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Antitumor activity of a novel *bis*-aziridinylnaphthoquinone (AZ4) mediating cell cycle arrest and apoptosis in non-small cell lung cancer cell line NCI-H460[1]

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Key words

cell cycle; apoptosis; Bcl-2 protein; aziridinylnaphthoquinone

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Abstract

Aim: The cytotoxic activities of a series of *bis*-aziridinylnaphthoquinone, AZ1 to AZ4, on human lung carcinoma cell lines, H460, and normal lung cells fibroblast cell line, MRC-5, and the mechanisms of H460 cells induced by AZ4 were investigated. **Methods:** The MTT assay was used to determine the cell proliferation. Cell cycle was analysed by FACS. The activity of caspase 3, 8 and 9 was determined by cell-permeable fluorogenic detection system. Western blot assay was used to evaluate the regulation of cyclin B, Cdc-2, p53, p21, and the Bcl-2 protein. **Results:** AZ1 to AZ4 displayed various cytotoxicity activities against H460 and MRC-5 cells. Compared to those compounds, AZ4 was with the most effective agent among the 5 tested analogues at reducing H460 cell viability with an IC_{50} value of 1.23 μmol/L; it also exhibited weak cytotoxicity against MRC-5 cells with an IC_{50} value of 12.7 μmol/L. The results show that growth arrest on the G_2-M phase of H460 cells induced by AZ4 for 24 h was discovered, and this might be altered with the reduced Cdc-2 protein expression of 47% at 2.0 μmol/L AZ4, but not with cyclin B protein expression. The AZ4 treated cells were then led to apoptosis after 48 h. This was associated with the activation of apoptotic enzyme caspase 3 and mediated by caspase 8, but not caspase 9 at various concentrations of AZ4 after being cultured for 48 h and 30 h, respectively. The anti-apoptotic protein (Bcl-2) expression in H460 cells altered by 39% with downregulation, and the p53 protein by 25% with upregulation after being cultured with 2.0 μmol/L AZ4 for 48 h. In a time-dependent wanrer, the expression of the p53 and p21 proteins were increased to the maximum at 24 h, and then decreased at 48. **Conclusion:** AZ4 represents a novel antitumor aziridinylnaphthoquinone with therapeutic potential against the non-small cell lung cancer cells.

Introduction

Despite advances in cancer treatment in the past 2 decades, the prognosis of lung cancer patients has improved minimally. Lung cancer is the leading cancer killer in both men and women. There was an estimated 171 900 new cases of lung cancer and an estimated 157 200 deaths from lung cancer in the Unite States in 2003[1]. There are 2 types of lung cancer cells, small cell lung cancer and non-small cell lung cancer. Non-small cell lung cancer (NSCLC) heterogeneously aggregates at least 3 distinct histological lung cancers, including epidermoid or squamous carcinoma, adenocarcinoma, and large cell carcinoma. NSCLC is the most common histological cell type of all lung cancers, and there is no curative treatment available for the advanced stages of these diseases. Although chemotherapy is an established treatment for advance NSCLC, it offers only a limited

survival benefit at the expense of substantial toxicity, drug resistance, and poor target specificity. Thus, there is a need for innovative strategies that effectively treat patients and exhibit more favorable safety profiles in advanced NSCLC.

The ideal bioreductive drug should be administered as an inactive prodrug that is only activated under low-oxygen conditions by 1 or 2 electron reductases[2–4]. Aziridine-substituted benzoquinone, such as mitomycin C, RH1, and tirapazamine (TPZ), are 3 principal aziridinyl quinone classes of hypoxia-specific cytotoxins that are being developed for clinical use[5–7]. In the case of di-aziridinyl-substituted quinone, this highly cytotoxic, bifunctional, alkylating agent can cross-link DNA in cells which results in inducing complicated cellular mechanisms leading to cell death by apoptosis such as TPZ and CI-1010 or necrosis[8,9].

Tumor tissue had lower oxidative reduction (redox) potential relative to most normal tissues which could increase the reductive activation of these quinone derivatives in tumors[2]. Therefore, the selectivity of bioreductive drugs is governed not only by the difference in oxygen tension between tumors and normal tissues, but also by levels of enzymes catalyzing bioreductive activation such as DT-diaphorase[3,10–11]. This fact led to the 1990 publication of the concept of "enzyme-directed bioreductive development" by Workman and Walton[12].

In this study, one of these *bis*-aziridinylnaphthoquinone analogues, designated as AZ4 (Figure 1) was found to inhibit NSCLC cell growth *in vitro*. The mechanism of AZ4 to the NSCLC cell H460 was investigated, including cell cycle arrest and apoptosis.

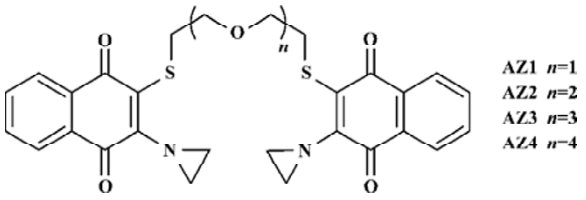

Figure 1. Chemical structures of AZ1 to AZ4.

Materials and methods

Synthesis of AZ1 to AZ4 AZ1 to AZ4 were prepared by following our previously published methods[13]. Stock solutions (10 mmol/L) were freshly prepared in DMSO (Sigma, St Louis, MO, USA) and the series was diluted and directly added into the cultured cells from 20 μmol/L to 0.2 μmol/L.

Cell culture The cell line H460 (human NSCLC) was cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), 2 mmol/L *L*-glutamine, 25 mmol/L hydroxyethyl-1-piperazine-ethanesulfonic acid (HEPES). The normal cell MRC-5 (human fetal lung fibroblast cell line) was cultured in Dulbecco's modified eagle's medium (DMEM) with 10% FBS, 2 mmol/L *L*-glutamine, and miminal essential medium (MEM) non-essential amino acid. The cell culture medium for those two cell lines all contained penicillin-streptomycin and fungizone. All the medium and supplements were purchased from Gibco Laboratories (Grand Island, NY, USA). All of the cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The amount of cells was counted after trypsinization by a Neubauer hemocytometer (VWR, Scientific Corp, Philadelphia, PA, USA).

Cytotoxicity assay (MTT assay) The MTT assay was according to the method of Skehan *et al*[14]. Two cell lines were seeded in 96-well flat-bottomed microtiter plates (3000–5000 cells/well), respectively. The cells were incubated for 24 h with drugs, applied as serial 1:2 dilutions (100 μL/well) ranging from 20 μmol/L down to 0.2 μmol/L or 0.1% DMSO as control. Twenty microliters of MTT (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. The formazan product was dissolved by adding 100 μL DMSO to each well, and the plates were read at 550 nm. All measurements were performed in triplicate and each experiment was repeated at least 3 times. The IC_{50} was calculated from the 50% formazan formation compared with the control without the addition of drugs.

Cell cycle analysis The H460 cells were serum starved without FBS overnight to synchronize cell phase, then the cells were treated with various concentrations of AZ4 for 24 h. The cells were harvested and incubated with hypotonic staining buffer [0.1% sodium citrate, 0.3% triton X-100, 0.01% propidium iodide (PI), and 0.01% ribonuclease A] for 15 min on ice in the dark. The DNA content was measured by a Becton Dickinson FACScan using Cell Quest software and analyzed using EXPO 32 and MultiCycle software (Beckman Coulter, High Wycombe, UK).

Microscopy image of caspase 3, 8, and 9 activity in H460 cells The method was according the report of Komoriya *et al*[15] with minor modifications. Briefly, the H460 cells were incubated with fluorogenic caspase substrates (PhiPhhiLux-G1D2, CaspaLux 8-L1D2, and CaspaLux 9-M1D2, OncoImmunin Inc, Gaithersburg, MD, USA), GDEVDGI, IETDGI, and LEHDGI at 10 μmol/L respectively, while suspended in RPMI-1640 plus 10% FBS, 10 mmol/L HEPES, and various concentrations of AZ4 for 24 h, 30 h(for caspases 8 and 9 only), and 48 h incubation. Substrates were present at 10 μmol/L throughout the course of the induction and imaging. The cells were viewed on a Nikon fluorescence microscope sys-

tem (Tokyo, Japan). Samples were excited using a 488/518 nm krypton/argon laser, and fluorescent images were acquired. The brightness/contrast settings were adjusted so that the fluorescent signal of cells without AZ4 was near the background. As the fluorogenic caspase substrates are cleaved in apoptotic cells, cellular fluorescence shifts from below to above the fluorescence of the bulk solution in the same plane.

Western blot analysis of cyclin B, Cdc-2, p53, p21, and the Bcl-2 protein The method used was that of Bacus *et al* and slightly modified[16]. Briefly, the cells were collected from a 100 mm cultured dish after being challenged by various concentrations of the AZ4 compound at 24 and 48 h, respectively. The cell pellets were spun down by centrifugation at 1000×*g* for 20 min. The pellets were resuspended in cold buffer (10 mmol/L HEPES, pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L diethiotreitol (DTT), 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, 30 mg/mL leupeptin, 5 mg/mL aprotinin, and 5 mg/mL pepstatin A, and incubated on ice for 5 min. The cells were broken down by lysis buffer and a sonicator. Cell lysates (25 μg) was separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Amersham, Buckingshamshire, UK). The blots were incubated with blocking buffer (11 mmol/L Tris-vase pH7.4, 154 mmol/L NaCl, and 5% skim milk), washed with washing buffer (11 mmol/L Tris-vase pH 7.4, 154 mmol/L NaCl, and 0.1% Tween-20), and incubated with specific antibodies against specific proteins cyclin B, Cdc-2, p53, p21, and Bcl-2. Each membrane was blocked in blocking buffer prior to incubation with antibodies. The primary antibody against cyclin B, Cdc-2, p53, p21, and Bcl-2, all belong to the mouse antihuman monoclonal antibody (Imgenex Co, San Diego, CA, USA). The secondary antibody (Jackson Immuno Research Lab Inc, West Grove, PA, USA) conjugated with horseradish peroxidase was added at an appropriate dilution by blocking buffer. The dilution factors for the primary antibodies were 1:500–1:1000 depending on the amount of proteins, and the dilution factors for the secondary antibodies were 1:2000. The primary β-actin antibody used was a mouse monoclonal antibody with the dilution factor of 1:10 000 (Biogenesis, England, UK). Immunodetec-tion was carried out using the enhanced chemiluminescence (NEN, Boston, MA, USA) detection system. To quantify the amount of protein expression achieved, we measured the intensity of chemiluminescence of the second antibody using a densitometer (BioRad Gel Doc 2000 software) and analyzed using Gel Doc (Hercules, CA, USA). The values in the relative protein interested expression-quantifying table represent the relative amount of protein expression in respect to β-actin expression divided by its control.

Results

Cytotoxic activity evaluation The chemical structures of AZ1 to AZ4 are shown in Figure 1. The chemical structure of AZ1 to AZ4 was composed of 2 aziridinylnaphthoquinones bridging by ethylene glycol units. AZ1 had the shortest ethylene glycol units; in retrospect, AZ4 contained the 4 ethylene glycol units. The cytotoxic activities of AZ1 to AZ4 against NSCLC H460 cells were evaluated; the human fetal lung fibroblast cell line (MRC5) was used as a normal cell control, and their IC_{50} values are listed in Table 1. The *bis*-aziridinylnaphthoquinone AZ1 to AZ4 were effective cytotoxic agents against H460 cells with IC_{50} values ranging from 1.23 to 7.21 μmol/L. The normal lung fibroblast cells MRC-5 were less sensitive to AZ1 to AZ4 with IC_{50} values of 2.8 to 12.7 μmol/L, respectively. The correlation between the cytotoxic activities and the linker distance between 2 aziridinylnaphthoquinone moieties against H460 cells was directly in proportion, but not with the MRC-5 cells. Initial characterization indicated that AZ4 was the most effective compound at inhibiting the growth of H460 cells and was less effective with MRC5 cells.

Table 1. Cytotoxicity of H460 and MRC-5 cell lines induced by AZ1 to AZ4 analyzed by MTT assay and expressed as IC_{50} (μmol/L). n=4. mean±SD.

	AZ1	AZ2	AZ3	AZ4
H460	7.21±0.15	2.10±0.11	1.87±0.02	1.23±0.12
MRC-5	10.22±0.23	2.82±0.13	3.02±0.16	12.71±0.18

Induction of the G_2-M cell cycle arrest and the effects on the expression of cell cycle-related proteins in H460 cells treated with AZ4 An analysis of DNA content was used to determine whether cell arrest was induced by AZ4. A DNA content analysis by a flow cytometer indicated that H460 cells had a significant population of cell arrest in G_2-M treated with AZ4 in 24 h compared with 0.1% DMSO alone (Figure 2). The peak area under the G_2-M phase started to ascend as low as 0.5 μmol/L with AZ4. The cell cycle-related proteins associated with mitotic, cyclin B and Cdc-2, in the AZ4-treated H460 cells for 24 h were also investigated (Figure 3, Table 2). The expression pattern of cyclin B in the cells remained basically unaltered with increased concentrations of AZ4 treatment; however, the expression pattern of Cdc-2 was

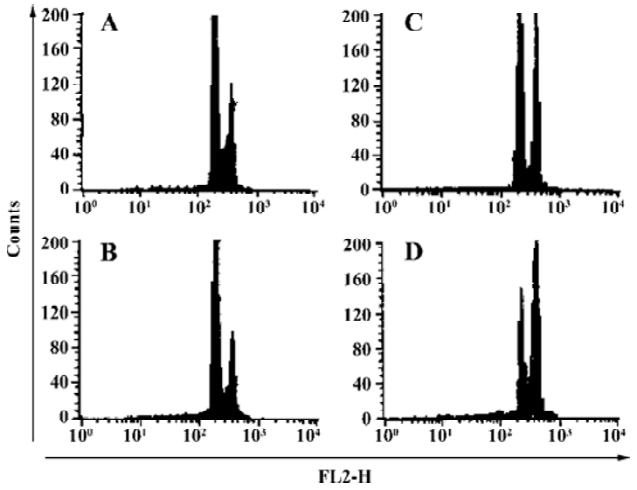

Figure 2. G_2-M cell cycle arrest is induced by AZ4. H460 cells were synchronized and treated for 24 h culture with (A) 0.1% DMSO control, (B) 0.25 μmol/L, (C) 0.50 μmol/L, or (D) 2.0 μmol/L AZ4 before PI staining and analysis of DNA content. Cell cycle arrest in G_2-M is apparent by the large population of cells with increased DNA content. The data shown were representative of 3 separate experiments. FL2-H is the fluorescence intensity.

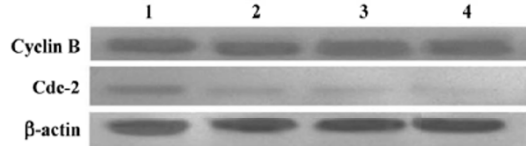

Figure 3. AZ4 alters the expression of cell cycle proteins, cyclin B and Cdc-2. The expression of cyclin B and Cdc-2 were assessed by immunoblotting. The H460 cells treated with various concentrations of AZ4 (lanes 1–4: 0, 0.25, 0.5, and 2 μmol/L, respectively) for 24 h, and cell lysate was prepared for analysis. The immunoblots shown have been equilibrated for protein loading. The data shown were representative of 3 separate experiments.

Table 2. Relative cyclin B and Cdc-2 protein expression quantitation.

	Lane 1	Lane 2	Lane 3	Lane 4
Cyclin B	1.00	0.88	1.12	1.01
Cdc-2	1.00	0.81	0.64	0.53

decreased relative to the control in a dose-dependent manner. The expression of Cdc-2 decreased by 47% at 2.0 μmol/L with AZ4. We proposed that the growth arrest on H460 cells induced by AZ4 might be altered with reduced protein expression of Cdc-2, but not cyclin B.

AZ4 triggers H460 cell apoptosis via caspase 3, 8, and 9-dependent routes and is associated with altered expression of p53 and Bcl-2 proteins We investigated whether apoptosis of H460 cells induced by AZ4 was mediated by caspase 3, 8, and 9 activation for 48 and 30 h after treatment. The activation of caspases 3, 8, and 9 was assessed by cell permeable fluorogenic caspase substrate, GDEVDGI, IETDGI, and LEHDGI, respectively. The induction of caspases 3, 8, and 9 would cleave the cell permeable to fluorogenic substrate individually, and then release the green fluorogenic substance. From those results, there was a large increase of the green peripheral clump as indicated by the arrows in Figures 4 and 5. The upstream enzyme of caspases 3 and 8 were activated in H460 cells induced by AZ4, but not incaspase 9 (data not shown). According to the visual observa-tion, the green peripheral clump appeared as low as the concentration at 0.625 μmol/L AZ4 at 30 h when observing caspase 8 (Figure 4) and 48 h for caspase 3 (Figure 5). However, enzyme activation of caspase 3 was not found to be as high as treated with the 2 μmol/L AZ4 (data not shown).

The apoptosis related proteins, tumor suppressor protein p53, and the anti-apoptotic protein Bcl-2 are two important components of the apoptotic pathway which act as regulators of apoptosis. From the results of the Western blot, AZ4 treatment altered both p53 and Bcl-2 expression in H460 cells in a dose-dependent manner after 48 h incubation (Figure 6, Table 3). The AZ4 induced overexpression of the p53 protein 25% more than the control when the H460 cells were exposed to 2.0 μmol/L AZ4. It is of interest that Bcl-2 protein expression was strongly reduced by 39% compared with the control when the H460 cells were treated with 2.0 μmol/L AZ4. In the time-dependent effect, the expression of p53 and p21 proteins were increased to a maximum at 24 h, and then decreased at 48 h (Figure 7, Table 4).

Table 3. AZ4 alters the protein expression of p53 and Bcl-2.

	Lane 1	Lane 2	Lane 3	Lane 4
p53	1.00	1.05	1.12	1.25
Bcl-2	1.00	0.96	0.75	0.61

Discussion

Bioreductive drugs such as AZQ, mitomycin C, RH1 and TPZ, have been developed to exploit the oxygen deficiency in the hypoxic fraction of solid tumors on the premise that hypoxic cells should show a greater propensity for reduc-

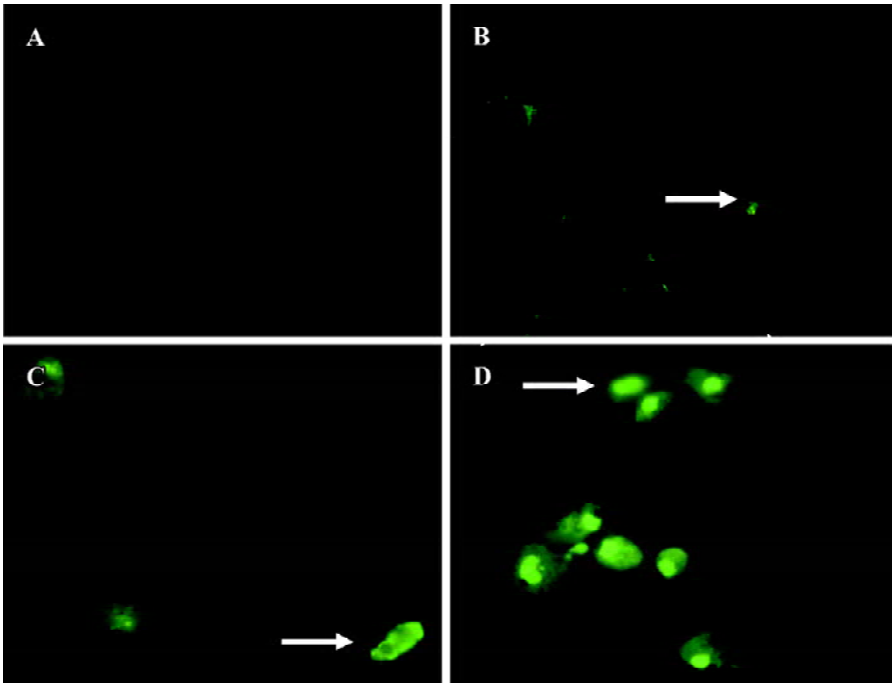

Figure 4. Caspase 8-mediated apoptosis is induced by AZ4. H460 cells were treated for 30 h with (A) 0.1% DMSO control, (B) 0.625 μmol/L, (C) 1.25 μmol/L, or (D) 2.0 μmol/L AZ4 before being incubated with cell permeable fluorogenic caspase substrate, GDEVDGI. Activated caspase 8 showed green peripheral clumps as indicated by the arrows. The data shown are representative of 3 separate experiments.

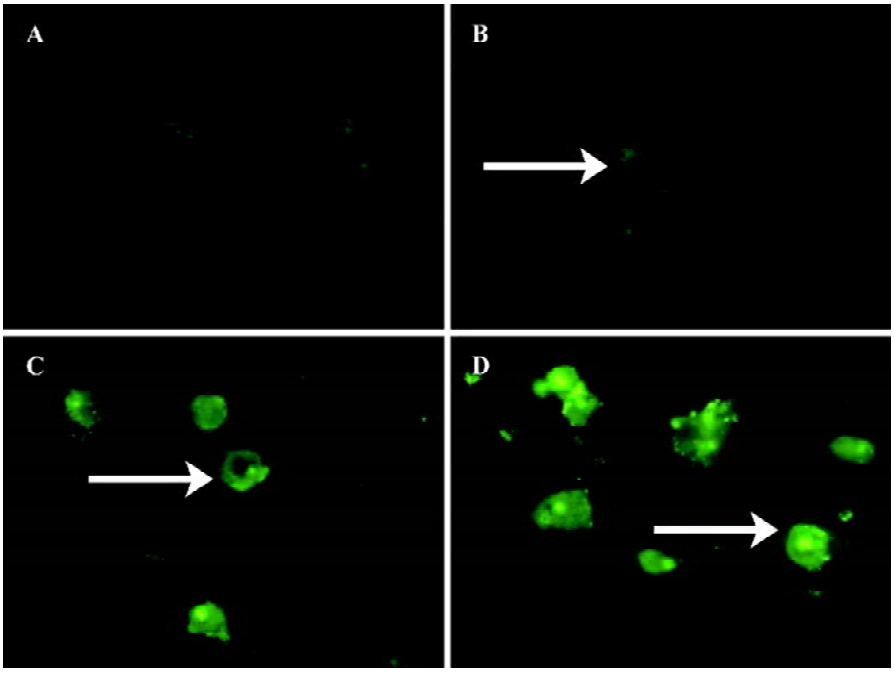

Figure 5. Caspase 3-mediated apoptosis is induced by AZ4. H460 cells were treated for 48 h with (A) 0.1% DMSO control, (B) 0.625 μmol/L, (C) 1.25 μmol/L, or (D) 2.0 μmol/L AZ4 before being incubated with cell permeable fluorogenic caspase substrate, GDEVDGI. Activated caspase 3 showed green peripheral clumps as indicated by the arrows. The data shown were representative of 3 separate experiments.

Table 4. AZ4 alters the protein expression of p53 and p21.

	0 h	4 h	8 h	12 h	24 h	48 h
p53	1.0	1.15	1.17	1.19	1.27	1.21
p21	1.0	1.0	1.0	1.06	1.12	1.04

tive metabolism than well-oxygenated cells[3,5–7]. Our pervious study on different series of *bis*-aziridinylnaphthoquinone compounds showed that these compounds exhibit more potent responses toward the solid tumors than the circulation tumors[14]. These results are supported by other reports indicating that there are differences in the reductive metabolism between the solid tumors and the circulation

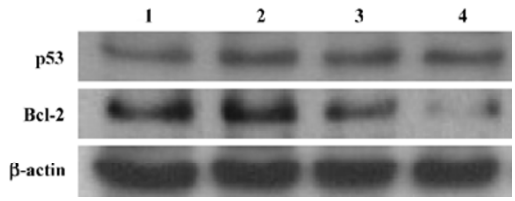

Figure 6. AZ4 alters the protein expression of p53 and Bcl-2. The expression of p53 and Bcl-2 were assessed by immunoblotting. H460 cells treated with various concentrations of AZ4 (lanes 1–4: 0, 0.625, 1.25, and 2 μmol/L) for 48 h, and cell lysates were prepared for the analysis. The immunoblots shown have been equilibrated for protein loading. The data shown are representative of 3 separate experiments.

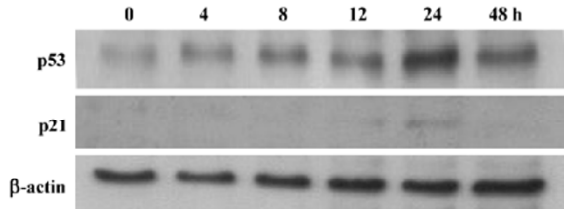

Figure 7. AZ4 alters the protein expression of p53 and p21. Expression of p53 and p21 were assessed by immunoblotting. H460 cells were treated with 1.25 μmol/L AZ4 for various timing (lanes 1–6: 0, 4, 8, 12, 24, and 48 h). Cell lysates were prepared for the analysis. The immunoblots shown have been equilibrated for protein loading. The data shown are representative of 3 separate experiments.

tumors[3]. Considering the importance of all the cellular reductases (eg NADPH cytochrome P450 reductase, cytochrome b5 reductase, NADP(H) oxidoreductase, NQO1) in response to the whole cellular reductive metabolism; these reductases are also responsible for the bioactivation of AZ4. The results from Table 1 suggest that AZ4 is a novel class of *bis*-aziridinylnaphthoquinone and is cytotoxic against H460 cells.

As shown in Figure 2, the cell cycle arrest at the G_2-M phase was observed at 0.5 μmol/L AZ4. The G_2-M phase-arresting proteins of H460 cells induced by AZ4 was related with the reduced expression of the Cdc-2 protein, but not cyclin B (Figure 3).

Apoptosis plays an important role in the removal of aberrant cells that might otherwise cause the development of tumors[17,18]. Normally, the process of cell reproduction is an ordered process known as cell cycle. The tumor suppressor gene p53 is a multifunctional protein mainly responsible for maintaining genomic integrity, and is the most frequently mutated gene in human tumors[19]. In response to DNA damage, aberrant growth signals or the chemotherapeutic drug p53 is stabilized and induces apoptosis and/or cell cycle arrest[20–22]. Therefore, p53 is a tumor suppressor gene with key regulator effects on both cell cycle and apoptosis. p53 also exerts its control on apoptosis by interacting with other important apoptotic molecules, such as members of the Bcl-2 family[23].

The protein from the Bcl-2 gene family, which is an anti-apoptotic associated protein, plays an important role in the regulation of apoptosis[24]. In the apoptotic pathway, some drugs will activate the cysteine protease family, such as caspases which specifically cleave their substrates after aspartic acid, then activate or inactivate their cellular protein targets by a process of limited proteolysis. The caspases play central roles in the execution of apoptosis[25–27]. Pro-apoptotic therapeutic strategies require, in turn, the exact knowledge of the very specific downstream pathway of apoptosis and of each single step in the targeted cell. In some cells, the signaling cascade is mediated by the caspases. Several proteins are known to regulate or interfere with this step of apoptosis, that is, proteins of the Bcl protein family, acting either anti-apoptotically (Bcl-2, Bcl-x etc) or pro-apoptotically (bax, bid etc)[28], and the pro-apoptotically-acting transcription factor p53.

The antitumor mechanism of AZ4 to lung cancer cell H460 was mediated with cell cycle arrest and the apoptosis pathway. According to the results shown in Figures 2 and 3, the cytotoxic activity of H460 cells induced by AZ4 was associated with G_2-M phase arrest at 24 h. Then, the H460 cells steered into the apoptotic pathway after prolonged treatment with AZ4 for 48 h. We observed significant apoptosis phenomena in H460 cells associated with the activation of caspase 3 which mediated the caspase 8 enzymes, but not caspase 9, the upregula-tion of p53 protein, and Bcl-2 down regulation by various concentrations of AZ4 (Figures 4–6). Bcl-2 was inactivated and made the G_2-M cells of H460 induced by AZ4 more susceptible to apoptosis which is supported by Yamamoto *et al* who proposed that stress response kinases phosphorylate Bcl-2 during cell cycle progression as a normal physiological process to inactivate Bcl-2 at the phase of G_2-M[29]. To observe the timing relationship of protein expression from cell cycle arrest to apoptosis, the time-dependent effect (Figure 7), the expression of p53 and p21 proteins were increased to the maximum at 24 h, then decreased at 48 h. p21 is a member of the Cip/Kip family of proteins, which promote cell cycle arrest by binding to and inhibiting cyclin-dependent kinases. The latter, when coupled with specific cyclins, facilitates the orderly procession of the cell cycle. p21 is also tightly regulated at the transcriptional level by p53 and probably serves as the

effector of the p53 cell cycle control[30]. This is in agreement with the above results, which support cell cycle arrest at 24 h, then apoptosis at 48 h.

In conclusion, AZ4 displayed selective cytotoxic activity against H460 cells and low cytotoxic activity when cultured with non-neoplastic human adherent lung fibroblasts. The cytotoxic effect induced by AZ4 on H460 cells may correlate with the induction of the G_2-M cell cycle at 24 h which increased p53, p21, and Bcl-2 protein expression and decreased the expression of the Cdc-2 protein. The cells were then steered the apoptotic pathway for 48 h treatment, where the anti-apoptotic protein Bcl-2 and cell checkpoint proteins p53 and p21decreased, while the apoptosis enzymes caspase 3 and 8 were activated. It is believed that this cytotoxic mechanism study holds promise for the development of a new generation of potent antitumor agents.

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