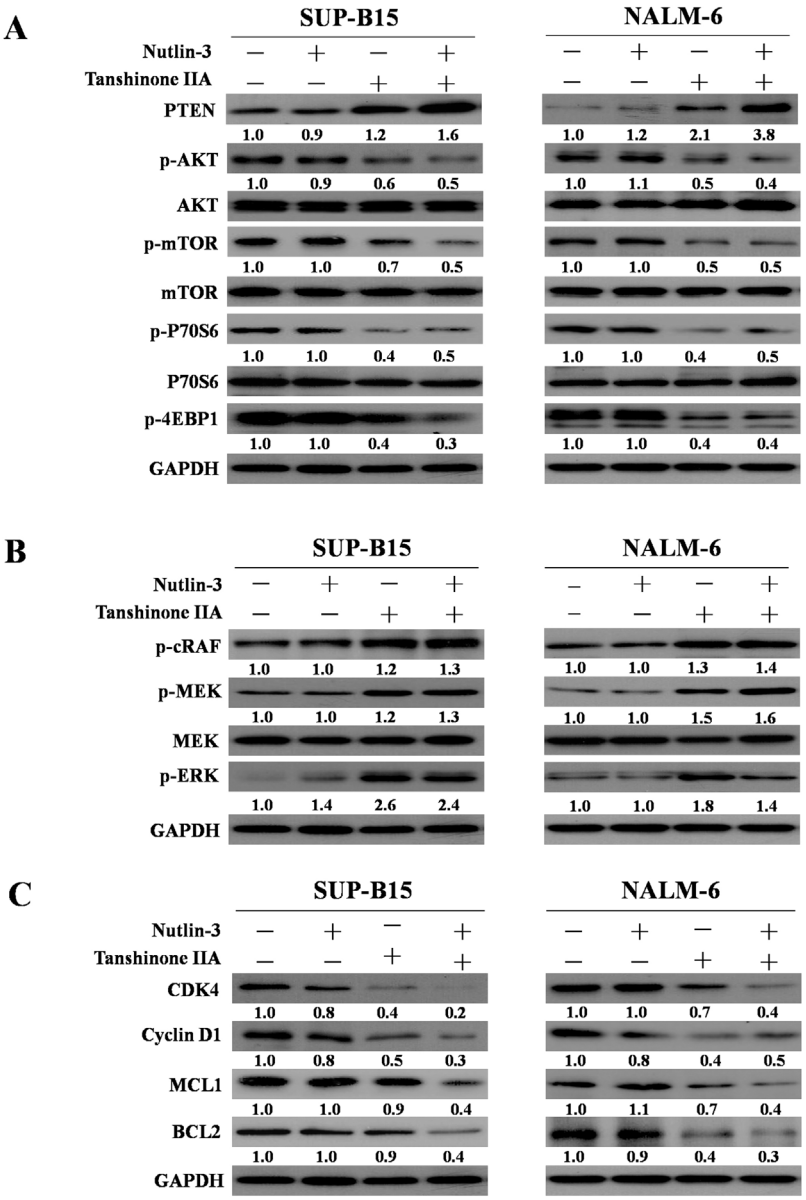


Fig. 6. Effects of Nutlin-3 plus Tanshinone IIA combined with imatinib on the activation of AKT/mTOR and RAF/MEK pathways and apoptotic and cell cycle regulatory proteins in SUP-B15 and NALM-6 cells. SUP-B15 cells were treated with 0.1% DMSO (control), 2.5 μM nutlin-3 (Nu), 10 μM Tanshinone-IIA (Tan) alone, or in combination for 24 h. NALM-6 cells were treated with 0.1% DMSO (control), 2.5 μM Nutlin-3 (Nu), 15 μM Tanshinone-IIA (Tan) alone, or in combination for 24 h. At the end of the treatments, the cells were collected and total proteins were extracted for Western blot analysis using corresponding antibodies. One representative Western blot of two independent experiments was presented. The numbers labeled below each blot represent relative intensity of the bands. GAPDH was used as the loading control. (A) Effects on the activation of the AKT/mTOR pathway. (B) Effects on the activation of RAF/MEK pathway. (C) Effects on cell cycle regulatory proteins cyclin D1, CDK4, and anti-apoptotic proteins MCL1, BCL2.

restricts Nutlin-3-mediated apoptosis in tumor cells. Although Tanshinone IIA had a weak effect on p53 activation, it can undermine the Nutlin-3-mediated upregulation of MDM2 when combined with Nutlin-3, impairing the negative feedback loop of the p53 pathway, thus resulting in higher and longer p53 activation, which could explain why Nutlin-3 combined with Tanshinone IIA activated p53 more effectively than Nutlin-3 alone. Second, the Nutlin-3 plus Tanshinone IIA combination relocated p53 in the nucleus to better exert its transcriptional activity by inhibiting AKT phosphorylation. The activation efficiency of the p53 pathway is not only related to the abundance of p53, but it also depends on the localization of p53 in the cell. Only p53 in the nucleus can play the transcriptional activity. Tanshinone IIA had certain inhibitory effects on AKT phosphorylation (Ser473) when combined with Nutlin-3; the inhibitory effect on AKT phosphorylation (Ser473) increased significantly, whereas phosphorylated AKT(Ser473) was responsible for the phosphorylation of MDM2 (Ser166). The phosphorylated MDM2 at the Serine 166 site is more likely to enter the nucleus than MDM2, and more effectively export p53 from the nucleus to the cytoplasm, and then mediate ubiquitination degradation of p53 (Mayo and Donner, 2001; Ogawara et al., 2002). Our results show that Nutlin-3 not only mediates the upregulation of MDM2, but also that of phosphorylated MDM2 (Ser166), whereas the addition of Tanshinone IIA inhibits Nutlin-3-mediated upregulation of phosphorylated MDM2 (Ser166) by inhibiting AKT phosphorylation on the Serine 473 site, which explains why Nutlin-3 combined with Tanshinone IIA can better locate p53 in the nucleus to better exert its transcription activity. Third, the Nutlin-3 plus Tanshinone IIA combination enhances the positive feedback loop of the p53 pathway, namely, the P53-PTEN-AKT loop. AKT regulates the activation of p53, and in turn p53 regulates the activation of AKT. The P53 protein induces the transcription of the PTEN gene, PTEN inhibits phosphorylation of PIP-3, which in turn inhibits phosphorylation of AKT kinase, thus inhibiting phosphorylation of MDM2, finally enhancing p53 activity, which then forms a positive feedback loop of the p53 pathway (Gottlieb et al., 2002; Mayo and Donner, 2002). Our results show that Nutlin-3 plus Tanshinone IIA synergistically induces p53 activation, which then upregulates PTEN, thus inhibiting AKT phosphorylation synergistically. In summary, the Nutlin-3 plus Tanshinone IIA combination better activated p53 by inhibiting the negative feedback loop P53-MDM2 and enhancing the positive feedback loop P53-PTEN-AKT of the p53 pathway to exert a better and more effective apoptosis-inducing effect.

In addition to inducing apoptosis, Nutlin-3 plus Tanshinone IIA also

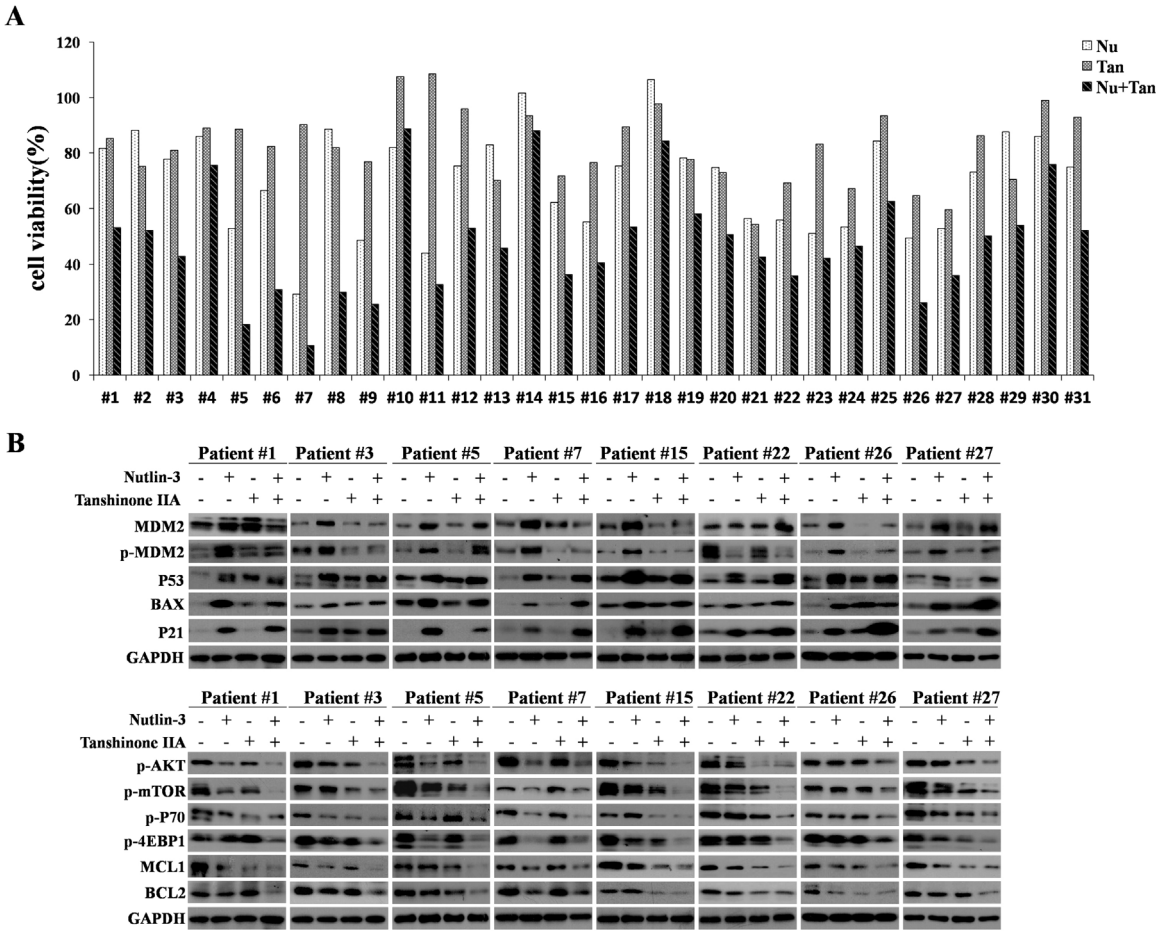


Fig. 7. The combination of Nutlin-3 plus Tanshinone IIA exhibits cytotoxicity effects in leukemia primary cells by activating p53 pathway and inhibiting the AKT/mTOR pathway. The mononuclear cells from 31 acute leukemia patients were treated with Nutlin-3, Tanshinone IIA alone, or in combination for 36 h. We selected 2.5 μM and 15 μM as the working concentrations of Nutlin-3 and Tanshinone IIA, respectively, and using 0.1% DMSO treatment as control. At the end of the treatment period, cell viability was determined using the MTT assay (A). As for the number of cells enough samples (#1, #3, #5, #7, #15, #22, #26, and #27), total proteins were extracted for Western bolting analysis of MDM2/p-MDM2, p53, BAX, p21, p-AKT, p-mTOR, p-P70S6, p-4EBP1, MCL1, and BCL2 (B).

exerted an anti-leukemic effect by inducing autophagic cell death. The AKT/mTOR, RAF/MEK/ERK, and p53 pathways are frequently associated with the regulation of autophagy, and the inhibition of AKT/mTOR and the activation of the RAF/MEK/ERK pathway are used to facilitate autophagy (Ellington et al., 2006; Ogier-Denis et al., 2000; Pattingre et al., 2003), whereas p53 regulates autophagy depending on its subcellular localization. P53 in the nucleus induces autophagy, whereas p53 in the cytoplasm suppresses autophagy (Green and Kroemer, 2009; Maiuri et al., 2010; Tasdemir et al., 2008). Nutlin-3 plus Tanshinone IIA induces autophagy by synergistically inhibiting the AKT/mTOR pathway, activating the RAF/MEK pathway, and relocating p53 in the nucleus. Autophagy is a double-edged sword; it not only contributes to the growth of tumor cells but can also suppress them (Degenhardt et al., 2006; Eskelinen, 2005; Gozuacik and Kimchi, 2004; Kon et al., 2011; Mathew et al., 2007; Notte et al., 2011). In our study, inhibiting autophagy induced by Nutlin-3 plus Tanshinone IIA reduced their cytotoxic effect; therefore, we conclude that Nutlin-3 plus Tanshinone IIA-induced autophagy is a mechanism of inhibiting the growth of leukemia cells and is one of the mechanisms of Nutlin-3 plus Tanshinone IIA against leukemia.

In primary leukemia cells, the Nutlin-3 plus Tanshinone IIA combination imparted a stronger cytotoxic effect than a single drug in approximately 70% of the cell specimens. In line with the cell lines, the Nutlin-3 plus Tanshinone IIA combination also worked mainly through the synergistic regulation of the p53 and AKT pathways. Since we did not routinely detect the mutation of the p53 gene in the primary leukemic cells, it was not clear whether there was a relationship between the inefficiency of the Nutlin-3 plus Tanshinone IIA combination and p53 gene mutations in the other 30% of the cell specimens. We speculate that there may be a p53 mutation in the specimens in which both Nutlin-3 and the Nutlin-3 plus Tanshinone IIA combination could not exert cytotoxicity. The Nutlin-3 plus Tanshinone IIA combination showed cytotoxic effects in relapsed leukemia primary cells, including Ph + ALL with ABL kinase T315I mutation and AML with FLT3-ITD mutation, suggesting a potential alternative therapy for relapsed and refectory leukemia patients.

In summary, we demonstrated that the combination of Nutlin-3 and Tanshinone IIA exerts synergistic anti-leukemia effects by regulating the p53 and AKT/mTOR pathways. Our findings provide a strong warrant for the further investigation of the combination of Nutlin-3 and Tanshinone IIA in leukemia, especially in relapsed refractory leukemia. Small-molecule MDM2 antagonists plus Tanshinone IIA lead to significant apoptosis, which may represent a valuable strategy for the treatment of acute leukemia.

Conflict of interest

The authors declare that they have no competing interests.

Authors' contributions

YG designed the experiments, coordinated the study, and drafted

the manuscript. YL involved in data analysis and drafting the manuscript. FFW and XOH conducted the experiments. BX and HBM participated in collection of the clinic samples. YPG conceived the study and participated in designing of the experiments and helped to draft the manuscript. All authors read and approved the final manuscript.

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