



Studying the enhancement of programmed cell death by combined AG1024 and paclitaxel in a model of chronic myelogenous leukemia



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ABSTRACT

Aims: Chronic myelogenous leukemia is a clonal malignancy of the pluripotent hematopoietic stem cells that is characterized by the uncontrolled proliferation and expansion of myeloid progenitors. Myeloid progenitors express the fusion oncogene BCR–ABL, which has uncontrollable activity in malignant cells and prevents the cell apoptosis caused by some antineoplastic agents, such as paclitaxel. Targeting these abnormalities by blocking the tyrosine kinase enzymes of BCR–ABL is a promising approach for chronic myelogenous leukemia therapy.

Main methods: Conventional Liu's staining is an auxiliary technique used in microscopy to enhance the contrast in microscopic images, aiding the observation of cell morphology. The MTT assay, flow cytometry of the sub-G1 analysis and the TUNEL assay were applied to estimate the apoptosis levels. RT-PCR and western blot methods were used to evaluate the key molecules conferring anti-cell-death properties.

Key findings: The effects of the tyrosine kinase inhibitor AG1024 were evaluated with regard to the regulation of BCR–ABL expression, inhibition of cell proliferation, and enhanced paclitaxel-induced apoptosis in BCR–ABL-expressing K562 cell lines. AG1024 downregulated the expression of BCR–ABL and anti-apoptosis factors, such as Bcl-2 and Bcl-xL, which were present in K562 cells. Moreover, the combination of AG1024 with paclitaxel inhibited cell proliferation and enhanced paclitaxel-induced apoptosis within 24 h.

Significance: In summary, the present study shows that the combination of AG1024 with paclitaxel inhibited model cancer cell proliferation, suggesting a new use of paclitaxel-based chemotherapy for cancer control.

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Introduction

Chronic myelogenous leukemia (CML) belongs to a group of diseases referred to as myeloproliferative disorders. This clonal disorder is generally easily diagnosed because the leukemic cells of more than 95% of patients have a distinctive cytogenetic abnormality, the Philadelphia chromosome (Ph1) (Kurzrock et al., 2003; Goldman & Melo, 2003). The Ph1 results from a reciprocal translocation between the long arms of chromosomes 9 and 22 and is demonstrable in all hematopoietic precursors (Deininger et al., 2000). This translocation results in the transfer of the ABL1 gene from the chromosome 9 oncogene to an area on chromosome 22 called the breakpoint cluster region (BCR) (Deininger et al., 2000). This translocation results in a fused BCR–ABL gene and the production of an abnormal tyrosine kinase fusion protein that causes the disordered myelopoiesis found in CML. Recent advances

in CML research have highlighted the role of the BCR–ABL oncoprotein as a molecular abnormality that activates certain signal pathways and alters the cells (Deininger et al., 2000). Paclitaxel (taxol) is a natural product that is crudely extracted from the bark of the Pacific yew *Taxus brevifolia*. It is an antimicrotubule agent that is active against a broad range of cancers generally considered refractory to conventional chemotherapy, particularly in advanced ovarian and breast carcinomas (Mabuchi et al., 2002). Paclitaxel can increase tubulin polymerization, stabilize microtubules and prevent tubulin depolymerization by binding to β tubulin and, consequently, arresting the cell cycle during the G2/M phase. Therefore, the microtubules formed in the presence of paclitaxel are extraordinarily stable and dysfunctional (Rowinsky & Donehower, 1995). Microtubules and their self-assembly of α and β tubulin heterodimers are important cytoskeleton components involved in the regulation of cell proliferation, differentiation, and apoptosis (Wang et al., 1999). The polymerization and depolymerization of tubulin essentially regulate microtubular dynamics (Wang et al., 1999). Numerous ligands bind to tubulin and affect its assembly properties. Microtubule targeting agents are important ligands that are effective as chemotherapeutic drugs for treating various types of tumors (Wang et al., 1999). Although paclitaxel has demonstrated antitumor activity against several cancers, it has limitations against certain resistant malignant tumors. For

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example, the BCR–ABL-positive erythroleukemia K562 cell line is resistant to paclitaxel (Jaffrezou et al., 1995). A family of low-molecular-weight compounds referred to as tyrosinostats have been synthesized and identified as potent protein tyrosine kinase (PTK) inhibitors. Different members of the tyrosinostat family recognize the PTKs of different growth factor receptors, such as the epidermal growth factor receptor (EGFR) and the insulin-like growth factor receptor (IGF1R), in a selective manner (Gazit et al., 1989). AG1024 is a tyrosine kinase inhibitor that specifically targets the IGF-1 receptor (Parrizas et al., 1997; Wen et al., 2001; Ohmichi et al., 1993). IGF1R is a tyrosine kinase membrane receptor that is ubiquitously expressed in all cell types except mature B cells and hepatocytes (Valentinis & Baserga, 2001). Its insulin-like growth factors (IGFs) have been shown to strongly stimulate cell proliferation and inhibit cell death (Komatsu et al., 1997). Several intracellular signaling pathways that are activated in response to IGF stimulation have been identified. The binding of IGFs to IGF1R activates the tyrosine kinase, which triggers numerous reactions among several molecules involved in the signal transduction pathway. Phosphoinositide 3-kinase (PI3K) is a key molecule that is activated in this manner (Jones & Clemmons, 1995; LeRoith et al., 1995). In response to the IGF stimulation, the activated PI3K converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate. This conversion results in the activation of the pleckstrin homology domain-containing serine/threonine kinases PDK1 and Akt due to cell anti-apoptosis. However, tyrosinostat AG1024 can induce apoptosis and enhance radiosensitivity by downregulating the PI3K/Akt signal pathway. The oncogene product BCR–ABL constitutively activates the PI3K/Akt signal pathways (Vivanco & Sawyers, 2002; Sordet et al., 2003; Deutsch et al., 2004). Several studies show that BCR–ABL initiates a protective response to paclitaxel by influencing the expression or activity of downstream anti-apoptotic proteins, including Bcl-2, Bcl-xL, cytochrome c, and perhaps others (Amarante-Mendes et al., 1998; Samali et al., 1997; Van Antwerp et al., 1996). The aberrant expression of BCR–ABL-mediated PI3K/Akt signaling pathways (required for the control of cellular survival) may convey paclitaxel resistance. Thus, the use of selective inhibitors on specific signals to increase drug sensitivity and circumvent this type of resistance is the focus of this study. BCR–ABL-positive K562 is used as a CML model, and the therapeutic effects of the tyrosine kinase inhibitor AG1024 combined with paclitaxel are compared with the use of paclitaxel alone.

Material and methods

Cell culture

The human chronic myelogenous leukemia K562 cell line (ATCC, CCL-243) has the Philadelphia chromosome, which transcribes a gene that leads to the production of the 210-kD BCR–ABL fusion protein. Conversely, the promyelocytic leukemia HL-60 cells (ATCC, CCL-240) lack the Philadelphia chromosome and, therefore, produce no BCR–ABL fusion protein. The K562 and HL-60 cell lines were maintained in RPMI1640 culture mediums containing 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and supplemented with 10% heat-inactivated fetal calf serum (FCS). These cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂.

Drug preparation

Paclitaxel (Biomol GmbH, Hamburg, Germany) was dissolved in dimethylsulfoxide (DMSO) to make a 10 mM stock solution, which was diluted to the desired concentration using the complete medium. The DMSO concentration was kept below 0.05% (v/v) in all experiments without detectable effects on cell growth or apoptosis. The AG1024 (EMD Millipore Co., Billerica, MA, USA) was diluted with the 1%-FCS-containing culture medium to the desired concentration. When

AG1024 was used, the FCS concentration of the culture medium was limited to 1% in all combined treatments.

Liu's stain

After treatment, the cells were cytospun (500 rpm, 5 min) onto a glass slide and air dried. First, Liu A was added to the sample spot for 20 s and mixed with twice the volume of Liu B for another 2 min. Then, the slides were washed with water and air dried for observation using a microscope. Liu A and B were purchased from Yeong Jyi Chemical Apparatus Co., Ltd., Taipei, Taiwan.

MTT assay

The cells were harvested and seeded at 1×10^6 cells/well into 24-well plates to investigate the cell viability efficiency. The cells were treated with 0, 0.1 and 1 µM paclitaxel alone or in combination with 0, 2, 5 and 10 µM of AG1024 and incubated for up to 72 h. The old mediums were removed by centrifugation at 2000 rpm for 2 min, and the cells were supplemented with 500 µl/well MTT (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) working solutions (1 mg/ml). The plates were incubated at 37 °C for 4 h until the solution color changed from yellow to blue. Next, 2 ml of 0.04 N HCl in isopropanol was added to each well to prevent reactions and dissolve formazan. The 200 µl solution was transferred to a 96-well plate and the cell viability was quantified by measuring the absorbance ratio at 570/630 nm.

Flow cytometry of Sub-G₁ analysis

All cells (approximately 1×10^6 cells) were fixed in 70% ice-cold ethanol for at least 2 h and harvested by centrifugation at 1500 rpm for 5 min at 4 °C. The pellet was treated with 200 µg/ml RNase A (Sigma-Aldrich Co. LLC., USA) at room temperature for 30 min and incubated with 10 µg/ml propidium iodide for at least 10 min. The data acquisition was accomplished using the Cell Quest Pro software package after gating the cell populations, which were defined by their FL2-A characteristics. The fluorescence intensity was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using the Cell Quest Pro software (Becton Dickinson, San Jose, CA, USA). A minimum of 10,000 events were analyzed for each sample.

TUNEL assay

The TUNEL assay was used to measure the percentage of apoptosis. All cells (approximately 2×10^6 cells) were fixed in a 1% ice-cold paraformaldehyde for 15 min and re-fixed in 70% ice-cold ethanol for at least 2 h. The cells were washed with 1-fold PBS and re-suspended in a 50-µl terminal deoxynucleotidyl transferase (TdT) reaction mixture [0.5 µl TdT enzyme (Thermo Fisher Scientific Inc., Waltham, MA, USA), 10 µl of 5-times TdT buffer, 0.5 µl of biotin-11-dUTP (NEN Life Science Products, Inc., Boston, MA, USA), and 39 µl of ddH₂O]. This mixture was incubated at 37 °C for 30 min. Next, the cells were rinsed with cold 1-fold PBS and re-suspended in a 100 µl of a FITC-labeled avidin staining solution [2.5 µg/ml FITC-avidin (Sigma-Aldrich Co. LLC., St. Louis, MO, USA), 4-times standard saline citrate (SSC), 0.1% Triton X-100, and 5% nonfat milk] and incubated in the dark for 30 min at room temperature. Subsequently, the cells were rinsed with 1-fold PBS containing 0.1% Triton X-100 and treated with 1 ml of 1-fold PBS containing propidium iodide (5 µl/ml) and RNase A (100 µg/ml) for 30 min in the dark. The apoptotic cell populations were defined through their FL1 characteristics. The fluorescence intensity was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using Cell Quest Pro software (Becton Dickinson, San Jose, CA, USA). A minimum of 10,000 events were analyzed for each sample.

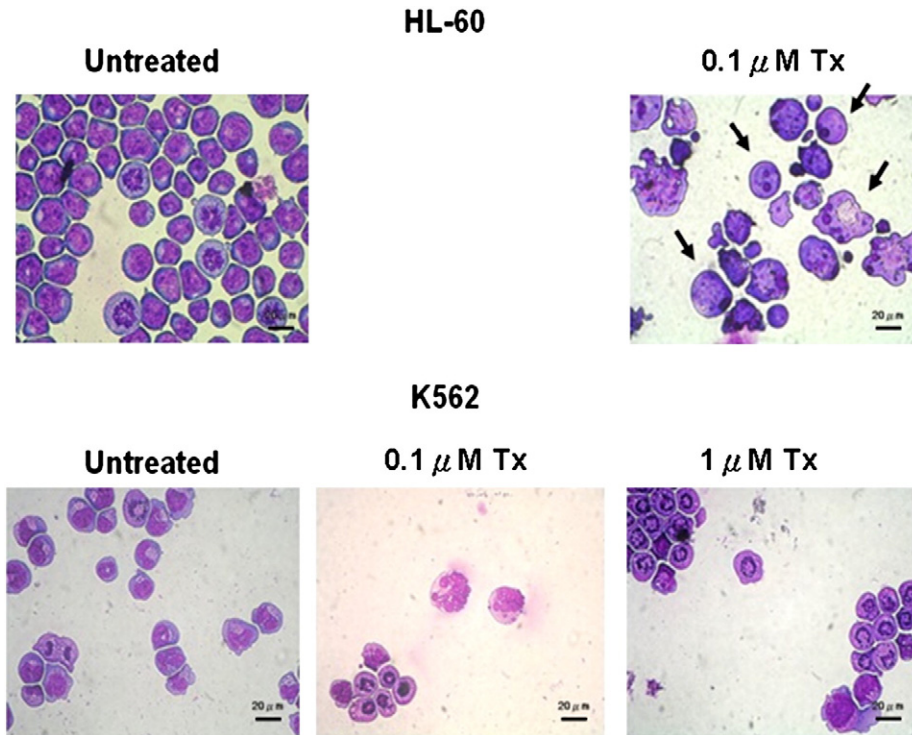


Fig. 1. Different effects of paclitaxel on the HL-60 and K562 cell lines. First, 1×10^6 cells of HL-60 or K562 were seeded into each well of a 24-well plate. The HL-60 cells were treated with 0 or 0.1 μM paclitaxel. The K562 cells were treated with 0, 0.1 or 1 μM paclitaxel. After 24 h of incubation, the cells were cytospun onto a glass slide and stained with Liu's stain. Untreated HL-60 and K562 cells with regular morphologies and mitotic figures were used as control groups. After the 0.1 μM paclitaxel treatment, the HL-60 cells showed clear signs of apoptotic bodies, nuclear condensation, fragmentation, and cell shrinkage (arrows). The K562 cells did not show apoptotic bodies even after a 1 μM paclitaxel treatment. Objects were magnified 400 \times , and all scale bars are 20 μm .

DAPI staining

The samples were collected and centrifuged at 1800 rpm for 5 min. The pellets were re-suspended in 1 ml of cold 1-fold PBS, transferred to an Eppendorf tube, and spun down at 4000 rpm for 5 min at 4 °C. The supernatant was discarded and the pellets were re-suspended in 1 ml of cold 1-fold PBS. Next, 10 μl of a 10% albumin solution (final conc. is 0.1%) was added, and the sample was incubated for 5 min at room temperature. The 100- μl sample was cytospun onto slides at a speed of 1200 rpm for 3 min. When the slides dried, 1% ice-cold paraformaldehyde was added and incubated for 30 min. The slides were washed three times with cold 1-fold PBS and dried. Thereafter, 50 μl of a DAPI (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) working solution (2.5 $\mu\text{g}/\text{ml}$) was added to each spot for 30 min in the dark. The slides were washed three times with cold 1-fold PBS and observed under the fluorescence microscope. The apoptotic cells exhibited the characteristic morphological changes, such as chromatin condensation and nuclear fragmentation, making them brighter than non-apoptotic cells after DAPI staining.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The samples were collected, and 1 ml of TRIzol® (Invitrogen Co., Carlsbad, CA, USA) was added to each sample to homogenize the cells. Additionally, 0.2 ml of chloroform was added to each sample and shaken vigorously by hand for 15 s. A total of 5 μg RNA was mixed with 1 μl oligo dT primer (Promega Co., Fitchburg, WI, USA) and an appropriate volume of DEPC water (maximum is 11.5 μl). The RNA samples were denatured at 70 °C for 10 min. Then, 4 μl of a 5 \times RT buffer, 2 μl of dNTP (Promega Co., Fitchburg, WI, USA), 0.5 μl of ribonuclease inhibitor (Thermo Fisher Scientific Inc., Waltham, MA, USA), and 1 μl of RevertAid™ M-MuLV

reverse transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA) were added to the mixture. The reaction was performed at 42 °C for 1 h followed by 95 °C for 5 min and cooled at 4 °C for 10 min. The cDNA product was stored at -20 °C for the PCR reaction. Next, 3 μl of cDNA were mixed with 0.5 μl of dNTP (Promega Co., Fitchburg, WI, USA), 2 μl Taq DNA polymerase 10 \times buffer containing 15 mM MgCl_2 (Promega Co., Fitchburg, WI, USA), 4 nmol of each 5' primer and 3' primer, and 0.5 μl Taq DNA polymerase (Promega Co., Fitchburg, WI, USA). Then, water was added to a final volume of 20 μl for the PCR reaction. The PCR products were examined using a 2% agarose gel with ethidium bromide. The oligonucleotide primer sequence designs were as follows: BCR-ABL primer *Forward*: 5'-ggagctgcagatgctgaccaac-3', *Reverse*: 5'-tcagacctgagctcaaagtc-3'; G3PDH primer *Forward*: 5'-accacagtcctatgccatcac-3', *Reverse*: 5'-tcaccacctgtgtgctga-3'.

Western blot assay

The cells were collected and washed with ice-cold 1-fold PBS, incubated in a lysis buffer at 4 °C for 30 min, and centrifuged at 12,000 rpm for 10 min at 4 °C. Then, the supernatant was transferred to a new tube and stored at -80 °C. The protein concentration was determined using the standard Bradford method (Bio-Rad Laboratories Inc., Hercules, CA, USA). Equal amounts of the total protein were prepared from different cell lysates. The samples were mixed using a vortex mixer, and the proteins were denatured in boiling water for 5 min. The SDS-polyacrylamide gel (5% or 12% separating gel and 4% stacking gel) was prepared using a Hoefer Dual gel caster. The proteins were separated on 5% (for BCR-ABL) or 12% (for caspase 3, Bcl-xL and Bcl-2) SDS-polyacrylamide gel with different markers (206 kD for the BCR-ABL; 170 kD for the others). Electrophoresis was performed

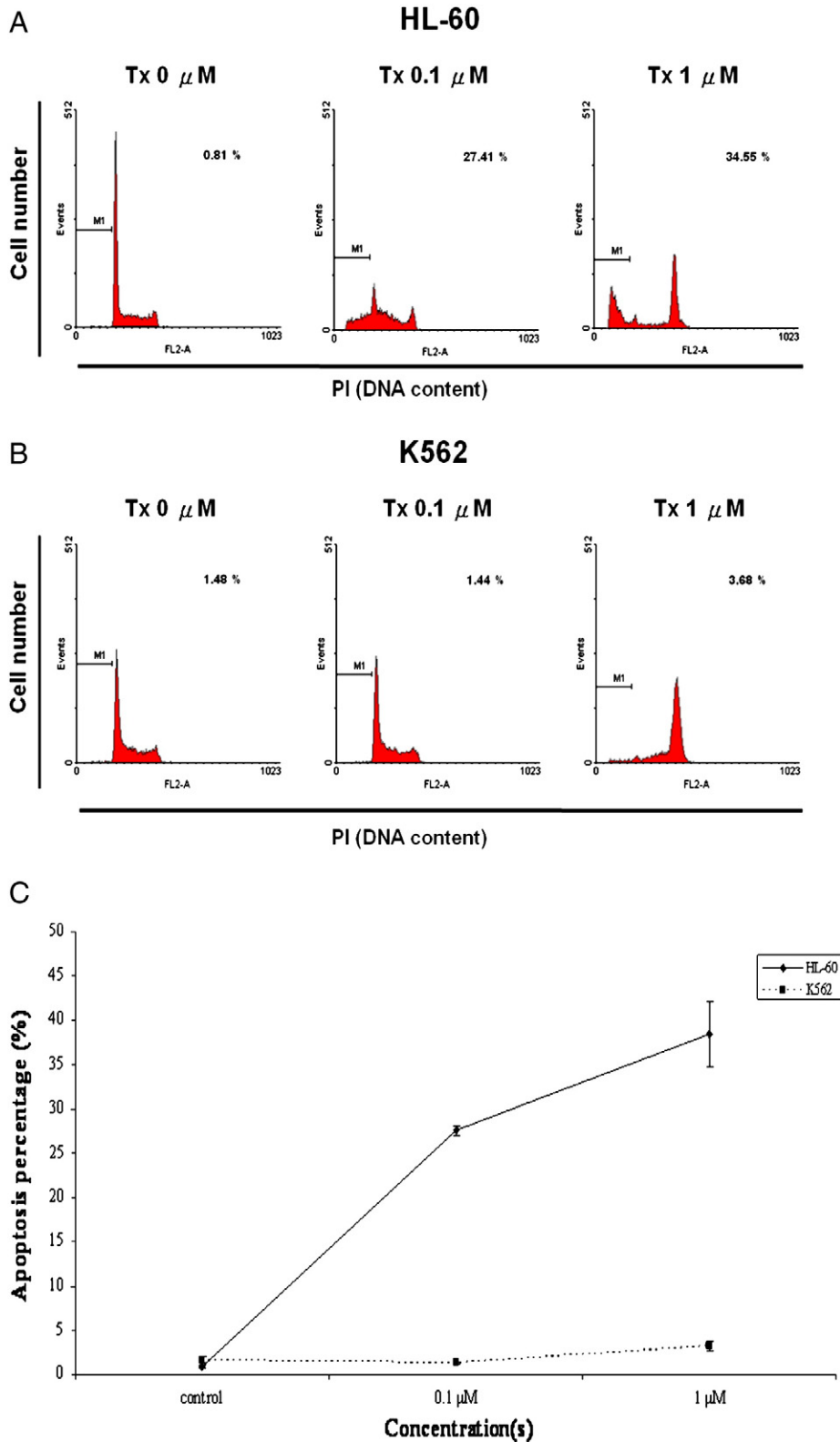


Fig. 2. Paclitaxel-induced apoptosis in HL-60 and K562 detected using flow cytometry. First, 1×10^6 cells of HL-60 or K562 were seeded in each well of a 24-well plate. Both groups were only treated with paclitaxel at different concentrations (0, 0.1 or 1 μ M) for 24 h. The cells were collected and analyzed using FACS to detect their apoptosis percentage through the sub-G₁ population: (A) HL-60; (B) K562; and (C) the percentage of apoptosis in both cell lines. The data are shown as the means \pm S.D., and the experiments were performed at least three times.

at room temperature and 80 V for 15 min and 100 V for 1 h. The proteins were transferred to Immobilon™-P transfer membranes via a wet transfer system. The primary antibodies for those targets were followed by datasheet working dilution from 1/500 to 1/1000, and the second

antibodies were applied at 1/10,000 dilution. Then, the antibodies were detected using the ECL Western Blot Chemiluminescence Reagent Plus (PerkinElmer Inc., Waltham, MA, USA) and quantified using the Image Quant software and Personal Densitometer SI.

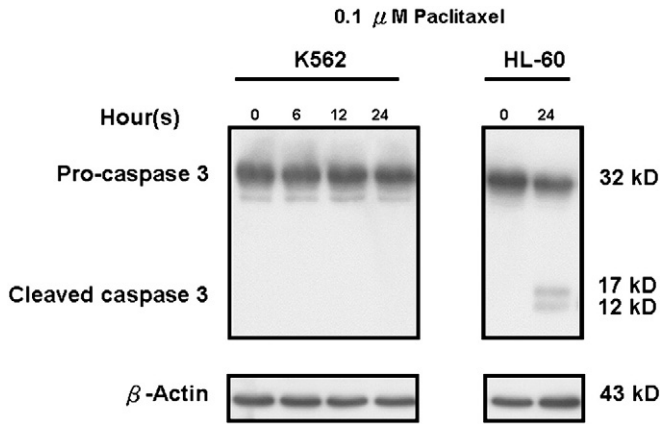


Fig. 3. The activation of caspase 3 after treatment with 0.1 μM paclitaxel in both cell lines. First, 2×10^6 cells of HL-60 or K562 were treated with 0.1 μM paclitaxel and incubated for 24 h. The cell lysates and 20 μg of the total proteins were loaded into each well of the SDS-PAGE. The protein lysates were analyzed with anti-caspase 3 antibodies. The detection of the cleaved 17 kD and 12 kD fragments indicated caspase 3 activation.

Statistical analysis

The data were presented as the mean values ± S.D. The statistical validity was evaluated using Student's *t*-test, and *p* values ≤ 0.001 were considered significant.

Results

The human chronic myelogenous leukemia K562 cell line is resistant to paclitaxel-induced apoptosis

Two human leukemic cell lines differing in sensitivity to paclitaxel-induced apoptosis were used to determine whether the level of BCR-ABL expression is a key determinant of resistance to paclitaxel-induced apoptosis. HL-60 cells are sensitive to paclitaxel undergoing dose-dependent apoptosis. Most HL-60 cells are apoptotic (arrows) after 24

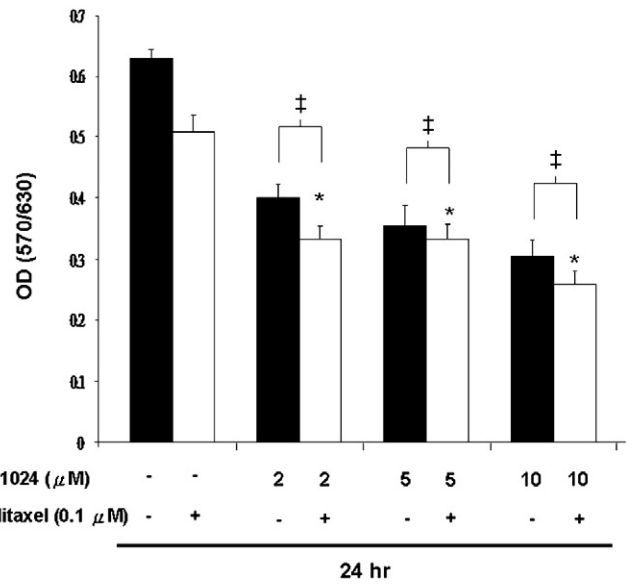
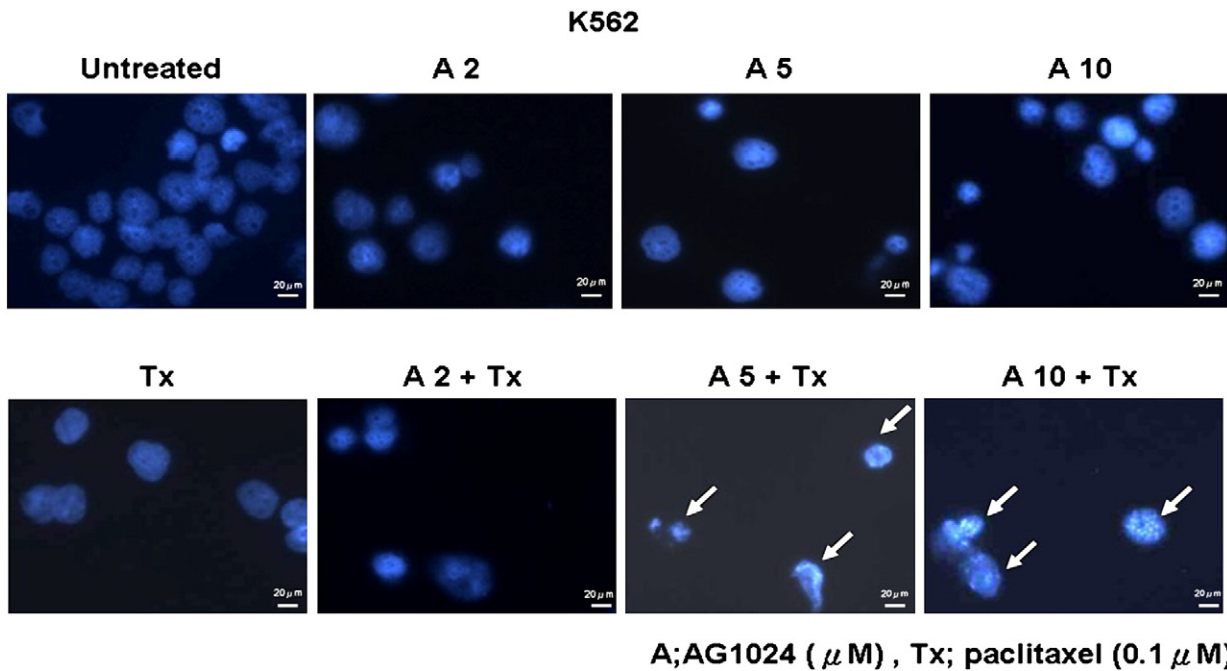


Fig. 5. The survival of the K562 cells under the treatment of both paclitaxel and AG1024. First, 1×10^6 cells of K562 were seeded in each well of a 24-well plate and were treated with only paclitaxel (0.1 μM) or mixed with AG1024 (2, 5 or 10 μM) for 24 h. The proliferation activity of the cells was detected using an MTT assay. *: Only paclitaxel, *p* < 0.001; †: Only AG1024, *p* < 0.001; the data are shown as the means ± S.D., and the experiments were performed at least three times.

h of Liu's staining. Conversely, the K562 cells are highly resistant to paclitaxel-induced apoptosis, showing little or no apoptosis after 24 h treatment, even in the presence of 0.1 μM paclitaxel (Fig. 1).

The BCR-ABL positive cells show a low percentage of apoptosis during paclitaxel-induced apoptotic stress

Two paclitaxel concentrations (0.1 or 1 μM) were used to treat the HL-60 and K562 cells. At 24 h post-treatment, the apparent sub-G₁



A; AG1024 (μM), Tx; paclitaxel (0.1 μM)

Fig. 4. The morphological changes in K562 after paclitaxel treatment combined with AG1024. At 24 h post-treatment, cells with AG1024, paclitaxel, or both were harvested and stained with DAPI. The K562 cells were only treated with 0.1 μM paclitaxel or mixed with AG1024 (2, 5 or 10 μM) for 24 h. The addition of AG1024 to the paclitaxel treatment significantly enhanced the cell death program (arrows) compared to the paclitaxel-only treatment. Objects were magnified 400×.

populations were detected in the DNA-content based assay of BCR–ABL-negative HL-60 cells (Fig. 2A). The percentage of apoptotic HL-60 increased in a dose-dependent manner and was significantly higher than the apoptosis percentage of the BCR–ABL-positive K562 (Fig. 2C). Conversely, significant sub-G₁ populations were not observed in the K562 cell line (Fig. 2B). The percentage of apoptosis remained below 5% even after treatment with 1 μM paclitaxel (Fig. 2C). The western blot results indicated that caspase 3 was not activated in the K562 cells during the 24 h treatment (Fig. 3).

Combination of tyrphostin AG1024 with paclitaxel sensitizes human chronic myelogenous leukemia K562 cell line to paclitaxel-induced apoptosis

After 24 h of incubation with AG1024 (2, 5 or 10 μM), paclitaxel (0.1 μM), or both, the cells were harvested and stained with DAPI. The HL-60 cells were positive controls of paclitaxel-induced apoptosis in the presence of 0.1 μM paclitaxel. With combined treatment, paclitaxel induced apoptosis in the majority of K562 cells (Fig. 4) and significantly

suppressed cell proliferation activity with increased tyrphostin AG1024 concentration (Fig. 5). Furthermore, the TUNEL assay showed that as more tyrphostin AG1024 was added, more paclitaxel-induced apoptotic effects were observed (Fig. 6).

Tyrphostin AG1024 downregulates the BCR–ABL expression in a dose-dependent manner in K562 cells

The RT-PCR method was used to assess the expression level of the BCR–ABL mRNA in the K562 cells after treatment with different concentrations of tyrphostin AG1024. The expression of BCR–ABL mRNA was downregulated by tyrphostin AG1024 (Fig. 7A). Furthermore, the relative expression of the mRNA was significantly downregulated compared to the paclitaxel-only treatment (Fig. 7B). The immunoblot assay was used to confirm the expression of BCR–ABL fusion protein in the K562 cells (Fig. 8A). Furthermore, tyrphostin AG1024 combined with paclitaxel was more effective at lowering BCR–ABL expression in both the mRNA level and the protein product (Figs. 7B, 8B).

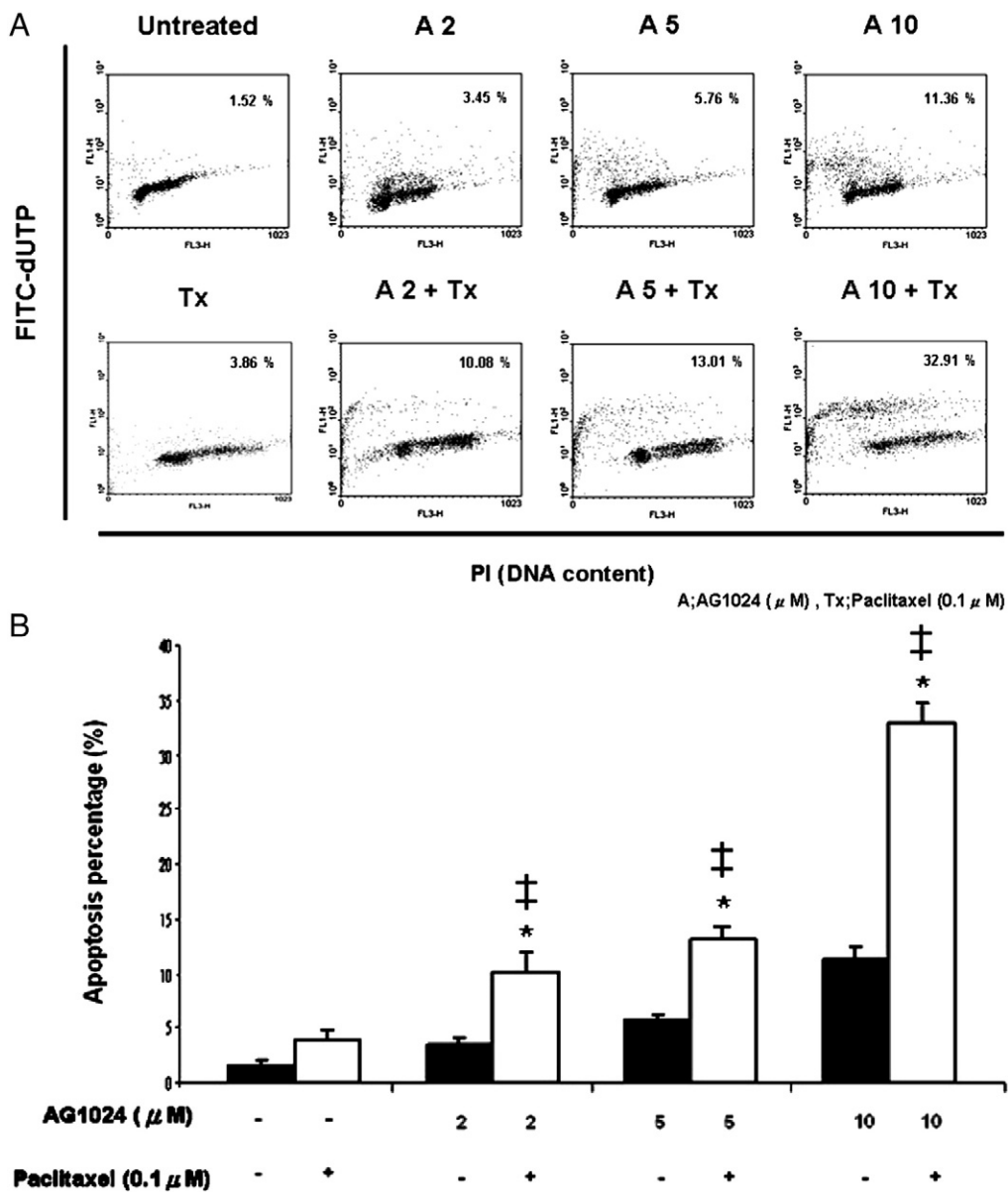


Fig. 6. Apoptosis induced by the combination treatment of paclitaxel and AG1024 detected using the TUNEL assay. First, 2×10^6 K562 cells were treated with paclitaxel (0.1 μM), AG1024 (2, 5 or 10 μM), or both and incubated for 24 h. The cells were harvested and stained with the TUNEL assay. The percentage of apoptosis was analyzed using FITC-dUTP-incorporated population analyses. (A) Flow cytometry analysis of apoptotic cells is shown as percentages in each plot. (B) Quantification of apoptotic cells is shown as percentages in the bar chart. *: Only paclitaxel, $p < 0.001$; †: Only AG1024, $p < 0.001$; the data are shown as the means \pm S.D. The assay was performed at least three times.

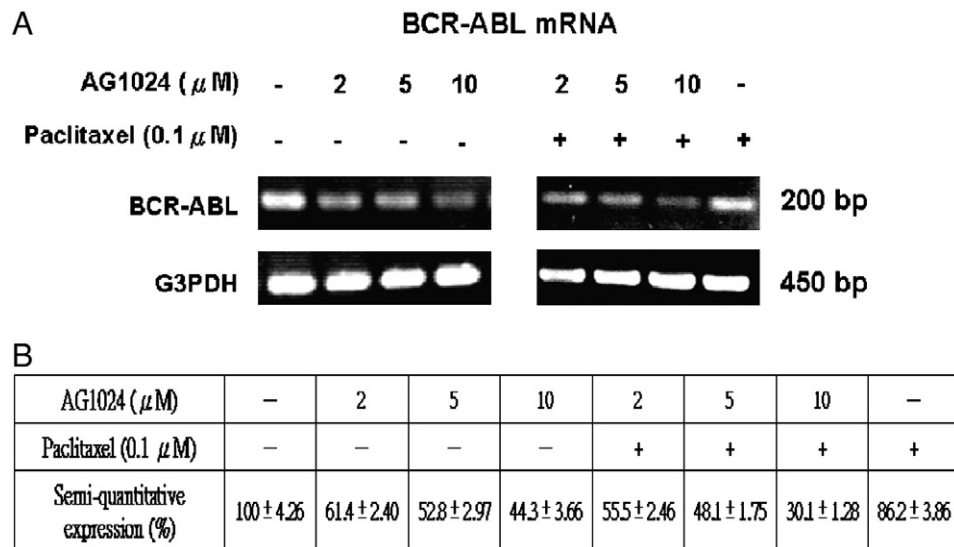


Fig. 7. The AG1024 inhibits BCR-ABL gene expression. (A) At 24 h post-treatment, the RT-PCR assay was used to detect the BCR-ABL gene expression (upper row) of the 1×10^6 K562 cells. The total RNA was purified, and RT-PCR was performed using the procedure described in the “Material and methods” section. The PCR product was separated using a 2% agarose gel, and the products were measured by the density in the photograph. (B) The percentage of semi-quantitative gene expression. The AG1024 downregulates BCR-ABL mRNA in a dose-dependent manner. The BCR-ABL gene expression level was not affected by paclitaxel (0.1 μM) treatment for 24 h. The data are shown as the means \pm S.D. The assay was performed at least three times.

The anti-apoptosis factors are dose-dependently downregulated by the tyrphostin AG1024 treatment

Further experiments were designed to measure the expression of anti-apoptosis factors downstream of BCR-ABL. The lower expression of anti-apoptosis factors conveys the sensitivity of leukemic cells to apoptosis induced by various stimuli. The western blot assay indicated that the Bcl-2 expression levels were downregulated by tyrphostin AG1024 in a dose-responsive manner (Fig. 8A). The expression of other anti-apoptosis factors such as Bcl-xL had similar results (Fig. 8A). The statistical analysis indicated that both had similar patterns of combination treatment (Fig. 8B), which may correlate to AG1024 inhibiting the BCR-ABL expression.

The combination of tyrphostin AG1024 with paclitaxel leads to dose-dependent sensitization of the K562 cells to paclitaxel-induced apoptosis

Cleaved caspase 3 was examined as a marker protein for cell apoptosis to further demonstrate whether the lower level of BCR-ABL expression induced by AG1024 can reverse the K562 cell resistance to paclitaxel-induced apoptosis. The immunoblotting indicated that the combination with tyrphostin AG1024 induced remarkable caspase 3 activation at 24 h post-treatment (Fig. 9). The TUNEL assay showed that as more tyrphostin AG1024 was added, more paclitaxel-induced apoptotic effects were observed. These results suggest that paclitaxel-induced apoptosis is consistent with the BCR-ABL expression level.

Discussion

The t(9;22) chromosomal translocation found in the human CML K562 cell line expresses the fusion oncoprotein p210 BCR-ABL, which exhibits dysregulated c-Abl tyrosine kinase activity (Ben et al., 1986; Mes-Masson et al., 1986). Substantial evidence indicates that BCR-ABL is an apoptotic suppressor. Its activity generates resistance to apoptosis induced by serum deprivation, irradiation, and chemotherapeutic agents (Cortez et al., 1995; Cortez et al., 1996; Jamieson et al., 1999; Murray & Fields, 1997). BCR-ABL can occasionally cause problems because of resistance to certain antineoplastic drugs, such as paclitaxel, especially for CML chemotherapy. A different strategy was used in this

study to overcome the K562 resistance: the combination of tyrosine kinase inhibitor AG1024 and paclitaxel.

First, two different cell models were studied: the BCR-ABL-negative cell, HL-60, and the BCR-ABL-positive cell, K562. Both cell lines were treated with various concentrations of paclitaxel for 24 h. The morphological manifestations indicated that the human K562 chronic myelogenous leukemia cells resisted the paclitaxel-induced apoptosis, but the BCR-ABL negative cell line HL-60 was not resistant to paclitaxel-induced apoptosis (Fig. 1). FACS showed the percentage of BCR-ABL that possessed resistance, and two doses of paclitaxel (0.1 or 1 μM) were added to treat the HL-60 and K562 cells for 24 h. The data showed that the BCR-ABL-negative cells were sensitive to paclitaxel-induced apoptosis, and nearly 40% of the cells were dead (Fig. 2A, C). Conversely, the percentage of paclitaxel-induced apoptosis was below 5% under the 1- μM paclitaxel treatment or not observed in the BCR-ABL-expressing cells (Fig. 2B, C). The immunoblot analysis using monoclonal anti-caspase 3 antibodies indicated that 0.1 μM paclitaxel did not trigger the caspase 3 activation in the K562 cells during the 24 h incubation, but this was clearly observed in the BCR-ABL-negative cells at 24 h post-treatment (Fig. 3). These data imply that BCR-ABL expression protected the cells from paclitaxel-induced apoptosis through an unknown mechanism.

A novel tyrosine kinase inhibitor, AG1024, was introduced to enhance the paclitaxel effect on K562. After 24 h of incubation with AG1024 (2, 5 or 10 μM), paclitaxel (0.1 μM) or both, the K562 cells were examined for signs of apoptosis through nuclear fragmentation. DAPI staining of the nuclear integrity indicated that the combination of tyrphostin AG1024 with paclitaxel sensitized the K562 cells to paclitaxel-induced apoptosis. This phenomenon was present in a dose-dependent manner (Fig. 4). The co-treatment of the inhibitor significantly suppressed cell proliferation and the proliferation ability decreased as tyrphostin AG1024 was increased (Fig. 5), and the TUNEL assay indicated that the combination treatment was more effective than the single treatment for inducing apoptosis (Fig. 6).

The BCR-ABL fusion protein has an important role in the resistance to paclitaxel. Therefore, BCR-ABL expression was examined under treatment. The RT-PCR method was used to detect the gene expression level of BCR-ABL. After 24 h of treatment with various concentrations, the AG1024 significantly downregulated the BCR-ABL gene expression. Conversely, paclitaxel exerted minor effects on the BCR-ABL gene

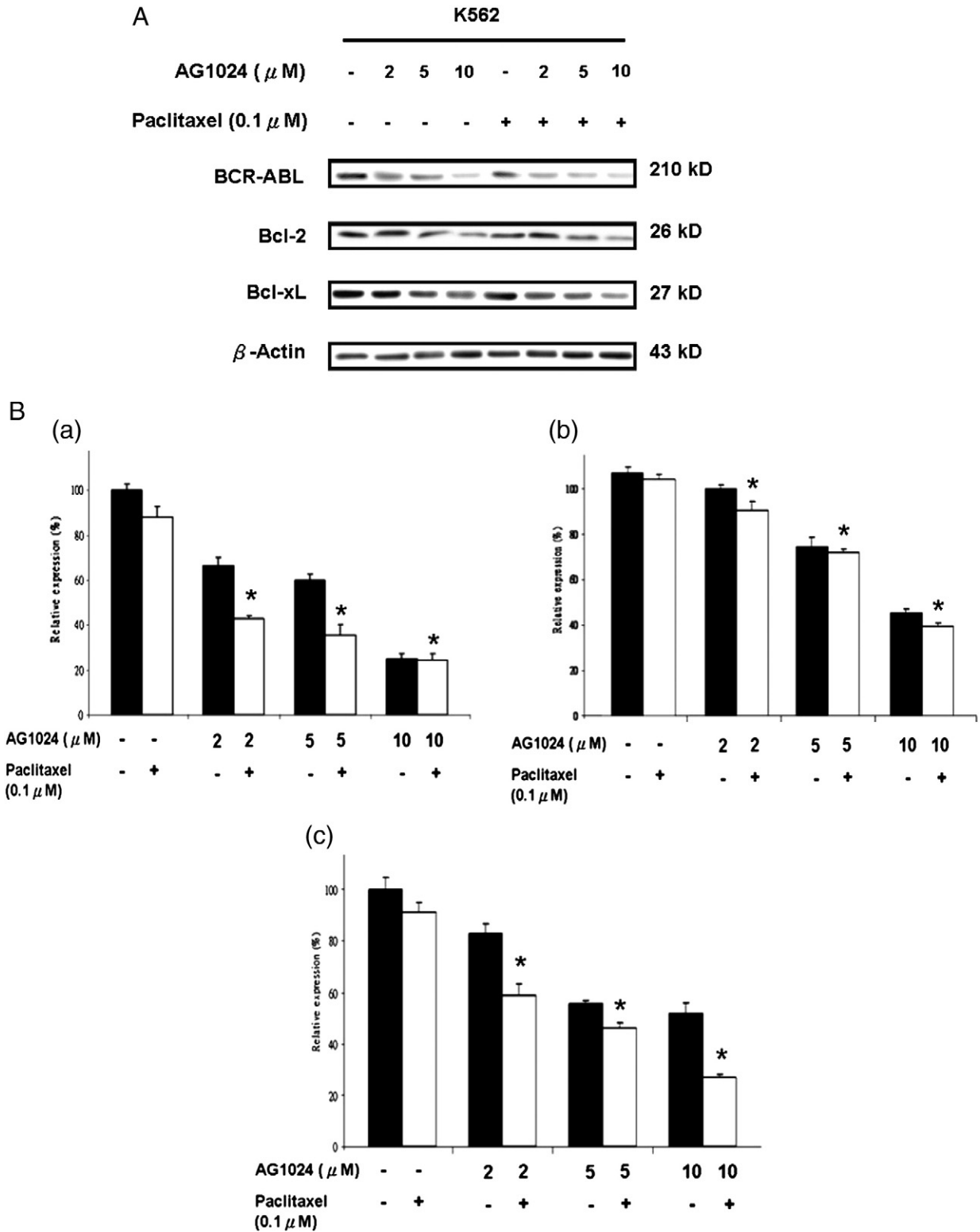


Fig. 8. Inhibitory effect of AG1024 on BCR-ABL, Bcl-2 and Bcl-xL protein expression. First, 2×10^6 K562 cells were treated with paclitaxel (0.1 μM), AG1024 (2, 5 or 10 μM), or both and incubated for 24 h. The protein lysates were analyzed with anti-BCR-ABL, anti-Bcl-2 and anti-Bcl-xL antibodies, respectively. (A) The western blot results of BCR-ABL, Bcl-2 and Bcl-xL, respectively. (B) A bar chart of the relative protein expression percentage. (a): BCR-ABL; (b): Bcl-2; (c): Bcl-xL; *: Only paclitaxel, $p < 0.001$. The data are shown as the means \pm S.D. The assay was performed at least three times.

expression in the K562 cells (Fig. 7). Although the mRNA was downregulated, the consistency of the protein expression with the mRNA level was not guaranteed because the protein was responsible for the biological effects of BCR-ABL. Thus, the western blot was used to analyze the BCR-ABL protein expression in the K562 cells after treatment. Western

blotting results showed similar patterns to those of the RT-PCR at 24 h post-combination treatment (Fig. 8). These data indicated that K562 cells resistant to paclitaxel-induced apoptosis can be reversed through the downregulation of the BCR-ABL gene and the suppression of its expression.

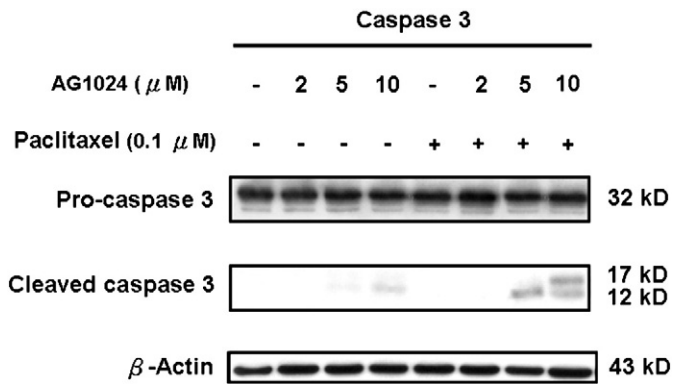


Fig. 9. The activation of caspase 3 under a combination treatment of paclitaxel and AG1024. The 2×10^6 K562 cells were treated with paclitaxel (0.1 μM), AG1024 (2, 5 or 10 μM), or both and incubated for 24 h. The cell lysates and 20 μg of the total proteins were loaded into each well of the SDS-PAGE. The protein lysates were analyzed using anti-caspase 3 antibodies. The detection of the 17 kD and 12 kD fragments indicated caspase 3 activation.

Recent studies indicated that the oncogene product BCR–ABL may constitutively activate the PI3K/Akt signal pathway (Sordet et al., 2003; Deutsch et al., 2004; Amarante-Mendes et al., 1998) and that the overexpression of a catalytically active subunit of PI3K in ovarian cancer cells induces paclitaxel resistance, which is reverted upon inhibition of the PI3K pathway using a selective inhibitor. Hence, the combination treatment's anti-apoptosis factor expression effect downstream of the PI3K/Akt signal pathway is a concern. Two major anti-apoptosis factors were examined: Bcl-2 and Bcl-xL. The lower expression of anti-apoptosis factors conveys the sensitivity of leukemic cells to apoptosis induced by various stimuli. The immunoblot analysis indicated that the expression levels of Bcl-2 and Bcl-xL were downregulated by tyrphostin AG1024 in a dose-responsive manner. Both factors exhibited similar trends and were correlated with the BCR–ABL expression level (Fig. 8). This result suggests that the inclusion of an inhibitor may not only suppress the BCR–ABL protein but also lower the PI3K/Akt signal activation.

Finally, the combination of the protein tyrosine kinase inhibitor AG1024 with paclitaxel enhanced cell program death in the K562 cells. Two approaches were used to show this result: the western blot assay of caspase 3 activation indicates that the combination treatment reversed the paclitaxel-resistance of K562 cells in the first 24 h (Fig. 9), and the TUNEL assay indicates that the combined treatment was more effective than the single treatment for inducing apoptosis.

This study provides a rational basis for circumventing the CML resistance during chemotherapy by combining the use of the tyrosine kinase inhibitor AG1024 with paclitaxel in curative or preventive settings.

Conflict of interest statement

The authors declare no conflict of interest.

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