

Effects of lapatinib on cell proliferation and apoptosis in NB4 cells

LU LIU^{1,2}, LIANG ZHONG², YI ZHAO¹, MIN CHEN¹, SHIFEI YAO¹, LIANWEN LI¹,
CHUNLAN XIAO¹, ZHILING SHAN², LIUGEN GAN¹, TING XU¹ and BEIZHONG LIU^{1,2}

¹Central Laboratory of Yong-Chuan Hospital, Chongqing Medical University, Chongqing 402160;

²Key Laboratory of Laboratory Medical Diagnostics, Ministry of Education,
Department of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, P.R. China

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Abstract. Acute promyelocytic leukemia (APL), characterized by the presence of the promyelocytic leukemia (PML)-retinoic acid α receptor (RAR α) fusion protein, responds to treatment with all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO). However, drug resistance and side effects restrict the application of these reagents. Hence, the development of novel therapeutic drugs for APL treatment is critical. Lapatinib, a small-molecule tyrosine kinase inhibitor, has been used in the treatment of different tumors. However, it is unclear whether lapatinib exerts antitumor effects on APL. The present study investigated the antitumor effects and potential mechanisms of lapatinib on NB4 cells derived from APL. Cell Counting Kit-8 assay and colony forming analysis indicated that lapatinib inhibited NB4 cell proliferation in a dose-dependent manner. Flow cytometry analysis revealed that lapatinib induced cell cycle arrest at the S phase and promoted cell apoptosis. Furthermore, Liu's staining and Hoechst 33258 staining revealed that lapatinib treatment induced an apoptotic nuclear phenomenon. Furthermore, lapatinib induced apoptosis by decreasing Bcl-2 and PML-RAR α levels, and by increasing the levels of Bax, cleaved PARP, cleaved caspase-3 and cleaved caspase-9. In addition, lapatinib increased the levels of phospho-p38 MAPK and phospho-JNK, and decreased the levels of phospho-Akt. The p38 inhibitor PD169316 partially blocked lapatinib-induced proliferation inhibition and apoptosis, whereas the JNK inhibitor SP600125 had no such effects. Therefore, treatment with lapatinib may be a promising strategy for APL therapy.

Introduction

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML) that is characterized by

a disease-initiating chromosome translocation of t(15;17) (q22;q21), leading to the generation of the promyelocytic leukemia (PML)-retinoic acid α receptor (RAR α) fusion protein (1). At present, the treatment of APL involves the use of all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO). ATRA primarily induces differentiation, whereas ATO promotes apoptosis in APL cells (2). Despite the significant progress in therapeutic strategies, 10-30% patients with APL are still resistant to ATRA or ATO (3). Hence, the development of new therapeutic drugs for APL treatment is critical.

Lapatinib is a small-molecule, tyrosine kinase inhibitor that targets the epidermal growth factor receptor and human epidermal growth factor receptor (HER-2) (4,5). Clinically, lapatinib is mainly used for the treatment of HER-2 positive metastatic breast cancer, and is approved for use by the Food and Drug Administration of the USA (1,6). In addition, since lapatinib is an oral drug and has few adverse reactions, it has been used in the treatment of several types of tumors, including triple-negative breast cancer, nasopharyngeal carcinoma, head and neck cancer, pancreatic cancer, cervical cancer, non-small cell lung cancer, and brain cancer (7-14). Moreover, several studies have reported the use of lapatinib in the treatment of leukemia; for example, lapatinib induces apoptosis in cell lines derived from acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) (15), and it has been shown to regulate autophagy, apoptosis, and megakaryocytic differentiation in K562 cells (16). In addition, lapatinib promotes autophagic cell death and differentiation in AML (17). Importantly, lapatinib inhibits cell proliferation and promotes cell apoptosis in Philadelphia chromosome-positive acute lymphoblastic leukemia (18). However, few studies have evaluated the effects of lapatinib in APL cells.

In the present study, we investigated the effects of lapatinib on NB4 cells, an APL cell line. We found that lapatinib inhibited cell proliferation and promoted apoptosis. Our observations suggested that lapatinib treatment may represent a novel therapeutic approach for the treatment of APL.

Materials and methods

Reagents. Lapatinib was purchased from Shanghai Selleck Chemicals Co., Ltd., (Shanghai, China). Liu's stain was purchased from BaSO Diagnostics Inc., (Zhuhai, China).

Correspondence to: Dr Beizhong Liu, Central Laboratory of Yong-Chuan Hospital, Chongqing Medical University, 1 Medical Road, Chongqing 402160, P.R. China
E-mail: liubeizhong@cqmu.edu.cn

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The inhibitor of P-38MAPK PD169316 was purchased from MedChemExpress (New Jersey, USA). Hoechst 33258 and the JNK inhibitor SP600125 were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Antibodies against caspase-3, caspase-9, cleaved caspase-9, p38MAPK, JNK, p-JNK, Akt, and p-Akt were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). Antibodies against Bax, Bcl-2, and PARP were purchased from Wanleibio (Shenyang, China). Antibody against PML-RAR α was purchased from Abcam (Cambridge, MA, USA). Antibody against p-p38 MAPK was purchased from EMD Millipore (Billerica, MA, USA). Goat-anti-rabbit antibody, goat-anti-mouse antibody and anti- β -actin antibodies were purchased from Zhongshan Golden Brige Biotechnology Co., Ltd., (Beijing, China).

Cell culture. NB4 cells (Institutes for Biological Science, Shanghai, China) were cultured in RPMI 1640 (Gibco Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco Life Technologies) supplemented with penicillin (100 mg/ml) and streptomycin (100 mg/ml) at 37°C in an environment containing 5% CO₂. The medium was refreshed every day.

CCK-8 assay. NB4 cells were seeded in 96-well plates at a density of 1.0×10^4 cells/well. Then, cells were treated with different concentrations of lapatinib for 24 h. After cultured 24 h, 10 μ l of CCK-8 (7Sea Cell Counting Kit; Sevenses Futai Bio-technology Co., Ltd., Shanghai, China) was added to each well followed by incubation for 2 h at 37°C. Cell viability was assessed by detection of absorbance at 450 nm using a spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiment was repeated at least three times.

Colony forming assay. NB4 cells were exposed to lapatinib or DMSO for 24 h. Then, cells were plated in 24-well plates in methylcellulose medium in triplicate. The numbers of colonies were determined following incubation for 14 days at 37°C and 5% CO₂.

Flow cytometry analysis. NB4 cells were fixed with pre-cold 70% ethanol overnight at 4°C and washed with PBS. Then, incubated with 50 μ l Propidium Iodide (PI) for 15 min at room temperature and cells were analysis in flow cytometer. Each experiment was repeated at least three times.

NB4 cells were treated of various concentrations of lapatinib for 24 h, and cells were harvested by centrifugation at 3000 r for 5 min. After washed three times with pre-cold PBS, cells were re-suspended in Binding Buffer (Sungene Biotech Co., Ltd., Tianjing, China), and stained by Annexin V-FITC and PI (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at room temperature and cells were analysis in flow cytometer. Each experiment was repeated at least three times.

Liu's staining. NB4 cells were treated with different concentrations of lapatinib for 24 h. Cells in each group were collected and plated onto the glass slides. First, Liu A was added to the sample spot for 20 s and mixed with twice the volume of Liu B for another 30 s. Then, the slides were washed with water and air dried for observation using a microscope.

Hoechst 33258 staining. NB4 cells were treated with different concentrations of lapatinib for 24 h. Cells in each group were collected and plated onto the glass slides, followed by fixative in 4% para-formaldehyde at room temperature. After 20 min, cells were washed twice with PBS, stained with Hoechst 33258 for 10 min and then washed twice with PBS. Nuclear morphological changes in the cells were observed under fluorescence microscope.

Western blot analysis. NB4 cells were lysed in radioimmunoprecipitation solution containing protease inhibitor phenylme-thanesulfonyl fluoride (PMSF), phosphatase inhibitor Na F and Na₃VO₃. Protein concentration was determined with BCA method. A total of 50 μ g of proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Membranes were blocked with 5% non-fat milk for 2 h, and incubated with specific antibodies (1:1,000) over night at 4°C. Then, membranes were incubated with goat-anti-rabbit and goat-anti-mouse secondary antibodies (1:4,000) for 1 h at 37°C. After washing with Tris-buffered saline containing Tween-20 (TBST), the immunoreactive complexes were visualized using an enhanced chemiluminescence system. Protein bands were analyzed by Quantity One Software (Bio-Rad Laboratories, Inc.). Each experiment was repeated at least three times.

Statistical analysis. Data were expressed as means \pm standard deviation (SD). Statistical analysis was performed with SPSS version 17.0. An independent samples t-test was used to compare the results of two groups. Statistical analysis of western blot results was performed using analysis of variance (ANOVA). $P < 0.05$ was considered to indicate a statistically significant difference. Each experiment was repeated at least three times.

Results

Lapatinib inhibits the proliferation of NB4 cells. The effect of lapatinib on the viability of NB4 cells was evaluated using CCK-8 assays. Treatment of NB4 cells with 0-20 μ M lapatinib for 24 h led to a concentration-dependent reduction in cell viability (Fig. 1A). Based on these observations, 15 and 20 μ M lapatinib were selected for our study. Exposure to 15 and 20 μ M lapatinib visibly inhibited the colony-forming capacity of NB4 cells compared to the cells of the dimethyl sulfoxide (DMSO) group (Fig. 1B).

Lapatinib induces cell cycle arrest at the S phase. The cell cycle of NB4 cells was detected by a flow cytometric assay. Compared to the cell cycle profile of the DMSO treated group, 15 and 20 μ M lapatinib treatment dramatically increased the percentage of cells in the S phase (Fig. 2).

Lapatinib promotes apoptosis in NB4 cells. NB4 cells were treated with 15 and 20 μ M lapatinib for 24 h and cell apoptosis was detected using flow cytometry. As shown in Fig. 3, cell apoptosis increased in the lapatinib treated groups compared to that of the DMSO-treated group (Fig. 3).

Effects of lapatinib on NB4 cell morphology. To further assess the effect of lapatinib treatment on NB4 cells' morphology,

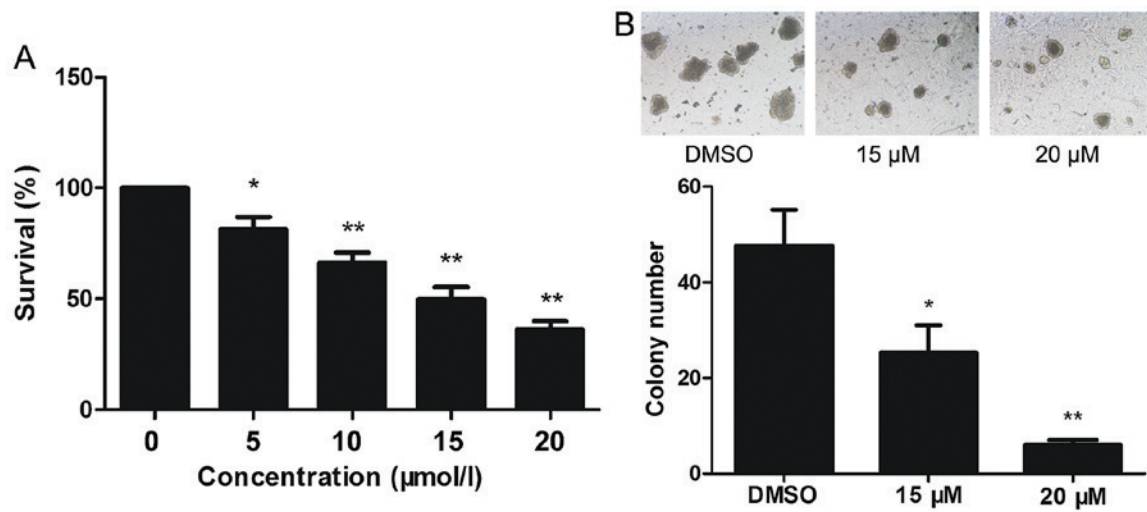


Figure 1. Lapatinib inhibited the proliferation of NB4 cells. (A) NB4 cells were cultured with 0-20 μM lapatinib for 24 h in 96-well plate. Cytotoxicity was detected by CCK-8 assays. (B) The colony-forming ability of NB4 cells was tested with or without the treatment of lapatinib, the magnification is 40x. Data was expressed as means ± SD. *P<0.05, **P<0.01.

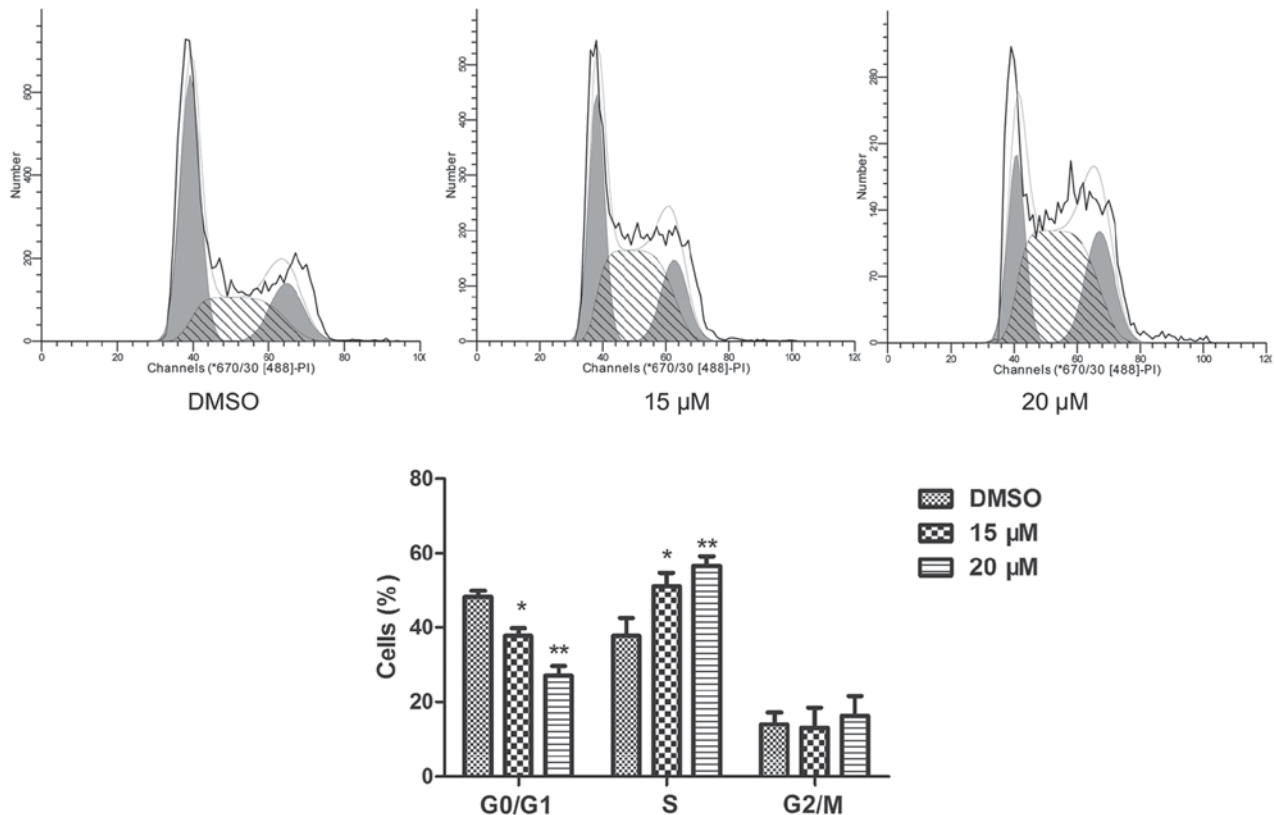


Figure 2. Lapatinib induced S phase arrest in NB4 cells. NB4 cells were treated with 15 and 20 μM lapatinib for 24 h, cells were harvested for cell cycle analysis by flow cytometry. Data was expressed as means ± SD. *P<0.05, **P<0.01.

cells were stained with Liu's stain after treatment with 15 and 20 μM lapatinib. Results showed that nuclear fragmentation was observed in the lapatinib-treated group (Fig. 4A). In addition, after incubation with 15 and 20 μM lapatinib, cells were harvested and stained with Hoechst 33258. The nuclear fluorescence intensities of the lapatinib treated groups were stronger than that of the negative control group (Fig. 4B), and nuclear fragmentation and condensation were observed in the lapatinib- treated group (Fig. 4).

Effects of lapatinib on apoptosis-related protein in NB4 cells. To investigate the effect of lapatinib on apoptosis in NB4 cells, the levels of apoptosis-related proteins were detected by western blotting. Bcl-2 was notably down-regulated with lapatinib treatment. In addition, the expression of Bax, cleaved caspase-3, cleaved caspase-9, and cleaved PARP were up-regulated in the lapatinib group but not in the DMSO group (Fig. 5A). The level of the fusion protein PML-RARα was down-regulated with lapatinib treatment (Fig. 5B).

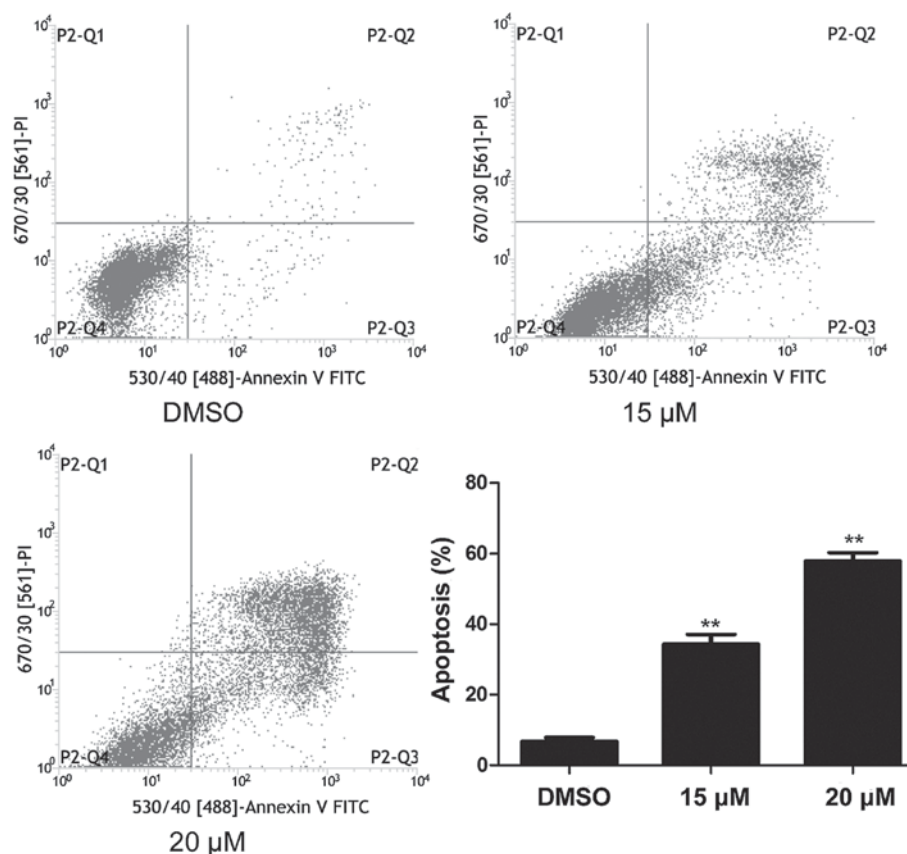


Figure 3. Lapatinib induced apoptosis of NB4 cells. Cells were treated with 15 and 20 μ M lapatinib for 24 h, and apoptosis was analyzed by flow cytometry using double staining with FITC-labeled annexin-V and PI. Cells undergoing early apoptosis are Annexin V-FITC⁺/PI⁻, while cells were undergoing late apoptosis are Annexin V-FITC⁺/PI⁺. The percentages of late and late apoptotic cells were summed to give the total number of apoptotic cells. Data was expressed as means \pm SD. **P<0.01.

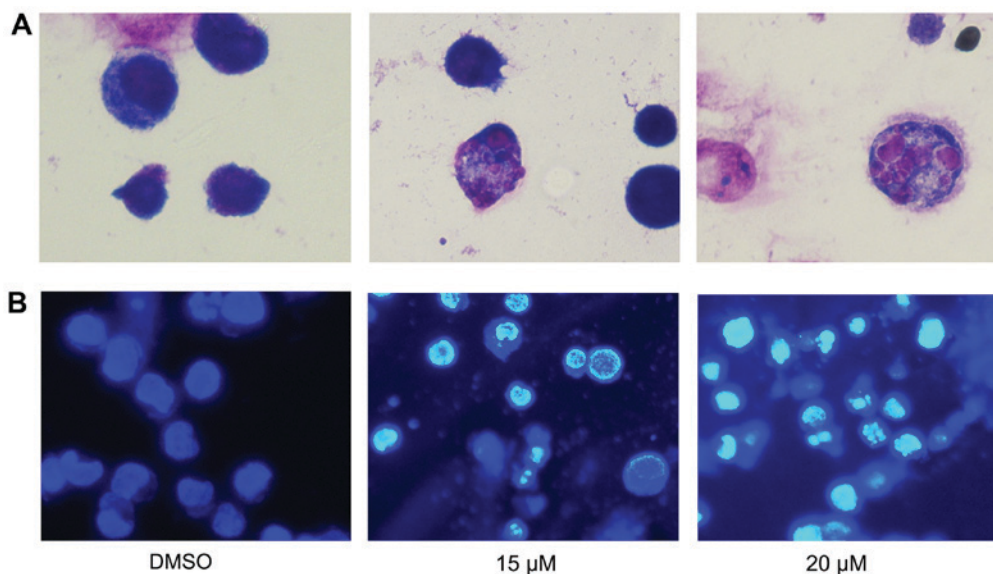


Figure 4. Effects of lapatinib on NB4 cell morphology. Cells were treated with 15 and 20 μ M lapatinib for 24 h. (A) Cells stained with Liu's staining were imaged with a microscope, the magnification is 1,000x. (B) Cells stained with Hoechst 33258 were imaged with a fluorescence microscope, the magnification is 400x.

Effects of lapatinib on MAPKs and AKT signaling pathways. To investigate the molecular mechanisms of lapatinib action on NB4 cells, we analyzed the levels of Akt, p-Akt, p38MAPK, p-p38MAPK, JNK, and p-JNK. Results showed that lapatinib notably down-regulated the expression of

p-Akt and up-regulated the expression of p-p38MAPK and p-JNK (Fig. 6).

PD169316 weakens the effect of lapatinib in NB4 cells. To further study the involvement of p38MAPK and JNK signaling

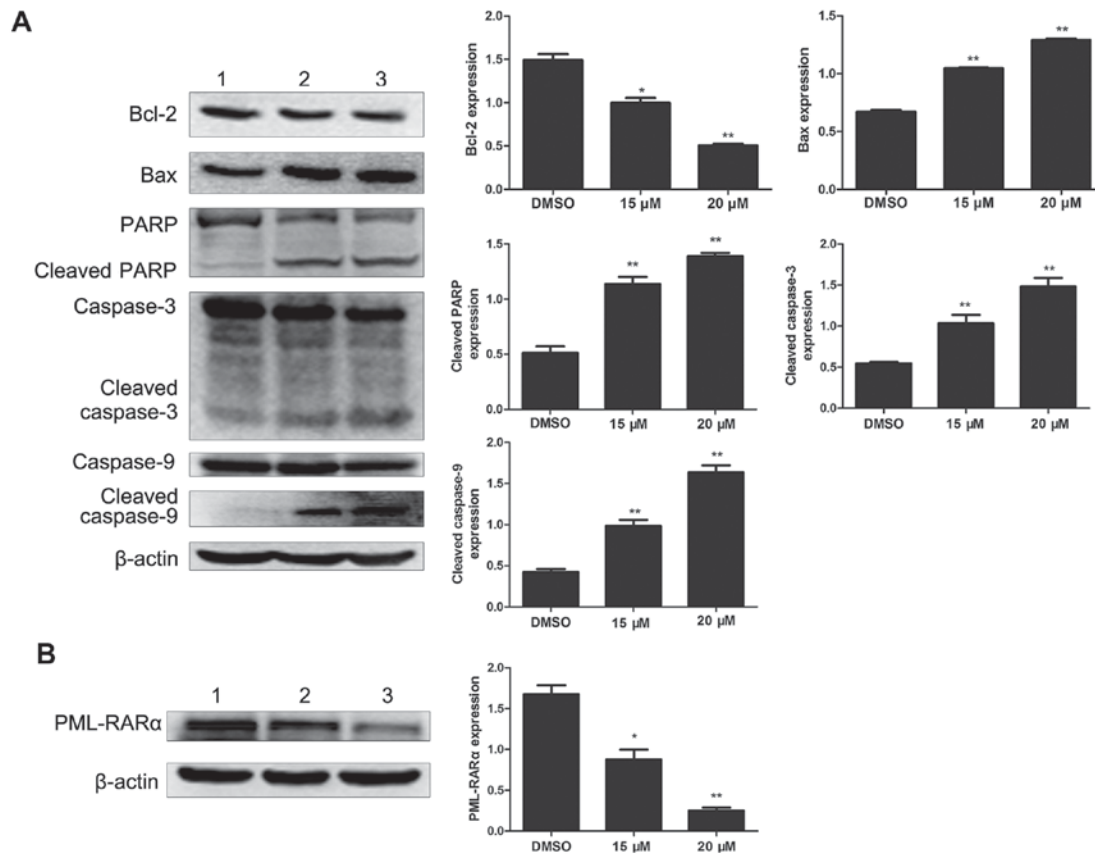


Figure 5. Protein expression levels in NB4 cells. Cells were treated with 15 and 20 μ M lapatinib for 24 h. (A) The levels of Bax, Bcl-2, PARP, caspase-3/9, and cleaved caspase-3/9 proteins were measured by western blotting. (B) The expression of PML-RAR α . Quantification analysis of western blotting, β -actin was served as a control. Data was expressed as means \pm SD. *P<0.05, **P<0.01 (1 NB4 cells treated with DMSO; 2 NB4 cells treated with 15 μ M lapatinib; 3 NB4 cells treated with 20 μ M lapatinib).

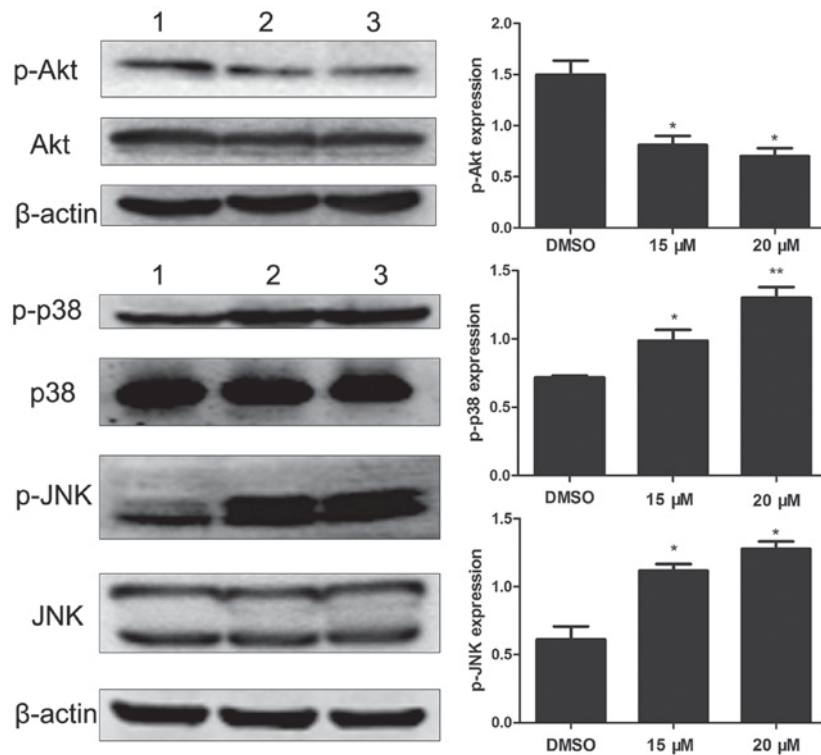


Figure 6. Effects of lapatinib on MAPKs and PI3K/AKT signaling pathways. Cells were treated with 15 and 20 μ M lapatinib for 24 h, the expression of p-AKT, AKT, p-p38 MAPK, p38 MAPK, p-JNK, and JNK were measured by western blotting. Quantification analysis of western blotting, β -actin was served as a control. Data were expressed as means \pm SD. *P<0.05, **P<0.01 (A: 1 DMSO; 2 15 μ M lapatinib; 3 20 μ M lapatinib. D: 1 DMSO; 2 PD169316; 3 15 μ M lapatinib; 4 PD169316+15 μ M lapatinib).

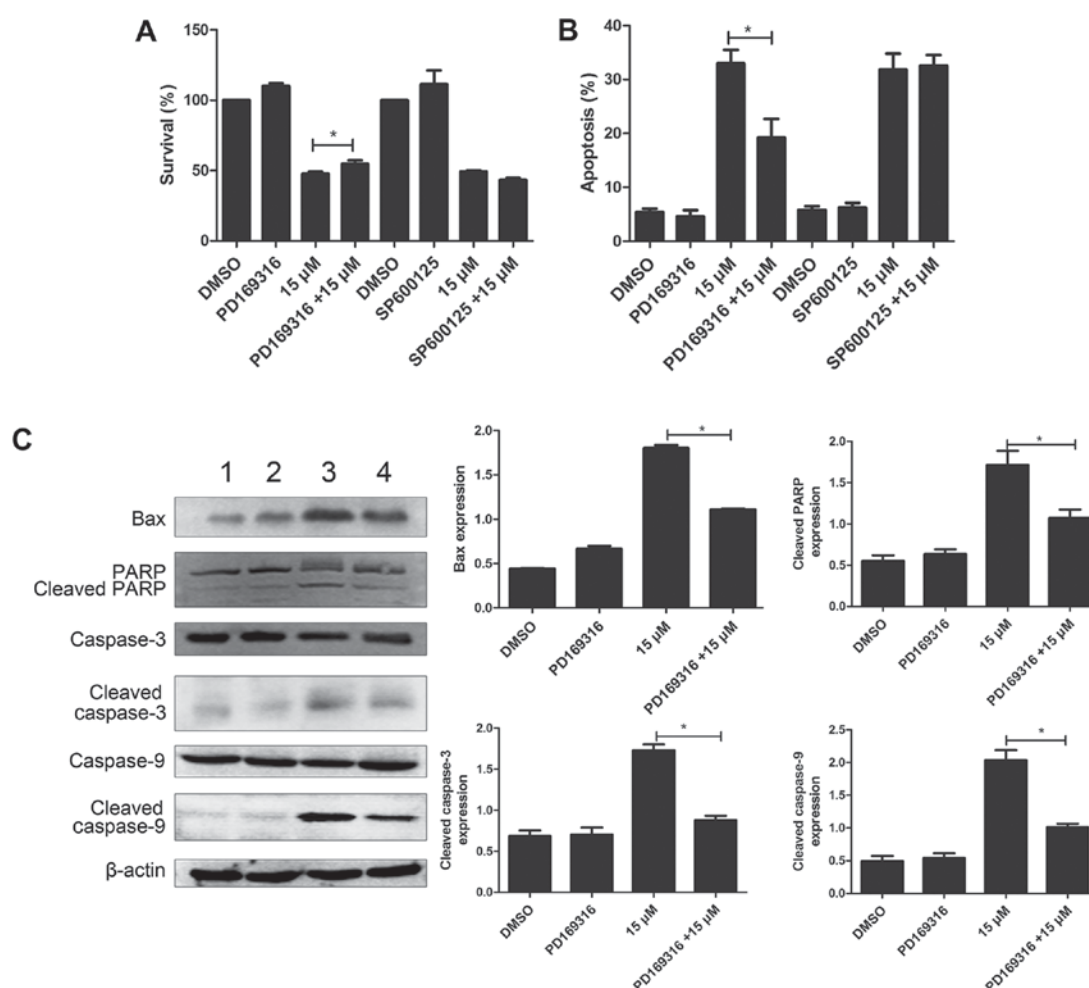


Figure 7. PD169316 reduces cell proliferation inhibition and apoptosis induced by lapatinib. NB4 cells were treated with 15 μ M lapatinib for 24 h in the presence or absence of PD169316 or SP600125. (A) Cell viability was measured by CCK-8 assay. (B) Cell apoptosis was analyzed by flow cytometry. (C) The levels of Bax, PARP, cleaved caspase-3/9, and caspase-3/9 were measured by western blotting. Quantification analysis of western blotting, β -actin was served as a control. Data was expressed as means \pm SD. * P <0.05 (A: 1 DMSO; 2 15 μ M lapatinib; 3 20 μ M lapatinib. D: 1 DMSO; 2 PD169316; 3 15 μ M lapatinib; 4 PD169316+15 μ M lapatinib).

pathways, in the mode of action of lapatinib, we treated the cells with 10 μ M each of PD169316 or SP600125 for 30 min before incubating with 15 μ M lapatinib. The results of the CCK-8 assay showed that PD169316 could partially reverse the growth inhibition induced by lapatinib treatment. FCM detections demonstrated that PD169316 partially reduced lapatinib-induced apoptosis. However, the JNK inhibitor, SP600125, had no such effect (Fig. 7A and B). In addition, the levels of Bax, cleaved caspase-3, cleaved caspase-9, and cleaved PARP were significantly reduced upon treatment with a combination of lapatinib and PD169316 than with lapatinib treatment alone (Fig. 7C).

Discussion

Previous studies showed that lapatinib decreased the viability of MOLM-13 and HL-60 cells (15), head and neck squamous carcinoma cells (19), gastric cancer cells (20), and breast cancer cells (21). In this study, the CCK-8 assay showed that proliferation of NB4 cells was inhibited by 5-20 μ M lapatinib in a concentration-dependent manner. Colony-forming assays showed that 15 and 20 μ M lapatinib dramatically reduced colony formation, indicating that lapatinib could suppress NB4 cell

proliferation. Moreover, some recent studies have reported that lapatinib promotes cell cycle arrest in the G₀/G₁ phase (19-21). However, our results showed that lapatinib reduced the number of cells in the G₁ phase and increased the number of cells in the S phase. Based on these results, we hypothesize that lapatinib inhibits cell proliferation by inducing S phase arrest.

Apoptosis is a process of programmed cell death and involves balancing of pro-survival and pro-cell death functions in the cell (22). Apoptosis is characterized by nuclear condensation, nuclear fragmentation, and formation of apoptotic bodies. Additionally, caspase-3 and PARP are cleaved during activation of apoptosis (23-25). In our study, flow cytometry analysis showed that lapatinib promoted cell apoptosis; indeed, nuclear fragmentation, nuclear condensation, and the presence of few apoptotic bodies were observed in NB4 cells after lapatinib treatment, and lapatinib induced cleavage of caspase-3 and PARP in a dose-dependent manner. Two apoptotic pathways exist: the extrinsic apoptotic pathway involving the death receptors is regulated by caspase-8, whereas the intrinsic apoptotic pathway involving the mitochondria is related to activation of caspase-3 and caspase-9 (26). Our data showed that treatment with lapatinib

induced the activation of caspase-9 rather than caspase-8 (data not shown); thus, we assumed that lapatinib may induce apoptosis via the intrinsic apoptotic pathway in NB4 cells. Indeed, our analysis showed that lapatinib decreased Bcl-2 and increased Bax levels, thereby demonstrating that lapatinib induces apoptosis through the intrinsic pathway mediated by the Bcl-2 family, which involves Bcl-2 and Bax. Interestingly, lapatinib also reduce the level of PML-RAR α , which blocks granulocyte cell maturation. This result showed that lapatinib-induced apoptosis was associated with the degradation of PML-RAR α . Taken together, our results suggested that lapatinib treatment may represent a therapeutic option for APL, although further studies are required to fully elucidate the mechanisms involved in this process.

MAPK family members can be activated by different factors and have important roles in apoptosis. MAPK signaling pathways, including ERK, p38 MAPK, and JNK, have been identified as chemotherapeutic targets for sensitizing cancer cells for apoptosis (27). Typically, p38 kinase and JNK are known as cell death signals, whereas ERK is a survival signal (28). Previous studies have shown that lapatinib induces apoptosis in breast cancer cells through the STAT5 and JNK signaling pathways (29). Indeed, after treatment with lapatinib, cells showed increased phosphorylation of p38 MAPK and JNK in a concentration-dependent manner. In addition, the p38 inhibitor PD169316 reduced the lapatinib-induced inhibition of cell viability and apoptosis, whereas the JNK inhibitor SP600125 did not affect these processes. Moreover, co-treatment with lapatinib and a p38 inhibitor significantly decreased the levels of Bax, cleaved PARP, cleaved caspase-3 and cleaved caspase-9 compared to those in cells treated with lapatinib alone. These results suggested that the p38 MAPK pathway was involved in lapatinib-induced apoptosis. In addition to the MAPK pathway, the PI3K/AKT signal transduction pathway plays a vital role in cell survival and prevents cancer cells from undergoing apoptosis during tumorigenesis (30). Several studies have reported that lapatinib induces apoptosis in triple-negative breast cancer cells by inhibiting the CIP2A/PP2A/P-AKT signaling pathways (11). Additionally, lapatinib can potentiate radiation-induced cell death in HER-2-overexpressing breast cancer cells via reduction of p-AKT levels (31). Consistent with this observation, our results showed that lapatinib exerted significant inhibitory effects on p-AKT levels. These results suggested that the AKT pathway was involved in lapatinib-induced apoptosis in NB4 cells.

Previous studies have reported that lapatinib induces autophagic cell death in acute myeloblastic leukemia-derived cell lines (17); however, we did not detect any changes in the levels of autophagy markers ATG5, ATG7, and LC3II following lapatinib treatment. These contradictory results could be attributed to variations in the concentrations of lapatinib, differences in the time points used in the experiments, cell status, and variations in other experimental factors.

In conclusion, our present study indicated that lapatinib inhibited NB4 cell growth and caused cell cycle arrest, and promoted apoptosis potentially through the p38MAPK and AKT signaling pathways. These results suggested that treatment with lapatinib may be an effective strategy for APL therapy. Further investigations are required for developing novel therapeutic approaches for the treatment of leukemia.

Acknowledgements

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