

Investigation of anti-tumor mechanisms of K2154: characterization of tubulin isotypes, mitotic arrest and apoptotic machinery

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Abstract Microtubules are crucial targets for cancer chemotherapeutic drugs, and new microtubule-directed agents are of continued interest in drug development. A novel microtubule-directed agent, ethyl-2-[N-*p*-chlorobenzyl-(2'-methoxy)]-anilino-4-oxo -4, 5-dihydro-furan-3-carboxylate, was identified. The compound, designated K2154, inhibited cell proliferation, with IC₅₀ values of 10.3, 15.3, 9.6, 11.2, 12.8 and 12.1 μM in prostate cancer PC-3, hepatocellular carcinoma Hep3B, non-small cell lung cancer A549, colorectal cancer HT29 and HCT116, and P-glycoprotein-rich breast cancer NCI/ADR-RES cells, respectively. Because NCI/ADR-RES cells were susceptible to inhibition by K2154, it indicated that this compound is a poor substrate for P-glycoprotein. In this study, PC-3 cells were used to identify the anticancer mechanisms of K2154. K2154 induced an arrest of the cell cycle at G2/M phase and a subsequent increase of hypodiploid phase in PC-3 cells, whereas it only induced a moderate level of G2/M arrest with little increase of hypodiploid phase in normal prostate cells. K2154 inhibited microtubule assembly in both in vitro turbidity assay and in vivo microtubule spin-down experiment. Immunochemical examination showed

that K2154 caused formation of abnormal mitotic characteristics with bipolar spindles, particularly, in β_{II}- and β_{III}-tubulin staining. It also induced several pathways, including cyclin B1 up-regulation, dephosphorylation on Tyr¹⁵ and phosphorylation on Thr¹⁶¹ of Cdk1 and Cdc25C phosphorylation, and roscovitine (a Cdk1 inhibitor) significantly inhibited K2154-induced apoptosis, suggesting a pro-apoptotic role of Cdk1. Phosphorylation of Bcl-2 and Bcl-xL and cleavage of Mcl-1, together with activation of caspase-9 and -3, indicated that mitochondrial pathway played a central role in K2154-mediated apoptotic cell death. Additionally, AIF contributed to a late phase of K2154-induced apoptotic pathway. In conclusion, it is suggested that K2154 displays an anticancer activity through a target on microtubules and a subsequent signaling cascade on cell cycle regulation and apoptotic machinery.

Keywords Microtubule · Tubulin isotype · Cdk1 · Bcl-2 family proteins · Caspase

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Introduction

Microtubules are structural proteins and composed of α- and β-tubulin heterodimers. There are at least six α-tubulin and seven β-tubulin isotypes that have been discovered in vertebrates (Ludueno 1998). The seven β-tubulin isotypes are encoded by a multigene family with highly homologous gene products, including β_I, β_{II}, β_{III}, β_{IVa}, β_{IVb}, β_V and β_{VI}, but differ in the last 15 carboxyl amino acid residues (Ludueno 1998; Cleveland and Sullivan 1986). Since the tissue distributions and cellular localizations of β-tubulin isotypes are diverse, their functions may be different,

although the details have not been fully elucidated (Walss-Bass et al. 2002; Aria and Matsumoto 1988; Burgoyne et al. 1988). It has been suggested that β_{III} -tubulin is a central target for taxanes. Both basic researches and clinical studies reveal that β_{III} -tubulin overexpression has been associated with microtubule destabilization and resistance to taxanes, and constitutes a prognostic factor in several solid tumors (Seve et al. 2005; Derry et al. 1997). Furthermore, there are several lines of evidence that β_{III} -tubulin is present in neoplastic but not in normal differentiated glial cells (Katsetos et al. 2003). Therefore, the investigation of molecular mechanisms responsible for the targeting on this isotype may provide significant insights into the regulation of the growth and progression of cancer cells. Importantly, microtubules are highly dynamic and play a crucial role during mitosis, such as the support for kinetochore attachment to chromosomes at the prometaphase, the proper position of chromosomes aligned at the metaphase plate and the segregation of the chromosomes at the anaphase and telophase (Jordan and Wilson 2004). The interruption of microtubule dynamics can result in mitotic arrest and inhibition of cell proliferation and, eventually, the induction of cell death. Accordingly, microtubules have become a potential target for the development of cancer chemotherapeutic agents (Sengupta et al. 2005; Orosz et al. 1997).

The activation of the Cdk1/cyclin B1 complex in the nucleus drives the progression from G2- to M-phase of the cell cycle. It has been suggested that Cdk1 could be a target for the induction of apoptosis. For example, the inactivation of Cdk1 increases apoptotic cell death induced by DNA damage (Ongkeko et al. 1995). Nevertheless, there are several lines of evidence that Cdk1 plays a pro-apoptosis other than an anti-apoptosis role in numerous tumor cell types. The tubulin-binding agents, such as taxol and vinca alkaloids, are able to increase the Cdk1 activity and induce apoptosis in tumor cells (Yu et al. 1998). However, the concern is how tubulin-binding agents drive the perturbation of cell cycle progression toward apoptotic cell death. It has been suggested that mitochondria may play a central role in the signaling pathway and that Cdk1 can trigger mitochondrial membrane permeabilization by targeting on Bcl-2 family proteins and subsequently induce cell apoptosis (Debatin et al. 2002).

Based on the basic researches and clinical studies, the target on tubulins can be an effective mechanism for cancer chemotherapy. Nonetheless, most of the tubulin-binding agents are derived from natural products and have complex chemical structures that limit chemical modification. Therefore, simple chemical structures with the aforementioned mechanisms could be valuable as lead structures for future therapeutic agents. K2154 (ethyl-2-[N-*p*-chlorobenzyl-(2'-methoxy)]anilino-4-oxo- 4,5-dihydro-furan-3-carboxylate) is a new synthetic compound with a key structure of 3-

furancarboxylate (a furancarboxylate derivative). It has been suggested that several furancarboxylate derivatives display the pharmacological effects. One is 2-furancarboxylate derivative, which exhibits the binding activity with protein kinase C (Lee et al. 2001). The other two are derivatives of 3-furancarboxylate and 4-furancarboxylate. These two compounds have been reported to display the inhibitory effect on platelet-activating factor (PAF) receptor (Peterson et al. 1989a,b). The structure of K2154 fits the criteria as a pharmacological probe. In this study, the action mechanisms of K2154 have been identified from the characterization of tubulin isotypes and cell cycle regulators to mitochondrial proteins and related apoptotic cascades.

Methods

Tissue explants and cell culture Human hyperplastic prostates were obtained from three males by transurethral resection of the prostate in the National Taiwan University Hospital. All patients had histories of prostatism and were diagnosed to have benign prostate hyperplasia by rectal digital examination, transrectal sonography of the prostate and urodynamic studies. Isolation of human prostatic cells from prostatic tissue explants was described in the previous study (Guh et al. 1998). 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 μ g/ml). Cultures were maintained in a humidified incubator at 37°C in 5% CO₂:95% air.

Sulforhodamine B (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 K2154 addition (T_0). After additional incubation of vehicle (0.1% DMSO) or K2154 for 48 h, cells were fixed with 10% TCA. 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 K2154 (Tx), the percentage growth was calculated at each of the compound concentrations. Percentage growth inhibition was calculated as $[(Tx - T_0)/(C - T_0)] \times 100$ for concentrations for which $Tx \geq T_0$. 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.

FACScan flow cytometric analysis After the treatment of cells with vehicle (0.1% DMSO) or K2154 for the indicated time courses, the cells were harvested by trypsinization, fixed with 70% (v/v) alcohol 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 NaHPO₄, 0.1 M citric acid, pH 7.8) for 30 min at room temperature. 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 using the FACScan and CellQuest software (Becton Dickinson, Mountain View, Calif.).

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 incubated with primary antibodies at 37°C for 1 h. The cells were washed twice with PBS and incubated with the fluorescein isothiocyanate (FITC, 1:100) or tetramethylrhodamine isocyanate (TRITC, 1:100)-conjugated secondary antibody at 37°C for 40 min. The nuclei were recognized by the staining with DAPI (1 mg/ml). The labeled targets in cells were detected by a confocal laser microscopic system (Leica TCS SP2).

In situ labeling of apoptotic cells Cells were seeded in 4-well chamber slides. After the treatment, cells were stained with Hoechst 33342 (0.1 µg/ml) at 37°C for 15 min and fixed with 4% paraformaldehyde for another 15 min at room temperature. The labeled targets in cells were detected by microscopic examination.

Western blotting Cells were seeded in 100-mm Petri dishes. After the treatment, cells were harvested with trypsinization, centrifuged and lysed in 0.1 ml of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptinin, 10 µg/ml aprotinin, 50 mM NaF and 100 µM sodium orthovanadate. In some experiments, the mitochondrial/cytosol fractionation kit (Biovision, Mountain View, Calif.) was used to separate mitochondrial and cytosolic fraction. Total protein was quantified, mixed with sample buffer and boiled at 90°C for 5 min. Equal amount of protein (30 µg) was separated by electrophoresis in either 10 or 12% SDS-PAGE, transferred to PVDF membranes and detected with specific antibodies.

The immunoreactive proteins after incubation with appropriately labeled secondary antibody were detected with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

In vitro tubulin turbidity assay The tubulin polymerization was detected by the use of CytoDYNAMIX Screen 03 kit (Cytoskeleton, Denver, Colo.). Tubulin proteins (>99% purity) were suspended in G-PEM buffer containing 80 mM PIPES, 2 mM MgCl₂, 0.5 mM EDTA, and 1.0 mM GTP (pH 6.9) 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, Sunnyvale, Calif.).

In vivo microtubule assembly assay After the treatment with the indicated compound, the cells were harvested by trypsinization and collected by centrifugation. The cells were lysed with 0.1 ml of hypotonic buffer (1 mM MgCl₂, 2 mM EGTA, 0.5% NP-40, 2 mM PMSF, 200 U/ml aprotinin, 100 µg/ml soybean trypsin inhibitor, 5.0 mM ε-amino caproic acid, 1 mM bezamidine and 20 mM Tris-HCl, pH 6.8). The cytosolic and cytoskeletal fraction of cell lysate were separated by centrifugation at 16,000×g for 15 min. The supernatant contained cytosolic tubulin. The pellet representing the particulate fraction of polymerized tubulin was resuspended in 0.1 ml hypotonic buffer. Tubulin contents in both fractions were detected by western blotting.

Reagents RPMI 1640 medium, FBS, penicillin, streptomycin, and all other tissue culture reagents were obtained from GIBCO/BRL Life Technologies (Grand Island, N.Y.). All of the chemical reagents and antibodies to β_I, β_{II}, β_{III}, and β_{IV} tubulin isotypes and FITC- or TRITC-conjugated secondary antibodies were obtained from Sigma (St Louis, Miss.). Antibodies to Bcl-2, Bcl-xL, Mcl-1, Survivin, Cyclin B1, Cdk1, Cdc25C, poly-(ADP-ribose) polymerase (PARP), cytochrome c, Smac, apoptosis inducing factor (AIF), and anti-mouse and anti-rabbit IgGs were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Antibodies to caspase-9, caspase-8, phospho-cdc2^{Tyr15}, phospho-cdc2^{Thr161}, phospho-Bcl-2^{Ser70} and Bid were from Cell Signaling Technologies (Boston, Mass.). Antibody to OxPhos Complex IV subunit 2 was from Molecular Probes (Leiden, The Netherlands). Antibody to caspase-3 was from Imgenex (San Diego, Calif.). Antibodies to α- and β-tubulins were from Serotec Products (Beverly, MA) and BD Biosciences PharMingen (San Diego, Calif.), respectively. Antibody to MPM-2 was from Upstate Biotechnology (Lake Placid, N.Y.). K2154 (ethyl-2-[N-ρ-chlorobenzyl-(2'-methoxy)]anilino-4-oxo-4,5-dihydro-furan-3-carboxyl-

ate) was synthesized and provided by our colleagues (Dr. Sheng-Chu Kuo).

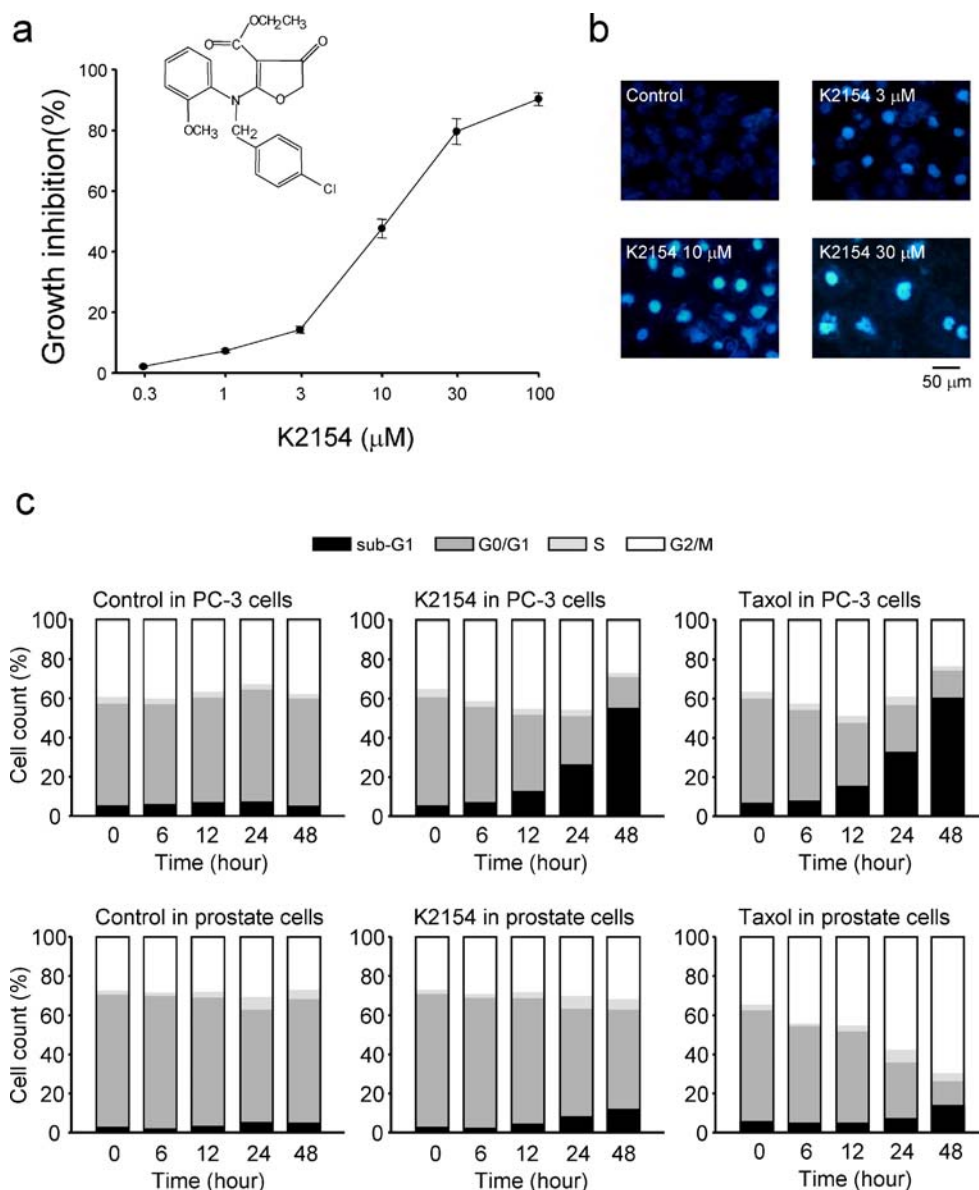
Results

Effect of K2154 on mammary cancer cell proliferation and cell cycle progression

Treatment of human hormone-resistant prostate cancer PC-3 cells with K2154 inhibited cell proliferation in a dose-dependent manner with an IC_{50} of 10.3 μ M (Fig. 1c). K2154 also displayed anti-proliferative effect in several types of cancer cells, including hepatocellular carcinoma Hep3B, non-small cell lung cancer A549, colorectal

cancer HT29 and HCT116, and P-glycoprotein (P-gp)-rich breast cancer NCI/ADR-RES cells, with IC_{50} values of 15.3, 9.6, 11.2, 12.8 and 12.1 μ M, respectively. Furthermore, K2154 was able to induce apoptotic cell death by detection of condensed DNA with Hoechst33342, a sign of apoptosis (Fig. 1b). Next, the effect of K2154 on cell cycle progression was determined by FACScan flow cytometric analysis of PI staining in PC-3 cells. The data demonstrated that K2154 induced a time-dependent increase of G2/M phase and a subsequent increase of hypodiploid (sub-G1) phase of the cell cycle, indicating the occurrence of mitotic arrest and apoptotic cell death (Fig. 1c). Furthermore, the selectivity of K2154 between tumor and normal prostatic cells was also examined. The data showed that K2154 only exhibited a modest effect on the induction of mitotic arrest of

Fig. 1 Effect of K2154 on cell proliferation and cell cycle progression. **a** The graded concentrations of K2154 were added to PC-3 cells for 48 h. Then, the growth inhibitory effect of K2154 was examined by SRB assays. **b** Cells were cultured in chamber slides for 24 h and then treated with graded concentrations of K2154 for another 24 h. After the incubation period, cells were washed and stained with Hoechst 33342, and examined under a fluorescence microscope. **c** PC-3 or primary prostatic cells were incubated in the control vehicle (0.1% DMSO), K2154 (30 μ M) or taxol (0.1 μ M) for the indicated time course. Then, cells were fixed and stained with propidium iodide to analyze DNA content by FACScan flow cytometry



the cell cycle and apoptotic cell death in normal prostatic cells (Fig. 1c). The doubling time of PC-3 and normal prostatic cells was 26.1 and 31.6 h, respectively, in this study.

Effect of K2154 on in vitro and in vivo microtubule assembly and characterization of β -tubulin isotypes

To determine whether tubulins were targets of K2154-induced anti-tumor effect, the in vitro tubulin polymer-

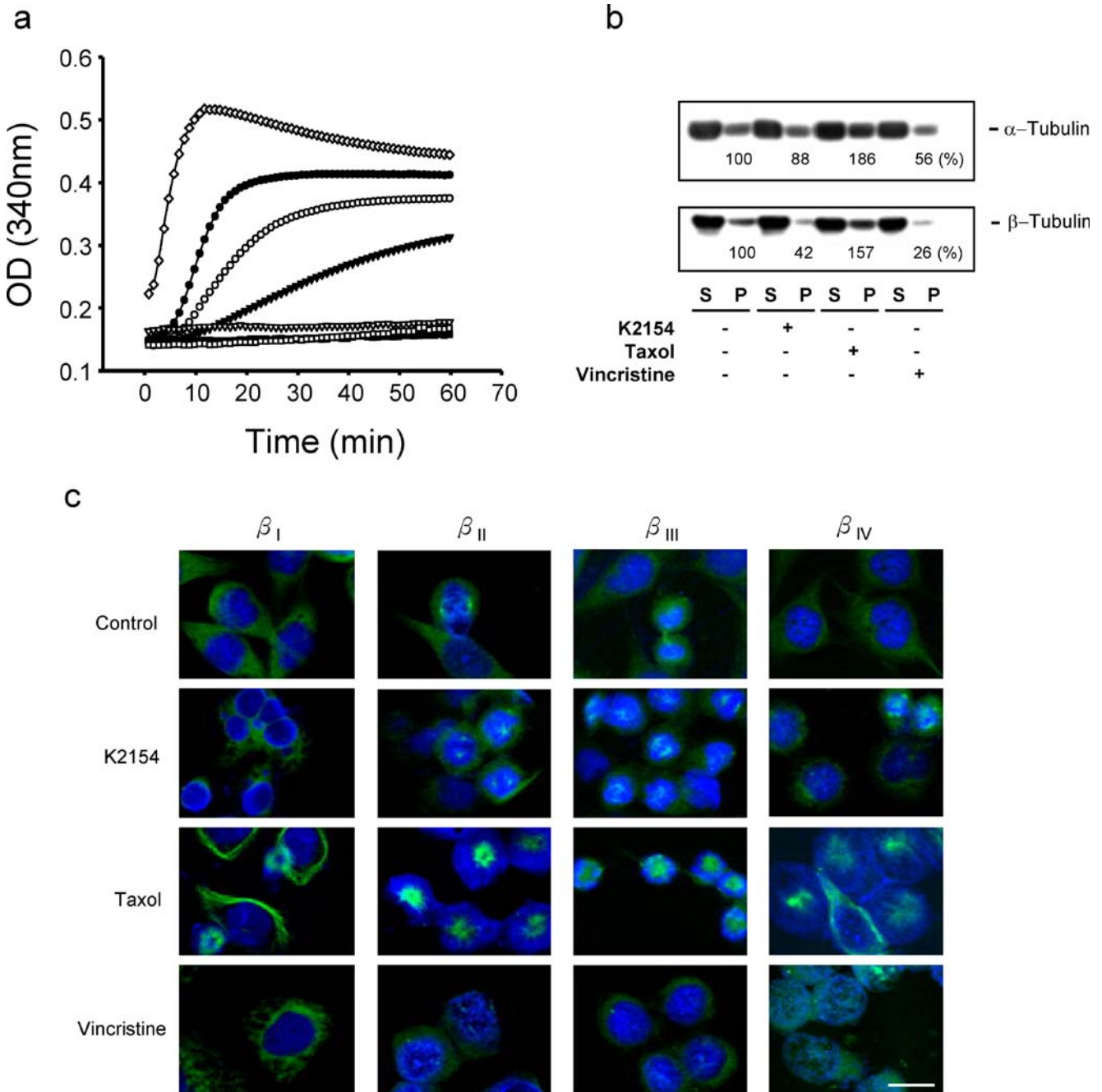


Fig. 2 Effect of K2154 on in vitro and in vivo tubulin assembly assays. **a** Tubulins were incubated in 37°C with GTP in the presence of 0.1% DMSO (●), K2154 (○, 10 μ M; ▼, 30 μ M; ▽, 100 μ M; ■, 300 μ M), taxol (□, 10 μ M) or vincristine (□, 10 μ M). Tubulin assembly was examined turbidimetrically. Change in absorbance at 340 nm is plotted as a function of time in minutes. **b** Cells were incubated in the presence of the indicated agent (K2154, 30 μ M; taxol 0.01 μ M; vincristine 0.1 μ M) for 9 h. Then, the cells were harvested

and separated into soluble (S, microtubule disassembly) and particulate form (P, microtubule assembly), and proteins were detected by western blot analysis. The relative expression of particulate fraction is also shown. **c** Cells were incubated in the vehicle or the indicated agent (K2154, 30 μ M; taxol 0.01 μ M; vincristine 0.1 μ M) for 15 h. Then, the cells were fixed for the detection of microtubule organization by immunochemical staining with antibodies of β -tubulin isotypes and DAPI for nuclear examination. Scale bar, 20 μ m

ization of turbidity assay was carried out. As demonstrated in Fig. 2a, the tubulins polymerized in a time-dependent manner in the presence of GTP at 37°C. As expected, taxol promoted, while vincristine inhibited, the tubulin polymerization. K2154 displayed an inhibitory effect on tubulin polymerization in a concentration-dependent manner (Fig. 2a). We also performed an *in vivo* microtubule assembly assay, which separated assembly and disassembly microtubules in particulate and soluble fractions, respectively. The data showed that K2154 markedly reduced the particulate fraction of β -tubulins by 58%, confirming the inhibitory effect on microtubule assembly (Fig. 2b). In addition, based on the target of β -tubulins, four different β -tubulin isotypes were further examined. The immunochemical examination showed that taxol promoted, whereas vincristine inhibited, the formation of multipolar spindles in all types of β -tubulin staining. In contrast, K2154 induced the formation of dipolar spindle with a characteristic of prometaphase arrest, in particular, by staining with β_{II} - and β_{III} -tubulin (Fig. 2c).

Regulation of the cyclin B1 and Cdk1 by K2154

Progression from G2- to M-phase of the cell cycle is driven by activation of the Cdk1/cyclin B1 complex. The perfect regulation of the Cdk1/cyclin B activity is crucial for the accurate progression of the cell cycle. After the exposure to K2154, the cyclin B1 expression was soon elevated. Furthermore, the dephosphorylation on inhibitory Tyr¹⁵ and phosphorylation on activating Thr¹⁶¹ of Cdk1 were induced. These data, together with the phosphorylation of Cdc25C, indicated that Cdk1 was activated in PC-3 cells when treated with K2154 (Fig. 3a). Additionally, the levels of the mitotic markers MPM2 were also dramatically increased, revealing the arrest of the cells at mitosis to K2154 action. Notably, the sudden decline of the MPM2 expression at a 36-h treatment of K2154 might result from the occurrence of apoptotic cell death (Fig. 3a).

An increase in Cdk1 activity has been found in cells during several apoptotic insults. In some cases the Cdk1 activation is suggested to be a consequence rather than a cause of apoptotic cell death (Zhou et al. 1998). Nonetheless, several studies have suggested that Cdk1 activation can be a mechanism of cell killing and lead to apoptosis (Konishi et al. 2002). To test the role of Cdk1, roscovitine (a pharmacological Cdk1 inhibitor) was used to suppress the Cdk1 activity. The data showed that K2154-induced apoptosis was significantly inhibited by roscovitine (10 μ M, Fig. 3b,c) suggesting that Cdk1 acted as a pro-apoptotic mediator in this study.

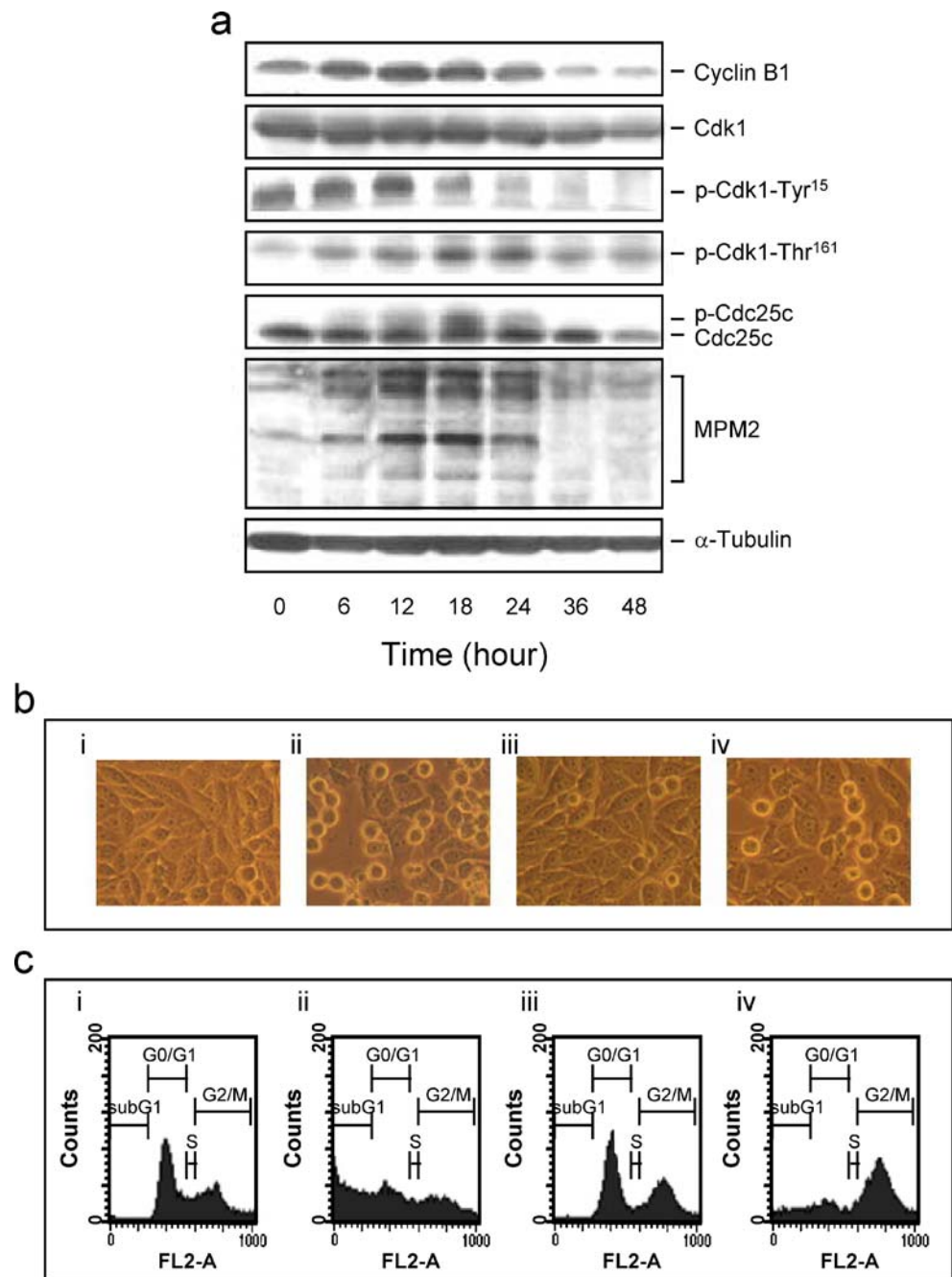
Effect of K2154 on numerous anti- and pro-apoptotic proteins

Recently, a lot of attention has been directed at investigating the role of apoptosis regulators in controlling survival decisions in tumor cells. Currently, two broad categories have been focused on. Within the first category, several pro-apoptotic proteins (e.g., Bax, Bak and Bim) promote release of cytochrome *c*, whereas other anti-apoptotic proteins (e.g., Bcl-2, Bcl-xL and Mcl-1) are able to antagonize the pro-apoptotic member proteins and/or preserve mitochondrial integrity by preventing loss of mitochondrial membrane potential (Abuharheid et al. 2005). Within the second category, proteins such as the inhibitors of apoptosis proteins (e.g., XIAP, CIAP-1, CIAP-2 and survivin) inhibit the activation of caspases, whereas Smac/DIABLO binds to several IAP proteins, thwarting their caspase-inhibitory functions (Holcik et al. 2001). In this study, K2154 had little effect on the pro-apoptotic members of Bcl-2 family proteins (data not shown). However, it induced the phosphorylation of Bcl-2 and Bcl-xL and the cleavage of Mcl-1 after 6- and 18-h treatments, respectively (Fig. 4). Furthermore, survivin was down-regulated while Smac/DIABLO was dramatically released from the mitochondria in a long-term treatment (48 h) with K2154 (Fig. 4). In addition to Smac/DIABLO, we also examined several apoptogenic factors including cytochrome *c* and AIF, which were also released from the mitochondrial intermembrane space. By using western blotting examination, there was no Complex IV expression indicating the cytosolic fraction without the existence of mitochondria (Fig. 5). The data also demonstrated that the release of cytochrome *c* and AIF from mitochondria was induced by K2154 after treatments for 18 and 48 h, respectively. (Fig. 5).

Effect of K2154 on the activation of caspases

Activation of caspases during apoptosis results in the cleavage and activation/inactivation of a range of critical cellular substrates, including the DNA repair enzyme PARP. In order to determine which caspases were involved in K2154-induced apoptosis, the expressions of activated caspases were detected by western blotting. In control cells, PARP and caspase-8, -9 and -3 were all present as uncleaved forms. After the treatment with K2154, the cleavage of PARP and caspases to catalytically active fragments was clearly detected in a time-dependent manner, suggesting the activation of these caspases. The data also showed that the time of caspase-9 activation, which preceded that of caspase-8, corresponded with the cytochrome *c* release (Fig. 6).

Fig. 3 Function examination of the involvement of Cdk1. **a** Cells were incubated in 0.1% DMSO or K2154 (30 μ M) for the indicated time courses. Then, the cells were harvested and lysed for the detection of protein expressions with antibodies of mitotic regulators by western blot analysis. **b, c** Cells were pre-incubated in 0.1% DMSO (i and ii) or roscovitine (iii and iv, 10 μ M) for 30 min and then K2154 (ii and iv, 30 μ M) was added for another 24 h. The cells were observed by microscope or stained with propidium iodide to analyze DNA content by FACScan flow cytometry

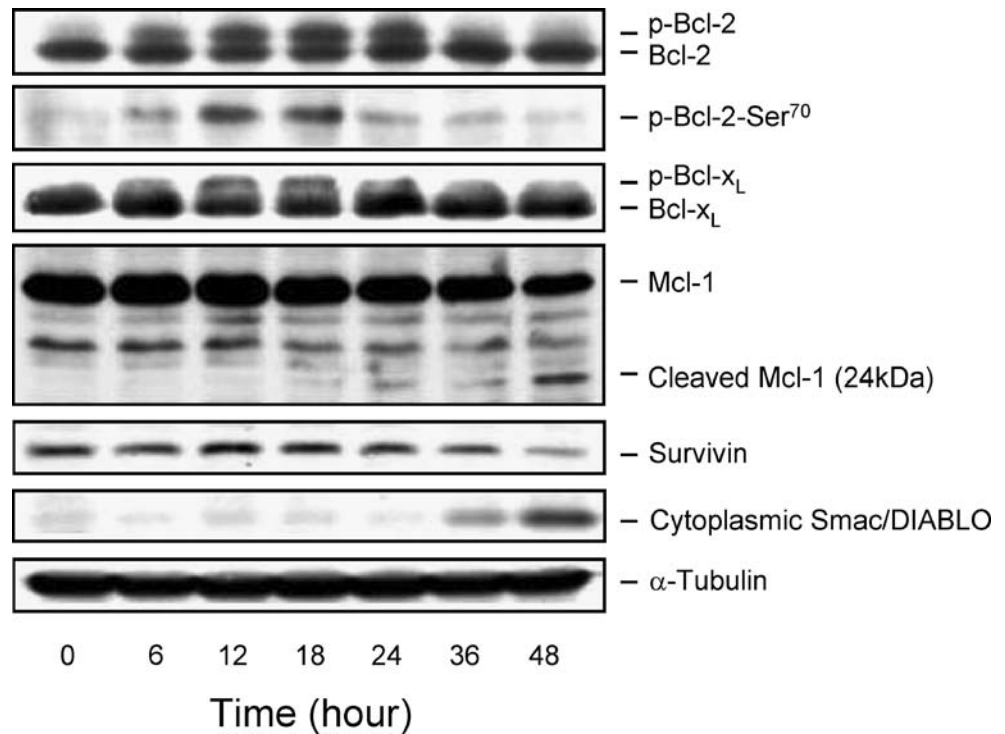


Discussion

Microtubules, composed of α - and β -tubulin heterodimers, are important cytoskeletal components involved in the regulation of cell proliferation, differentiation, and apoptosis. The microtubular dynamics are delicately regulated by polymerization and depolymerization of tubulin. Numerous natural products inhibit cell proliferation by acting on tubulin, affecting its assembly properties. Accordingly, tubulin-directed agents are potential ligands as chemotherapeutic drugs for the treatment of a variety of tumor types

(Ehrlichova et al. 2005; Sengupta et al. 2005; Jordan and Wilson 2004). K2154 is a chemically synthetic compound with a simple chemical structure. It displayed anti-proliferative activity in numerous types of human cancer cells. In particular, K2154 showed anticancer effect in P-gp-rich NCI/ADR-RES cells with similar potency to that in other cancer types. The data indicated that K2154 was not a P-gp substrate. It is meaningful for the development of cancer chemotherapeutic agents since P-gp confers multiple drug resistance by effluxing a diverse array of anticancer drugs, such as vincristine, vinblastine, docetaxel and taxol (Silverman

Fig. 4 Effect of K2154 on the expression of family proteins of Bcl-2 and IAP. Cells were incubated in 0.1% DMSO or K2154 (30 μ M) for the indicated time courses. Then, the cells were harvested and lysed for the detection of protein expressions with antibodies of family proteins of Bcl-2 and IAP by western blot analysis



1999; Wils et al. 1994). Furthermore, our data showed that K2154 displayed more potency in inducing mitotic arrest and apoptosis in PC-3 cells than those in normal prostatic cells. The shorter doubling time of PC-3 than normal prostatic cells may partly explain the selectivity.

To further characterize the anti-tumor effect, K2154 was found to inhibit the polymerization of tubulin (>99% purity) in an in vitro turbidity assay and reduce the particulate fraction of β -tubulin in an in vivo assay. These data suggested that the direct target on tubulin, especially on β -tubulin, leading to the inhibition of tubulin polymer-

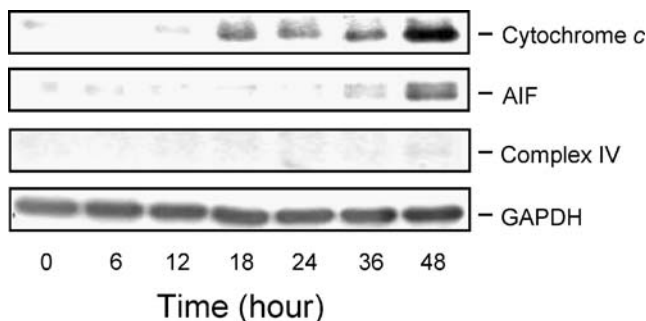


Fig. 5 Effect of K2154 on the expression of mitochondrial apoptogenic factors. Cells were incubated in 0.1% DMSO or K2154 (30 μ M) for the indicated time courses. After the treatment, the cells were harvested and separated into mitochondrial and cytosolic fractions as described in Methods. The cytosolic protein expression was detected with antibodies of mitochondrial apoptogenic factors by western blot analysis. Complex IV is used to detect the presence of mitochondria

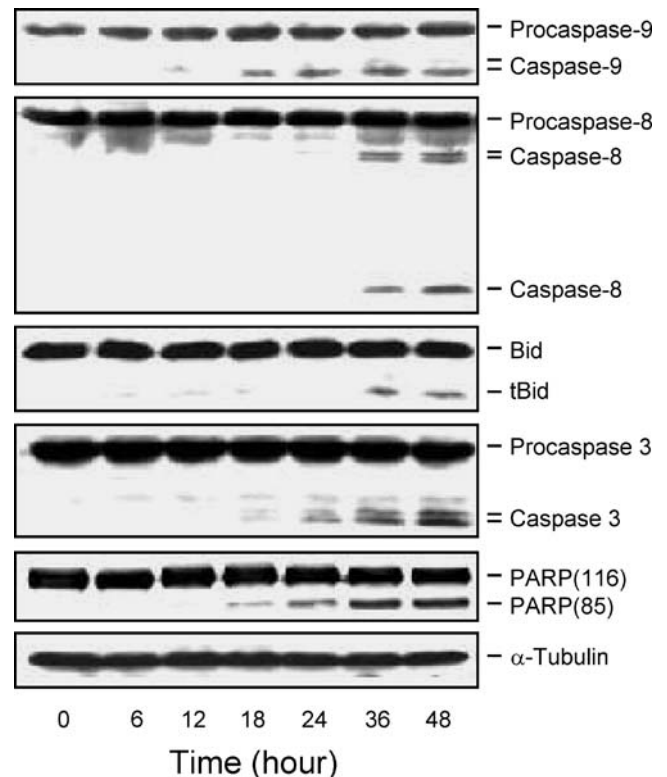


Fig. 6 Effect of K2154 on the cleavage of procaspases and related substrates. Cells were incubated in 0.1% DMSO or K2154 (30 μ M) for the indicated time courses. Then, the cells were harvested and lysed for the detection of protein expressions with antibodies of several caspases and related substrates by western blot analysis

ization might primarily contribute to K2154-induced effect. It has been suggested that the different affinity of the agents on tubulin isotype as well as various isotype compositions in different cancer types determine the efficacy and toxicity of the agents (Pellegrini and Budman 2005; Lobert et al. 1998; Correia 1991). After further investigation, our data demonstrated that K2154 exhibited more selectivity toward β_{II} - and β_{III} -tubulin, although it inhibited tubulin polymerization, an action similar to vinca alkaloids. Numerous studies suggest that vincristine triggers dissolution of microtubules, inducing formation of tubulin paracrystals and causing neurotoxicity (Muller et al. 1988). In contrast, although K2154 inhibited tubulin polymerization (Fig. 2a), the cells with a moderate degree of microtubule association were apparent (Fig. 2c). This discrepancy may, possibly, explain the lesser cytotoxic effect of K2154 on human normal prostatic cells and rat primary neuronal and microglial cells (data not shown). Furthermore, β_I - and β_{IV} -tubulin were found in most of the mammalian axonemal structures, including the olfactory sensory neurons and ciliated epithelial cells of the nose, trachea, ependyma, fallopian tube and testis, suggesting their functional involvement (Vent et al. 2005; Jensen-Smith et al. 2003). Our data showed that K2154 exhibited minor effect on β_I and β_{IV} -isotypes, suggesting a limited toxicity on cilia function.

It has been widely accepted that the mitotic arrest of the cell cycle or an aberrant mitotic exit into a G_1 -like multinucleate condition is a crucial causative factor for apoptosis induced by tubulin-binding agents (Chen and Horwitz 2002; Wang et al. 1999; Lin et al. 1998). Furthermore, an increase in Cdk1 activity has been found in cells during several apoptotic insults. Several studies have suggested that Cdk1 activation can be a mechanism of cell killing and lead to apoptosis (Konishi et al. 2002). In this study, the mitotic arrest induced by K2154 was found to precede the cell death. Accordingly, the activation of Cdk1 was identified to contribute to K2154-induced cell death by several observations, namely, (1) the elevation of cyclin B1 expression, (2) the phosphorylation of Cdc25C, (3) the dephosphorylation on inhibitory Tyr¹⁵ and phosphorylation on activating Thr¹⁶¹ of Cdk1, and (4) the suppression of cell death by roscovitine (a Cdk1 inhibitor). Mitotic catastrophe, a type of cell death thought to share some apoptotic pathways, is extensively characterized in the effect induced by tubulin-binding agents (Hyzy et al. 2005; Castedo et al. 2004). Mitotic catastrophe is controlled by numerous molecular regulators, such as cyclin B1-dependent Cdk1, aurora kinases, cell-cycle checkpoint proteins, survivin, p53, caspases and the Bcl-2 family of proteins (Hyzy et al. 2005; Castedo et al. 2004). In this study, K2154-treated cells appeared to stay arrested in the mitotic phase from which they consequently entered a cell death pathway or exited mitosis, but the nuclei were deformed (Fig. 2c). Beside the

morphological observations, K2154-treated cells showed various biochemical criteria, including the activation of Cdk1/cyclin B1, the dramatic appearance of mitotic epitope MPM-2, the activation of caspases and alteration of the Bcl-2 family of proteins (see below). It is likely that the cells treated with K2154 at least share some characteristics with those of undergoing mitotic catastrophe.

Next to the function in respiration, mitochondria play a crucial role in the control of apoptosis. Numerous stimuli and stresses are able to stimulate the release of apoptogenic factors from the mitochondrial intermembrane space, leading to the programmed demise of the cell. There is increasing evidence that the regulation of the Bcl-2 family of proteins shares the signaling pathways induced by tubulin-binding agents (Wang et al. 1999). Our data demonstrated that K2154 induced the phosphorylation of Bcl-2 at ser⁷⁰, which was widely reported to be associated with loss of its anti-apoptotic function (Wang et al. 1999; Haldar et al. 1995). Additionally, the Bcl-xL phosphorylation was also stimulated by K2154 and other tubulin-binding agents (Poruchynsky et al. 1998). It is believed that Bcl-xL phosphorylation may play a crucial role in regulating its function and may participate in the determination of tubulin/microtubule-related chemotherapeutic efficacy in human tumors (Poruchynsky et al. 1998). Interestingly, K2154 also stimulated the cleavage of Mcl-1 protein. It has been suggested that Mcl-1 is a substrate for caspases, in particular caspase-3, during induction of apoptosis. The Mcl-1 cleavage occurs after Asp¹²⁷ and Asp¹⁵⁷ and generates four products of 24, 19, 17 and 12 kDa (Michels et al. 2005; Herrant et al. 2004). Our data showed that the onset of Mcl-1 cleavage correlated with the activation of caspase-3, resulting in a predominant fragment of 24 kDa, suggesting the involvement of caspase-3 in Mcl-1 cleavage.

Caspases are intracellular cysteine proteases responsible for and associated with apoptosis. In this study, the western blot analysis revealed that the cytochrome *c* release and activation of caspase-9 and -3, which preceded caspase-8, were time-correlated, indicating that K2154-induced apoptosis might be primarily mediated by the mitochondrial (intrinsic) apoptosis pathway. Furthermore, the release of AIF was also induced by K2154. Recent studies have identified the relationship between PARP-1 activation and nuclear translocation of AIF, suggesting that AIF is a crucial downstream effector of PARP-1-mediated cell death (Hong et al. 2004). Although the link between PARP-1 activation and AIF release reaction could not be made in the present work, the AIF-mediated caspase-independent pathway might contribute to K2154-induced anti-tumor effect in this study. Taken together, our data suggest that the mechanism of action of K2154 involves an interaction with tubulin resulting in disturbance of

regular function of mitotic spindles, leading to mitotic arrest and activation of apoptotic signaling cascades. K2154 is an effective anti-tubulin agent with simple chemical structure, which allows further design and synthesis of derivatives to minimize toxic effect and maximize aqueous solubility for the development of cancer chemotherapeutic drugs.

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