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Quinolone analogue inhibits tubulin polymerization and induces apoptosis via Cdk1-involved signaling pathways

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ABSTRACT

Cancer chemotherapeutic agents that interfere with tubulin/microtubule function are in extensive use. Quinolone is a common structure in alkaloids and its related components exhibit several pharmacological activities. In this study, we have identified the anticancer mechanisms of 2-phenyl-4-quinolone. 2-Phenyl-4-quinolone displayed anti-proliferative effect in several cancer types, including hormone-resistant prostate cancer PC-3, hepatocellular carcinoma Hep3B and HepG2, non-small cell lung cancer A549 and P-glycoprotein-rich breast cancer NCI/ADR-RES cells. The IC_{50} values were 0.85, 1.81, 3.32, 0.90 and 1.53 μM, respectively. 2-Phenyl-4-quinolone caused G2/M arrest of the cell-cycle and a subsequent apoptosis. The turbidity assay showed an inhibitory effect on tubulin polymerization. After immunochemical examination, the data demonstrated that the microtubules were arranged irregularly into dipolarity showing prometaphase-like states. Furthermore, 2-Phenyl-4-quinolone induced the Mcl-1 cleavage, the phosphorylation of Bcl-2 and Bcl-xL (12-h treatment), and the caspase activation including caspase-8, -2 and -3 (24-h treatment). The exposure of cells to 2-phenyl-4-quinolone caused Cdk1 activation by several observations, namely (i) elevation of cyclin B1 expression, (ii) dephosphorylation on inhibitory Tyr-15 of Cdk1, and (iii) dephosphorylation on Ser-216 of Cdc25c. Moreover, a long-term treatment (36 h) caused the release reaction and subsequent nuclear translocation of AIF. In summary, it is suggested that 2-phenyl-4-quinolone displays anticancer effect through the dysregulation of mitotic spindles and induction of mitotic arrest. Furthermore, participation of cell-cycle regulators, Bcl-2 family of proteins, activation of caspases and release of AIF may mutually cross-regulate the apoptotic signaling cascades induced by 2-phenyl-4-quinolone.

1. Introduction

Microtubules are composed of a backbone of α- and β-tubulin heterodimers and microtubule-associated proteins [1]. They are major cellular components that play crucial roles in a lot of cellular functions, including the maintenance of cell shape, cell adhesion and movement, cell signaling, cell replication and cell division. Microtubules are in a highly dynamic process

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of polymerization and depolymerization in cells undergoing replication and division, while they are easily affected by numerous endogenous regulators and exogenous factors, and are considered as a susceptible target by numerous therapeutic drugs [2]. Several cancer chemotherapeutic drugs show their anticancer effects through the disturbance of microtubule dynamics, leading to dysregulation of mitotic spindles and the causative mitotic arrest in cancer cells. For example, paclitaxel and docetaxel bind to the microtubule lattice, stabilize microtubule bundles and impair cell mitosis in numerous types of cancer cells [3,4]. In contrast, *Vinca* alkaloids bind to and inhibit microtubule polymerization, resulting in a blockade in mitosis and causing apoptotic cell death [5].

Tubulins of α- and β-subunits are encoded in vertebrates by six and seven different genes, respectively. Each tubulin isotype can be distinguished by its different C-terminal sequence [6,7]. β-Tubulin isotypes, which are the predominant targets of anti-mitotic agents, can be identified by sequence alignment. To date, there are seven β-tubulin isotypes being identified in mammals. Among these isotypes, β_{III}-tubulin is a central target for taxanes. Several studies suggest that the overexpression of β_{III}-tubulin involves in microtubule destabilization and resistance to taxanes [8]. Furthermore, there are several lines of evidence that β_{III}-tubulin is one of the cytoskeleton components in neoplastic other than normal differentiated glial cells [9]. Therefore, the investigation of anticancer mechanisms responsible for tubulin-involved pathways may provide significant insights into the regulation of the growth and progression of cancer cells.

In recent decades, the agents that modulate the G2/M checkpoint are of particular interest in the development of cancer chemotherapeutic drugs. Several clinical tubulin-binding drugs, such as taxanes and *Vinca* alkaloids, act as inhibitors in G2/M phase transition, providing evidence that the dysregulation of tubulin dynamics may disrupt G2/M transition and, subsequently, induce the cell-cycle arrest and apoptosis in tumor cells [10]. The activation of the Cdk1/cyclin B1 complex in the nucleus triggers the progression of the cell-cycle from G2- to M-phase. The activity of Cdk1 is regulated by several factors, including the level of transcription of cyclin B1, Cdk1 phosphorylations on different residues, Cdk1 inhibitor $p21^{Cip1/Waf1}$ and Cdc25 phosphatase activity [11]. It has been suggested that Cdk1 could be a target for the induction of apoptosis. It was demonstrated that inactivation of Cdk1 increases apoptotic cell death induced by DNA damage [12]. Nevertheless, there are many lines of evidence that instead of anti-apoptotic role, Cdk1 acts as a pro-apoptotic mediator in numerous tumor cell types. As regards the tubulin-binding agents, taxol and *Vinca* alkaloids can induce the Cdk1 activity and apoptosis in tumor cells [13]. Recently, several lines of evidence suggest that mitochondria may play a central role in signaling pathways to drive the cell-cycle arrest toward apoptotic cell death caused by tubulin-binding agents. Several observations suggest that Cdk1 is able to trigger mitochondrial membrane permeabilization by targeting on Bcl-2 family proteins, such as Bcl-2 and Bad, and subsequently induce the apoptotic cell death [14]. However, the tubulin-binding agents have diverse expression patterns of Bcl-2 family proteins in varied tumor types.

Targeting tubulin leading to a subsequent apoptosis in cancer cells is an effective mechanism for cancer chemotherapy. However, most of the clinical tubulin-binding agents are derived or semi-synthesized from natural products, which have complicated chemical structures and are hardly synthesized. Accordingly, the tubulin-binding agents with simple chemical structures could be of value as lead pharmacophore for future therapeutic agents. Quinolone is a common structure in natural alkaloids. It has been suggested that quinolone alkaloids, in particular 4-quinolone alkaloids, exhibit several pharmacological activities, such as antimycobacterial activity, and inhibition of leukotriene biosynthesis and monoamine oxidase activity [15–17]. 2-Phenyl-4-quinolone fits the criteria as a pharmacological probe. In this study, the mechanism of 2-phenyl-4-quinolone has been identified from the characterization of tubulin isotypes and cell-cycle regulators to mitochondrial proteins and related apoptotic cascades. This study also provides a prototype structure that enables the investigation of tubulin-targeting strategy in cancer chemotherapy.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, and all other tissue culture reagents were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). EGTA, EDTA, leupeptin, dithiothreitol, reagent, phenylmethylsulfonnylfluoride (PMSF), sulforhodamine B (SRB), propidium iodide (PI), antibodies to β_I, β_{II}, β_{III}, and β_{IV} tubulin isotypes, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, and tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG were obtained from Sigma (St Louis, MO). Antibodies to Bcl-2, Bcl-xL, Mcl-1, Bak, Bax, XIAP, CIAP-1, Survivin, Cyclin B1, Cdk1, Cdc25C, apoptosis inducing factor (AIF), and anti-mouse and anti-rabbit IgGs were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies to caspase-9, caspase-8, phospho-$cdc2^{Tyr15}$, phospho-$cdc2^{Thr161}$, phospho-Bcl-2^{Ser70} and Bid were from Cell Signaling Technologies (Boston, MA). Antibody to caspase-3 was from Imgenex (San Diego, CA). Antibodies to α- and β-tubulins were from Serotec Products (Beverly, MA) and BD Biosciences PharMingen (San Diego, CA), respectively. Antibody to MPM-2 was from Upstate Biotechnology (Lake Placid, NY). 2-Phenyl-4-quinolone was synthesized and provided by one of our colleagues (Dr. Sheng-Chu Kuo). The purity is more than 98% by the examination of HPLC and NMR.

2.2. Cell culture

NCI/ADR-RES cell line was from DTP Human Tumor Cell Line Screen (Developmental Therapeutics Program, NCI). The other cancer cell lines were from American Type Culture Collection (Rockville, MD). Human cancer cells were cultured in RPMI1640 medium with 10% FBS (v/v) and penicillin (100 units/ml)/streptomycin (100 μg/ml). Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

2.3. SRB assays

Cells were seeded in 96-well plates in medium with 5% FBS. After 24 h, cells were fixed with 10% trichloroacetic acid (TCA) to represent cell population at the time of 2-phenyl-4-quinolone addition (T_0). After additional incubation of DMSO or 2-phenyl-4-quinolone for 48 h, cells were fixed with 10% TCA and SRB at 0.4% (w/v) in 1% acetic acid was added to stain cells. Unbound SRB was washed out by 1% acetic acid and SRB bound cells were solubilized with 10 mM Trizma base. The absorbance was read at a wavelength of 515 nm. Using the following absorbance measurements, such as time zero (T_0), control growth (C), and cell growth in the presence of 2-phenyl-4-quinolone (T_x), the percentage growth was calculated at each of the compound concentrations levels. Percentage growth inhibition was calculated as: $[(T_x - T_0)/(C - T_0)] \times 100$ for concentrations for which $T_x \geq T_0$. Growth inhibition of 50% (IC_{50}) is determined at the drug concentration which results in 50% reduction of total protein increase in control cells during the compound incubation.

2.4. FACScan flow cytometric analysis

After the treatment of cells with vehicle or 2-phenyl-4-quinolone for the indicated times, the cells were harvested by trypsinization, fixed with 70% (v/v) ethanol at 4 °C for 30 min and washed with phosphate-buffered saline (PBS). After centrifugation, cells were incubated in 0.1 ml of phosphate-citric acid buffer (0.2 M $NaHPO_4$, 0.1 M citric acid, pH7.8) for 30 min at room temperature. Then, the cells were centrifuged and resuspended with 0.5 ml propidium iodide solution containing Triton X-100 (0.1%, v/v), RNase (100 μg/ml) and propidium iodide (80 μg/ml). DNA content was analyzed with the FACScan and CellQuest software (Becton Dickinson, Mountain View, CA).

2.5. In vitro tubulin turbidity assay

The tubulin polymerization was detected by the use of CytoDYNAMIX Screen 03 kit (Cytoskeleton Inc., Denver, CO). Tubulin proteins (>99% purity) were suspended in G-PEM buffer containing 80 mM PIPES, 2 mM $MgCl_2$, 0.5 mM EDTA, and 1.0 mM GTP (pH6.9) and 5% glycerol with or without the compound. Then, the mixture was transferred to a 96-well plate and the absorbance was measured at 340 nm (37 °C) for 60 min (SpectraMAX Plus, Molecular Devices Inc., Sunnyvale, CA).

2.6. Confocal immunofluorescence microscopic examination

Cells were seeded in 8-well chamber slides. After the compound treatment, the cells were fixed with 100% methanol at −20 °C for 5 min and incubated in 1% bovine serum albumin (BSA) containing 0.1% Triton X-100 at 37 °C for 30 min. The cells were washed twice with PBS for 5 min and stained with primary antibodies at 37 °C for 1 h and then, the FITC or TRITC-conjugated secondary antibody at 37 °C for 40 min. Nuclear staining was performed by 1 mg/ml DAPI. The cells were analyzed by a confocal laser microscopic system (Leica TCS SP2).

2.7. Western blotting

After the indicated exposure time of cells to DMSO or the indicated agent, cells were washed twice with ice-cold PBS and reaction was terminated by the addition of 100 μl ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100). For Western blot analysis, the amount of proteins (40 μg) were separated by electrophoresis in a 10 or 15% polyacrylamide gel and transferred to a nitrocellulose membrane. After an overnight incubation at 4 °C in PBS/5% nonfat milk, the membrane was washed with PBS/0.1% Tween 20 for 1 h and immuno-reacted with the indicated antibody for 2 h at room temperature. After four washings with PBS/0.1% Tween 20, the anti-mouse or anti-rabbit IgG (dilute 1:2000) was applied to the membranes for 1 h at room temperature. The membranes were washed with PBS/0.1% Tween 20 for 1 h and the detection of signal was performed with an enhanced chemiluminescence detection kit (Amersham).

2.8. Data analysis

The compound was dissolved in DMSO. The final concentration of DMSO was 0.1% in cells. Data are presented as the mean ± S.E.M. for the indicated number of separate experiments.

3. Results

3.1. Effect of 2-phenyl-4-quinolone on cell proliferation and cell-cycle progression

The exposure of human hormone-resistant prostate cancer PC-3 cells to 2-phenyl-4-quinolone caused a concentration-dependent inhibition of cell proliferation with an IC_{50} of 0.85 μM. 2-Phenyl-4-quinolone also displayed similar effect in several types of human cancer cell lines, including hepatocellular carcinoma Hep3B and HepG2, non-small cell lung cancer A549 and P-glycoprotein (P-gp)-rich breast cancer NCI/ADR-RES cells, with IC_{50} values of 1.8, 3.3, 0.9 and 1.5 μM, respectively (Fig. 1A). The effect of 2-phenyl-4-quinolone on cell-cycle progression was evaluated by FACScan flow cytometric analysis of PI staining in asynchronized PC-3 cells. 2-Phenyl-4-quinolone caused an arrest of the cell cycle in G2/M phase as early as a 6-h treatment. The accumulation of mitotic cells showed a time-dependent manner, which was associated with a declined proportion in G1 phase and a subsequent increase in sub-G1 population (Fig. 1B). Similar results were obtained in the aforementioned types of cancer cells when exposed to 2-phenyl-4-quinolone (data not shown).

3.2. Effect of 2-phenyl-4-quinolone on tubulin polymerization and mitotic spindle organization

The in vitro tubulin polymerization of turbidity assay was performed to examine if 2-phenyl-4-quinolone has a direct interaction with tubulins. The data in Fig. 2A demonstrates that the tubulins polymerized in a time-dependent manner in the presence of GTP at 37 °C. Taxol (a microtubule

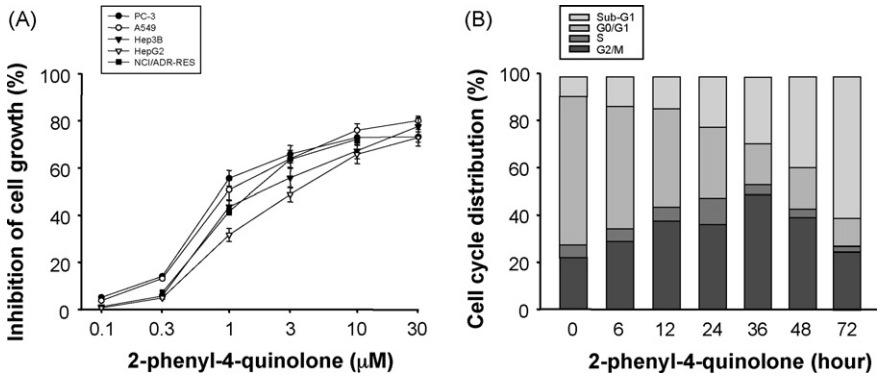

Fig. 1 – Identification of 2-phenyl-4-quinolone-induced anticancer effect and cell cycle progression. (A) Cells were treated with 2-phenyl-4-quinolone for the indicated concentration for 48 h. Then, the cells were fixed and stained with SRB. After a series of washing, bound SRB was subsequently solubilized and the absorbance was read at a wavelength of 515 nm. Data are expressed as mean ± S.E.M. of four determinations (each in triplicate). (B) PC-3 cells were treated with 2-phenyl-4-quinolone (3 μM) for the indicated times. Then, the cells were fixed and stained with PI to analyze DNA content by FACScan flow cytometry. Data are representative of three independent experiments.

stabilization agent) and vincristine (a microtubule depolymerizing agent) were used as reference compounds. Consequently, taxol promoted, while vincristine inhibited, the tubulin polymerization; 2-phenyl-4-quinolone showed an inhibitory effect on tubulin polymerization in a concentration-dependent manner (Fig. 2A). Furthermore, the organization of mitotic spindle was detected by immunofluorescence microscopic examination in PC-3 cells. The in situ labeling of tubulin and chromosomes showed that the exposure to 2-phenyl-4-quinolone caused the mitotic arrest of cells with characteristics that microtubules were arranged irregularly into a spindle-like status with predominant aster array, star-shaped microtubules, dipolarity or multipolarity showing prometaphase-like states. The data revealed that 2-phenyl-4-quinolone-treated cultures exhibited the increased numbers of mitotic figures when compared with control samples (Fig. 2B–E). As demonstrated in Fig. 2B, 2-phenyl-4-quinolone occasionally induced a major aster array of β_I-tubulin in some mitotic arrest cells; whereas caused dipolar mitotic spindles of β_{II}- and β_{III}-tubulin staining (Fig. 2C and D). In contrast, β_{IV}-tubulin contributed lesser extent to mitotic spindles (Fig. 2E). The reference compound, taxol, promoted the formation of multipolar spindles, while vincristine inhibited microtubule assembly in all isotypes of β-tubulin (Fig. 2B–E). The quantification data also demonstrated that 2-phenyl-4-quinolone induced a higher rate of formation of abnormal spindle with β_{II}- and β_{III}-tubulin staining (Table 1).

Table 1 – Effect of 2-phenyl-4-quinolone (5 μM), taxol (0.1 μM) and vincristine (0.1 μM) on the formation of abnormal mitotic spindles

β-Tubulin isotype	% Abnormal mitotic spindles[a]		
	2-Phenyl-4-quinolone	Taxol	Vincristine[b]
β_I	40.0	92.3	83.3
β_{II}	91.2	94.1	88.9
β_{III}	81.3	100	92.9
β_{IV}	38.9	89.3	73.3

The data are shown by the count of at least fifteen cells in an individual image.
[a] The microtubules were arranged irregularly into a spindle-like status with predominant aster array, star-shaped microtubules, dipolarity or multipolarity.
[b] The microtubules were depolymerized with slight tubulin staining.

3.3. *Effect of 2-phenyl-4-quinolone on cyclin B1 expression and Cdk1 activity*

The Cdk1/cyclin B1 complex plays a central role on the regulation of cell-cycle progression from G2- to M-phase. After the treatment with 2-phenyl-4-quinolone, the level of cyclin B1 expression dramatically increased associated with a reduced level of cyclin A expression in PC-3 cells (Fig. 3). Furthermore, the dephosphorylation on inhibitory Tyr-15 of Cdk1 was induced although the protein levels of Cdk1 were not changed. These data together with the dephosphorylation on Ser-216 of Cdc25c suggested that Cdk1 was activated in PC-3 cells when treated with 2-phenyl-4-quinolone (Fig. 3). Additionally, using monoclonal antibody MPM-2 to recognize mitotic phosphoproteins, the data showed that the exposure of cells to 2-phenyl-4-quinolone caused an increase of the MPM-2 expression (Fig. 3). The data corresponded to an arrest of the cell-cycle in G2/M phase (Fig. 1B) confirming the arrest of the cells at mitosis phase to 2-phenyl-4-quinolone action.

3.4. *Effect of 2-phenyl-4-quinolone on Bcl-2 family of proteins*

Recently, a lot of attention has been directed on Bcl-2 family of proteins to elucidate their roles in regulating survival decisions in tumor cells. Several pro-apoptotic family proteins (e.g., Bax, Bid and Bak) promote release of cytochrome c, whereas anti-apoptotic members (e.g., Bcl-2, Bcl-xL and Mcl-1) are capable of antagonizing the pro-apoptotic proteins and

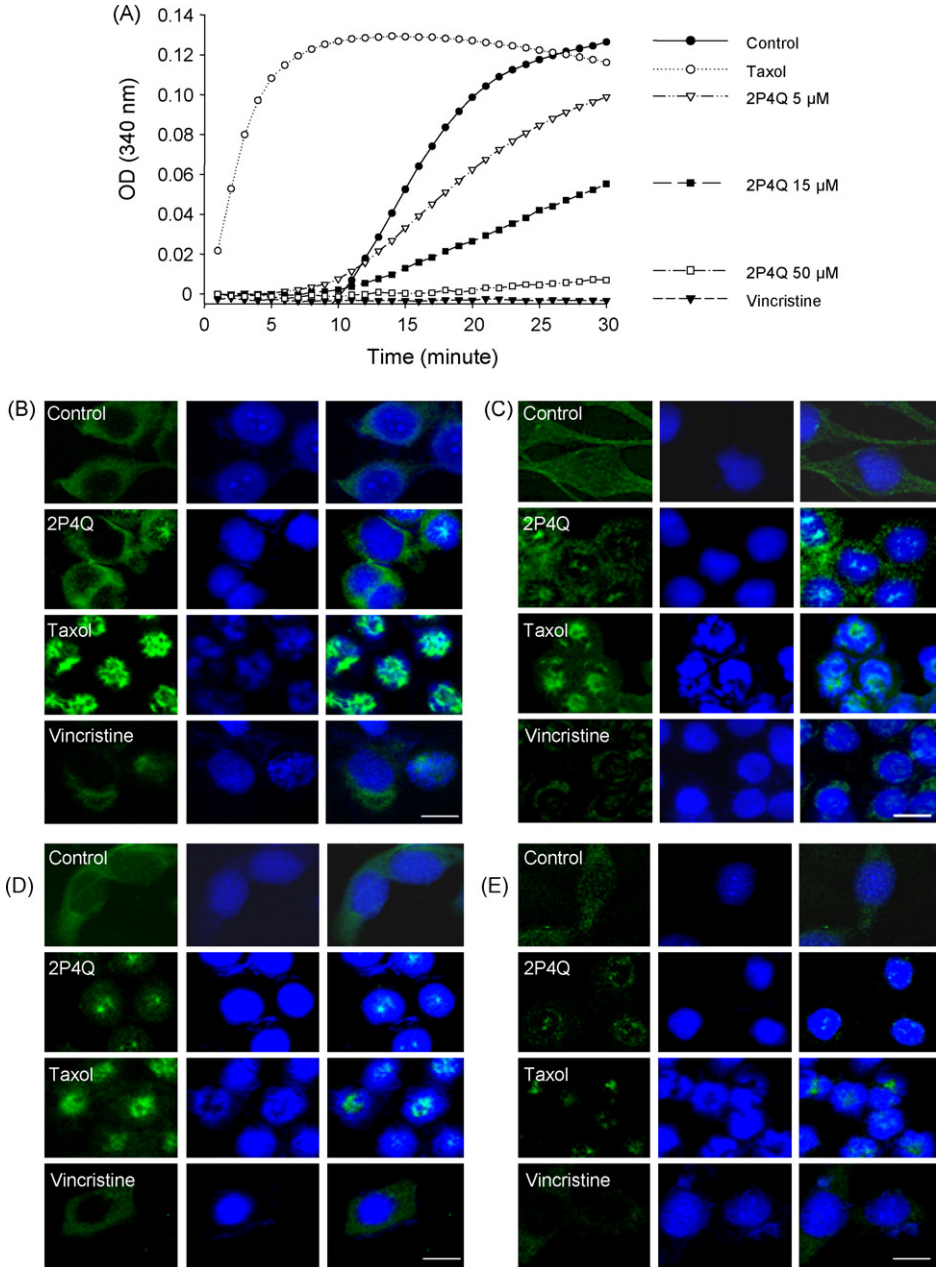

Fig. 2 – Effect of 2-phenyl-4-quinolone on in vitro and in situ tubulin assembly assays. Purified tubulin from bovine brain was incubated at 37 °C with GTP in the absence (control) or presence of the indicated agent. Tubulin polymerization was examined turbidimetrically. Change in absorbance at 340 nm is plotted as a function of time in minutes (A). PC-3 cells were incubated in the vehicle (0.1% DMSO, control), 2-phenyl-4-quinolone (2P4Q, 3 μM), taxol (0.1 μM) or vincristine (0.1 μM) for 18 h. Then, the cells were fixed and stained with primary antibody to tubulin (B, β_I; C, β_{II}; D, β_{III}; E, β_{IV}). Then, FITC-labeled secondary antibodies were used (green fluorescence) and the protein was detected by a confocal laser microscopic system. The nuclei were apparent by DAPI staining (blue fluorescence). Areas of colocalization between microtubule expression and nuclei in the merged panels are cyan. *Arrow*, mitosis-arrested cell with deformed nucleus; *Scale bar*, 20 μm.

preventing the loss of mitochondrial membrane potential. In this study, 2-phenyl-4-quinolone induced the band-shift of the expression of Bcl-2 and Bcl-xL in PC-3 cells, indicating the phosphorylation of these two members. Furthermore, the phosphorylation on Ser-70 of Bcl-2 was identified. Notably, 2-phenyl-4-quinolone also caused the cleavage of Mcl-1 after a 24-h treatment (Fig. 4A). However, 2-phenyl-4-quinolone had little effect on the pro-apoptotic member proteins, such as Bax and Bak (data not shown), but induced the cleavage of Bad into a 15-kDa truncated protein after a 24-h treatment (Fig. 4A).

3.5. *Effect of 2-phenyl-4-quinolone on the activation of caspases and expression of IAP family proteins*

The activation of caspase proteases plays a central role in the process of numerous apoptotic cell deaths. It also contributes

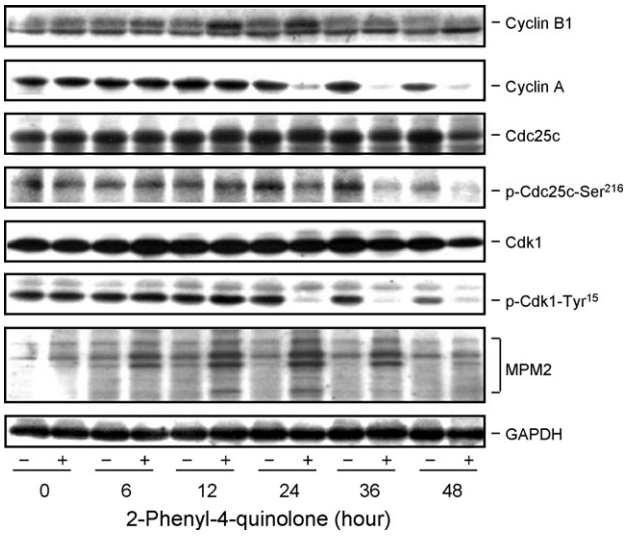

Fig. 3 – Effect of 2-phenyl-4-quinolone on the expression of cell cycle regulators. PC-3 cells were incubated in the vehicle (0.1% DMSO) or 2-phenyl-4-quinolone (3 μM) for the indicated times. Then, the cells were harvested and lysed for the detection of protein expressions with antibodies of mitotic regulators by Western blot analysis. For Western blotting, the amount of proteins (40 μg) was separated by electrophoresis in a 10–15% polyacrylamide gel, transferred to a nitrocellulose membrane and immuno-reacted with the indicated antibody. The data are representative of three independent experiments.

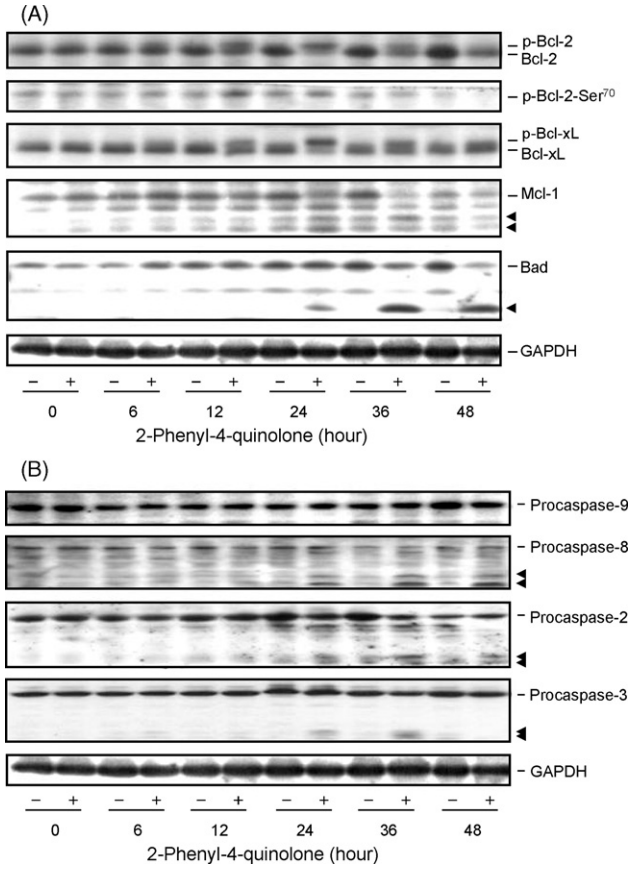

Fig. 4 – Effect of 2-phenyl-4-quinolone on the expression of Bcl-2 family member proteins and caspases. PC-3 cells were incubated in the vehicle (0.1% DMSO) or 2-phenyl-4-quinolone (3 μM) for the indicated times. Then, the cells were harvested and lysed for the detection of protein expression with specific antibody by Western blot analysis. For Western blotting, the amount of proteins (40 μg) was separated by electrophoresis in a 10 to 15% polyacrylamide gel, transferred to a nitrocellulose membrane and immuno-reacted with the indicated antibody. The data are representative of three independent experiments. The arrowhead indicates the cleaved form of parent protein.

to apoptosis induced by tubulin-binding agents in cancer cells [10,18]. To determine which caspases were involved in 2-phenyl-4-quinolone-induced apoptosis, the expressions of caspases were detected by Western immunoblot analysis. Caspases are synthesized primarily as inactive precursors. In control cells, all of the examined caspases were present as uncleaved forms. After the treatment of PC-3 cells with 2-phenyl-4-quinolone for 24 h, the cleavage of inactive precursors to catalytically active fragments was clearly detected in several caspases, including caspase-8, -2 and -3 (Fig. 4B). Of note, 2-phenyl-4-quinolone did not induce the activation of caspase-9, the major initiator caspase of intrinsic (mitochondrial) apoptosis pathway (Fig. 4B). To delineate this issue, the expression of IAP family proteins has been detected. The data showed that 2-phenyl-4-quinolone induced an increase of survivin expression but not the other family members (Fig. 5).

3.6. *Effect of 2-phenyl-4-quinolone on the release reaction of cytochrome c and AIF*

It is well known that mitochondria can release apoptogenic factors involved in caspase-dependent and -independent cell death [19]. Cytochrome *c* and AIF are two important apoptogenic factors released from the mitochondria. In this study, the immunochemical examination showed that 2-phenyl-4-quinolone induced the release reaction of cytochrome *c* and AIF, and the nuclear translocation of AIF in PC-3 cells (Fig. 6).

4. Discussion

Microtubules are important cytoskeletal components involved in a lot of cellular functions, in particular the cell mitosis. Antimicrotubule agents, including polymerizing and depolymerizing drugs, are widely used in oncochemotherapy. Currently, new antimicrotubule agents are of continued interest in the drug development. Unlike clinical antimicrotubule agents that have complex chemical structures, 2-phenyl-4-quinolone is a 4-quinolone analogue with a simple chemical structure, which fits the criteria as a pharmacological probe. 2-phenyl-4-quinolone displayed effective anti-proliferative activity in numerous types of cancer cells. Particularly, it showed anti-proliferative effect in P-gp-rich NCI/ADR-RES cells with similar efficacy compared to other cancer cell lines, indicating that 2-phenyl-4-quinolone was

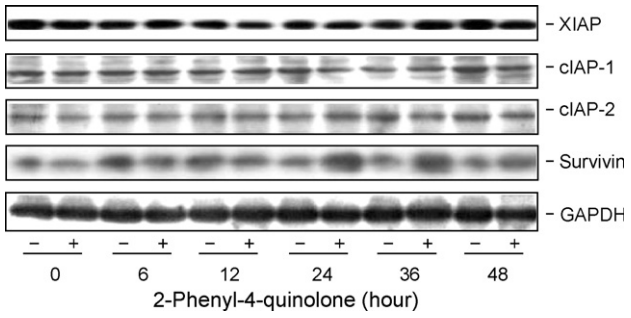

Fig. 5 – Effect of 2-phenyl-4-quinolone on the expression of IAPs. PC-3 cells were incubated in the vehicle (0.1% DMSO) or 2-phenyl-4-quinolone (3 μM) for the indicated times. Then, the cells were harvested and lysed for the detection of protein expression with specific antibody by Western blot analysis. For Western blotting, the amount of proteins (40 μg) was separated by electrophoresis in a 10–15% polyacrylamide gel, transferred to a nitrocellulose membrane and immuno-reacted with the indicated antibody. The data are representative of three independent experiments.

not a P-gp substrate. The data indicate the development advantage of 2-phenyl-4-quinolone, since P-gp confers multiple drug resistance by effluxing numerous antimicrotubule drugs, such as vincristine, vinblastine, docetaxel and taxol [20,21].

One of the key characteristics of antimicrotubule agents is the induction of mitotic arrest of the cell-cycle [10]. 2-phenyl-4-quinolone induced an arrest of the cell cycle in G2/M phase that preceded the increase of sub-G1 population, suggesting that the apoptotic cell death might result from the mitotic arrest of the cell cycle. To identify the antimicrotubule effect, the in vitro tubulin polymerization assay and confocal immunochemical examination were used. Consequently, 2-phenyl-4-quinolone was able to inhibit the tubulin polymerization, leading to the mitotic arrest in prometaphase with characteristics that microtubules were arranged irregularly into a spindle-like status with predominant aster array, star-shaped microtubules, dipolarity or multipolarity showing prometaphase-like states.

It has been suggested that the mitotic arrest of the cell cycle or an aberrant mitotic exit into a G_1-like multinucleate condition plays a central role in apoptotic cell death induced by antimicrotubule agents [10]. Moreover, Cdk1 activation may be involved in some apoptotic signaling pathways. In this study, 2-phenyl-4-quinolone induced the mitotic arrest of cells that preceded apoptosis. Therefore, several cell-cycle regulators, in particular Cdk1, were examined. Our data demonstrated that 2-phenyl-4-quinolone induced the activation of Cdk1 based on several observations, namely (i) the down-regulation of cyclin A and elevation of cyclin B1 expression, (ii) the de-phosphorylation of Cdc25c at Ser-216 that prevents the binding with 14-3-3 proteins, leading to the nuclear localization of Cdc25c and (iii) the de-phosphorylation on inhibitory Tyr-15 of Cdk1 by Cdc25c phosphatase, leading to the activation of Cdk1. Furthermore, it has been evident that the activity of Cdk1 must be sustained from prophase to metaphase. Subsequently, the entry into anaphase is dependent on a rapid decline of the Cdk1 activity [22]. The sustained activation of Cdk1 by 2-phenyl-4-quinolone may explain why the cells were arrested at prometaphase.

The downstream apoptosis pathways following the cell-cycle arrest caused by a variety of pharmacological agents have been widely explored. The mitochondria-mediated signaling pathways are the most identified mechanisms. Bcl-2 and several pro-survival family members associate with the mitochondrial outer membrane and maintain their integrity. Aberrant expression of pro-survival members of the family proteins, such as Bcl-2, Bcl-xL and Mcl-1, is believed to contribute to uncontrolled growth of malignant cells. In contrast, pro-apoptotic members such as Bax and Bak oligomerize in mitochondrial outer membrane and disrupt their integrity, causing the release of apoptogenic factors. The BH3-only proteins such as Bid and Bim antagonize and inactivate the pro-survival members [23]. There is increasing evidence that the regulation of Bcl-2 family of proteins shares the signaling pathways induced by antimicrotubule agents [10]. Our data showed that 2-phenyl-4-quinolone induced the phosphorylation of Bcl-2 at Ser-70. Several lines of evidence suggesting that Bcl-2 phosphorylation is associated with the loss of its anti-apoptotic function [10]. Cdk1 is one of the kinases capable of phosphorylating Bcl-2 on Ser-70 [24]. In contrast, several studies show that Bcl-2 phosphorylation is only a biochemical marker in M-phase event [25]. Moreover, several studies failed to demonstrate the direct Bcl-2 phosphorylation by Cdk1 in an in vitro assay [26]. In this study, the Bcl-2 phosphorylation induced by 2-phenyl-4-quinolone occurred earlier than Cdk1 activation excluding its regulation by Cdk1. Interestingly, the Bcl-xL phosphorylation was also induced by 2-phenyl-4-quinolone. The similar effect is also stimulated by several antimicrotubule agents [27]. It has been suggested that Bcl-xL phosphorylation may play a role in regulating its function and may participate in the determination of antimicrotubule-related chemotherapeutic efficacy in human tumors. Although our data and the recent studies [27] could not provide direct evidence to support that Bcl-xL phosphorylation can facilitate apoptotic cell death, the phenomenon may support that 2-phenyl-4-quinolone is an antimicrotubule agent. Mcl-1, another pro-survival member, was found to be cleaved in cells in response to 2-phenyl-4-quinolone. Numerous lines of evidence suggest that Mcl-1 is a caspase substrate, in particular caspase-3, during induction of apoptosis [28]. Our data showed that the onset of Mcl-1 cleavage correlated with the activation of caspase-3, suggesting the involvement of caspase-3 in Mcl-1 cleavage. Of note, 2-phenyl-4-quinolone had little effect on the pro-apoptotic member proteins, such as Bax and Bak, but induced the cleavage of Bad into a 15 kDa truncated protein, an onset correlated with the caspase-3 activation. Similarly, it has been evident that Bad is cleaved by caspase-3 at its N terminus to generate a 15-kDa truncated protein, which is a more potent inducer of apoptosis than the wild-type protein [29]. It is likely that the truncated Bad may facilitate 2-phenyl-4-quinolone-induced apoptotic cell death.

Caspases are intracellular cysteine proteases responsible for apoptosis. Once activated after apoptotic stimuli, caspases can stimulate intracellular signaling cascades and activate

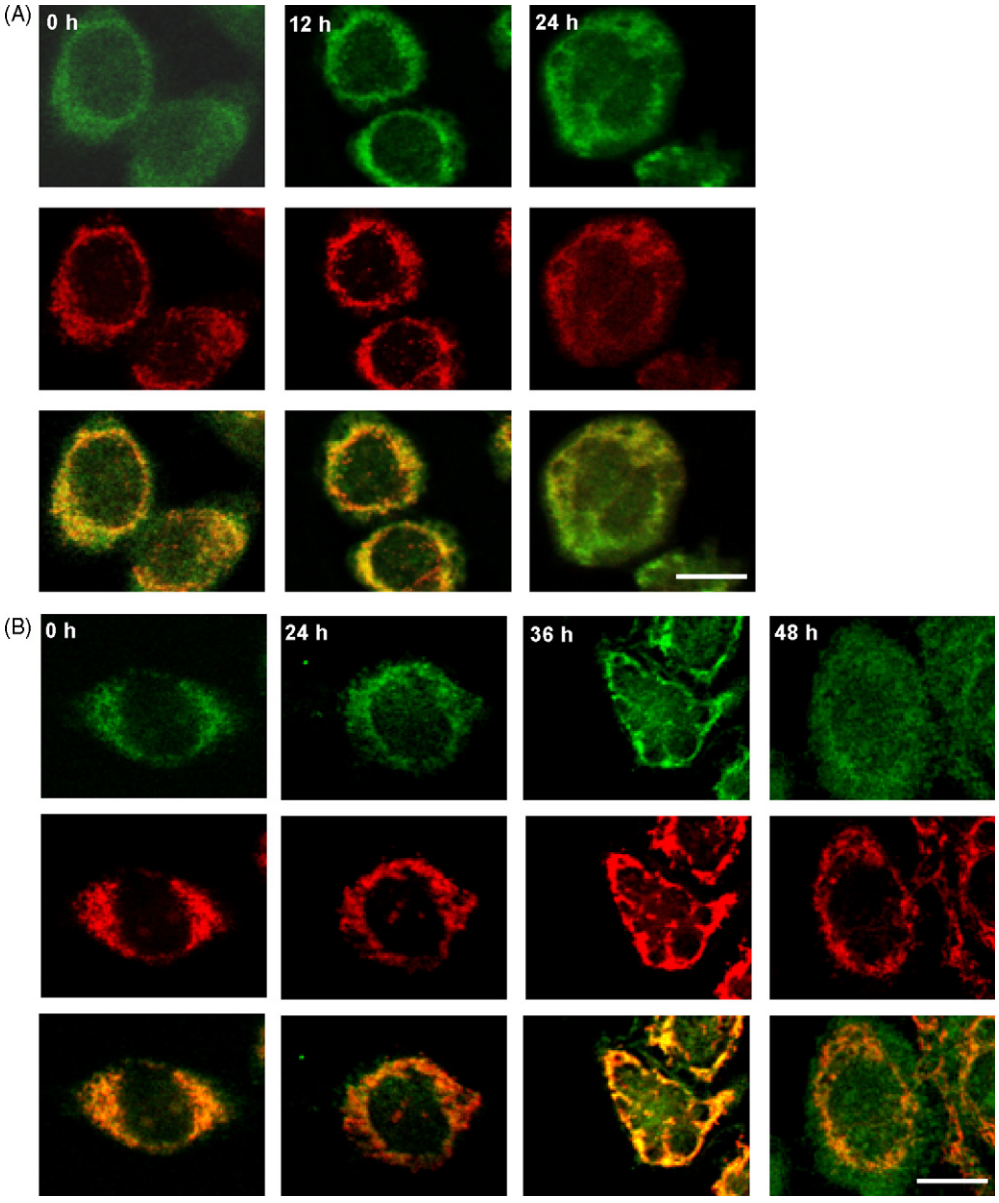

Fig. 6 – Effect of 2-phenyl-4-quinolone on the release reaction of cytochrome *c* and nuclear translocation of AIF. PC-3 cells were incubated in the vehicle (0.1% DMSO, 0 h) or 2-phenyl-4-quinolone (3 μM) for the indicated times. The cells were fixed and stained with primary antibody to cytochrome *c* (A) and AIF (B). Then, FITC-labeled secondary antibodies were used (green fluorescence) and detected by a confocol laser microscopic system. The mitochondria were apparent by MitoTracker staining (red fluorescence). Areas of colocalization between cytochrome *c*/AIF expression and mitochondria in the merged panels are yellow. *Scale bar*, 20 μm.

other caspase family members. In this study, 2-phenyl-4-quinolone induced the activation of caspase-8. However, the protein levels of both death ligands (FasL and TRAIL) and death receptors (Fas, DR4 and DR5) were not modified by 2-phenyl-4-quinolone (data not shown), suggesting that the extrinsic apoptosis pathway did not play a crucial role to 2-phenyl-4-quinolone action. Caspase-2 is a unique caspase with characteristics of both initiator and effector caspase. Recently, the compensatory caspase activation has been investigated during several apoptosis signaling cascades. It has been suggested that the elimination of caspase-9 (e.g., caspase-$9^{-/-}$) in cell death caused by apoptotic stimulus induces a compensatory activation of caspase-2 [30]. Furthermore, it has been proposed that caspase-2 may act as an initiator of mitochondrial apoptosis pathways, as Apaf-1-mediated activation of caspase-2 is indicated by a reduction of caspase-2 activation in Apaf-$1^{-/-}$ thymocytes exposed to dexamethasone [31]. It is possible that 2-phenyl-4-quinolone-induced caspase-2 activation is a compensatory effect of the absence of caspase-9 activation.

Recently, it has been discovered that in response to apoptotic insult, mitochondria can release several effectors to initiate a caspase-independent process in apoptosis. AIF is one of the effectors predominantly contributing to the

caspase-independent cell death. Moreover, AIF is capable of interacting with cytochrome c and caspases during several apoptosis processes, revealing that distinct apoptosis pathways may be reciprocally regulated to induce apoptotic cascades [19,32]. Our data showed that the long-term exposure to 2-phenyl-4-quinolone induced a dramatic release of AIF from mitochondria and a subsequent translocation into the nucleus. Given that a profound apoptosis was triggered in a long-term (more than 36 h) treatment with 2-phenyl-4-quinolone, it was suggested that AIF may play a role in facilitating 2-phenyl-4-quinolone-induced apoptotic cell death.

Taken together, it is suggested that 2-phenyl-4-quinolone displays anti-proliferative and apoptotic effects in human cancer cell lines. The mechanism of action involves an interaction with tubulin, leading to dysregulation of mitotic spindles and induction of mitotic arrest of the cell cycle. 2-Phenyl-4-quinolone also induces the Cdk1 activation by the dephosphorylation on Ser-216 of Cdc25c and on inhibitory Tyr-15 of Cdk1. Consequently, the participation of Bcl-2 family members including the phosphorylation of Bcl-2 and Bcl-xL, Mcl-1 cleavage and t-Bid formation, activation of caspases and release of AIF may mutually cross-regulate the apoptotic signaling cascades induced by 2-phenyl-4-quinolone.

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REFERENCES

[1] Dustin P. Microtubules. Sci Am 1980;243:66–76.
[2] Wilson L, Jordan MA. Microtubule dynamics: taking aim at a moving target. Chem Biol 1995;2:569–73.
[3] Manfredi JJ, Parness J, Horwitz SB. Taxol binds to cellular microtubules. J Cell Biol 1982;94:688–96.
[4] Rowinsky EK. The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Annu Rev Med 1997;48:353–74.
[5] Hadfield JA, Ducki S, Hirst N, McGown AT. Tubulin and microtubules as targets for anticancer drugs. Prog Cell Cycle Res 2003;5:309–25.
[6] Nielsen MG, Turner FR, Hutchens JA, Raff EC. Axoneme-specific β-tubulin specialization: a conserved C-terminal motif specifies the central pair. Curr Biol 2001;11:529–33.
[7] Vent J, Wyatt TA, Smith DD, Banerjee A, Luduena RF, Sisson JH, et al. Direct involvement of the isotype-specific C-terminus of β tubulin in ciliary beating. J Cell Sci 2005;118(Pt 19):4333–41.
[8] Seve P, Mackey J, Isaac S, Tredan O, Souquet PJ, Perol M, et al. Class III β-tubulin expression in tumor cells predicts response and outcome in patients with non-small cell lung cancer receiving paclitaxel. Mol Cancer Ther 2005;4:2001–7.
[9] Katsetos CD, Legido A, Perentes E, Mork SJ. Class III β-tubulin isotype: a key cytoskeletal protein at the crossroads of developmental neurobiology and tumor neuropathology. J Child Neurol 2003;18:851–66.
[10] Wang LG, Liu XM, Kreis W, Budman DR. The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review. Cancer Chemother Pharmacol 1999;44:355–61.
[11] Castedo M, Perfettini JL, Roumier T, Kroemer G. Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe. Cell Death Differ 2002;9:1287–93.
[12] Ongkeko W, Ferguson DJ, Harris AL, Norbury C. Inactivation of Cdc2 increases the level of apoptosis induced by DNA damage. J Cell Sci 1995;108:2897–904.
[13] Yu D, Jing T, Liu B, Yao J, Tan M, McDonnell TJ, et al. Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase. Mol Cell 1998;2:581–91.
[14] Debatin KM, Poncet D, Kroemer G. Chemotherapy: Targeting the mitochondrial cell death pathway. Oncogene 2002;21:8786–803.
[15] Adams M, Wube AA, Bucar F, Bauer R, Kunert O, Haslinger E. Quinolone alkaloids from Evodia rutaecarpa: a potent new group of antimycobacterial compounds. Int J Antimicrob Agents 2005;26:262–4.
[16] Adams M, Kunert O, Haslinger E, Bauer R. Inhibition of leukotriene biosynthesis by quinolone alkaloids from the fruits of Evodia rutaecarpa. Planta Med 2004;70:904–8.
[17] Lee MK, Hwang BY, Lee SA, Oh GJ, Choi WH, Hong SS, et al. 1-methyl-2-undecyl-4(1H)-quinolone as an irreversible and selective inhibitor of type B monoamine oxidase. Chem Pharm Bull (Tokyo) 2003;51:409–11.
[18] Jackson SJ, Singletary KW. Sulforaphane: A naturally occurring mammary carcinoma mitotic inhibitor, which disrupts tubulin polymerization. Carcinogenesis 2004;25:219–27.
[19] Van Gurp M, Festjens N, Van Loo G, Saelens X, Vandenabeele P. Mitochondrial intermembrane proteins in cell death. Biochem Biophys Res Commun 2003;304:487–97.
[20] Wils P, Phung-Ba V, Warnery A, Lechardeur D, Raeissi S, Hidalgo IJ, et al. Polarized transport of docetaxel and vinblastine mediated by P-glycoprotein in human intestinal epithelial cell monolayers. Biochem Pharmacol 1994;48:1528–30.
[21] Metzinger DS, Taylor DD, Gercel-Taylor C. Induction of p53 and drug resistance following treatment with cisplatin or paclitaxel in ovarian cancer cell lines. Cancer Lett 2006;236:302–8.
[22] Smits VA, Medema RH. Checking out the G2/M transition. Biochim Biophys Acta 2001;1519:1–12.
[23] Cory S, Adams JM. The Bcl-2 family: Regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2: 647–56.
[24] Pathan N, Aime-Sempe C, Kitada S, Haldar S, Reed JC. Microtubule-targeting drugs induce Bcl-2 phosphorylation and association with Pin1. Neoplasia 2001;3:70–9.
[25] Ling YH, Tornos C, Perez-Soler R. Phosphorylation of bcl-2 is a marker of M phase events and not a determinant of apoptosis. J Biol Chem 1998;273:18984–91.
[26] Scatena CD, Stewart ZA, Mays D, Tang LJ, Keefer CJ, Leach SD, et al. Mitotic phosphorylation of Bcl-2 during normal cell cycle progression and Taxol-induced growth arrest. J Biol Chem 1998;273:30777–84.
[27] Poruchynsky MS, Wang EE, Rudin CM, Blagosklonny MV, Fojo T. Bcl-xL is phosphorylated in malignant cells following microtubule disruption. Cancer Res 1998;58: 3331–8.
[28] Michels J, Johnson PW, Packham G. Mcl-1. Int J Biochem Cell Biol 2005;37:267–71.
[29] Condorelli F, Salomoni P, Cotteret S, Cesi V, Srinivasula SM, Alnemri ES, et al. Caspase cleavage enhances the

apoptosis-inducing effects of BAD. Mol Cell Biol 2001;21:3025–36.
[30] Zheng TS, Hunot S, Kuida K, Momoi T, Srinivasan A, Nicholson DW, et al. Deficiency in caspase-9 or caspase-3 induces compensatory caspase activation. Nat Med 2000;6:1241–7.
[31] Susin SA, Lorenzo HK, Zamzami N, Marzo I, Brenner C, Larochette N, et al. Mitochondrial release of caspase-2 and -9 during the apoptotic process. J Exp Med 1999;189:381–94.
[32] Cregan SP, Dawson VL, Slack RS. Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene 2004;23:2785–96.