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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.

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 tyrphostins have been synthesized and identified as potent protein tyrosine kinase (PTK) inhibitors. Different members of the tyrphostin 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, tyrphostin 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% CO_2.

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-G_1 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 ddH_2O]. 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.