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Microtubule Inhibitory Effects of Various SJ Compounds on Tissue Culture Cells

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SJ compounds (SJ8002 and related compounds) are a group of novel anticancer agents (Cho, Chung, Lee, Kwon, Kang, Joo, and Oh. PCT/KR02/00392). To explore the anticancer mechanism of these compounds, we examined the effect of SJ8002 on microtubules of six human cell lines. At a high concentration (2 μg/mL), SJ8002 effectively disrupted microtubules of the six cell lines within 1 h. At lower concentrations (0.05~1.0 μg/mL), the antimicrotubule activity of SJ8002 varied defending on cell lines. The inhibition of *in vitro* polymerization of pure tubulin by SJ8002 suggested that SJ8002 acts on free tubulin, inhibits the polymerization of tubulin dimer into microtubules, and hence induces the depolymerization of microtubules.

Key words: SJ compounds, Anticancer, Antimicrotubule, Tubulin

INTRODUCTION

Microtubules are involved in various cellular activities that are essential for the survival of eukaryotic cells. The formation of spindle fibers that enables an equal distribution of the duplicated genetic material of an eukaryotic cell to two daughter cells at every round of cell division is possibly the most important role of microtubules. In interphase cells, cytoplasmic microtubules are originated from a centrosome. Before each cell division, the centrosome is duplicated. At the onset of mitosis, cytoplasmic microtubules are depolymerized into free tubulin dimers and the dimers are assembled into spindle fibers that are elongated from the two duplicated centrosomes that now act as spindle poles. As the spindle fibers elongate, the two spindle poles move apart to opposite ends of the cell. In the mean time, some of the spindle fibers are attached to kinetochore of sister chromatids (kinetochore microtubules). When all kinetochores are attached with spindle fibers, sister chromatids begin to separate and each chromosome moves to opposite poles with the shortening of kinetochore microtubules. Inhibition of this microtubule dynamics during cell division results in the arrest of cell proliferation and cell death. Due to this critical role of microtubule in cell proliferation, chemical reagents that disrupt the formation and function of spindle fibers have been studied as one of the major sources for the development of therapeutic drugs for cancer treatment and some of them are currently used for clinical treatment (Jordan *et al.*, 1998; Nogales, 2001).

SJ8002 (1-[(5,6-dimethyl-2-methoxypyridin-3-yl) aminocarbonyl]-4-(3,5-xylyl)-piperazine hydrochloride) and several related compounds belong to a group of novel synthetic compounds (SJ compounds) that exhibit anticancer activities (Fig. 1) (Cho *et al.*, 2002). In this report, we present data that SJ8002 depolymerizes microtubules of various human cell lines. Inhibition of *in vitro* tubulin polymerization by SJ8002 suggests that SJ8002 directly acts on free-tubulin dimers and prevents these dimers from polymerizing into microtubules, hence induces the depolymerization of microtubules. In addition, we present data that other SJ compounds exhibit similar but not identical antimicrotubule activities on microtubules of HeLa cells.

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MATERIALS AND METHODS

SJ compounds

SJ8002 ((1-[(5,6-dimethyl-2-methoxypyridin-3-yl)amino-carbonyl]-4-(3,5-xylyl)-piperazine hydrochloride, MW 404.93) and related compounds (Fig. 1) were produced by Samjin Pharmaceutical Co. LTD. SJ compounds were dissolved in DMSO (10 mg/mL).

Cells

The human fibroblast cell line, 293, cervical carcinoma line, HeLa, human hepatoma lines, HeG2, Huh7, and Hep3B were grown in Dulbecco's modified Eagle medium (DMEM; GIBCO-BRL) supplemented with 10% FBS and 4 mM glutamine. The human prostate cancer cell line, DU145, was grown as described elsewhere (Hartley-Asp and Gunnarsson, 1982). Cultures were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Cell fixation and immunocytochemistry

Cells were washed three times with PBS and fixed with 4% paraformaldehyde (in PBS) for 15 min. Fixed cells were washed three times, 5 min each, with PBS. For immunostaining, mouse monoclonal anti-α-tubulin antibody (T5168; Sigma) in PBS-BT (1% BSA, 0.1% Triton X-100 in PBS) was added to the fixed cells and incubated for 1 h at room temperature. The cells were washed with PBS four times, 5 min each, and secondary antibody (TRITC-conjugated-anti-mouse IgG antibody) in PBS-BT was added. After 45 min at room temperature, the cells were washed with PBS as above. All samples were mounted in Mowiol mounting solution (10% Mowiol 4.88, Calbiochem, La Jolla, CA, 25% glycerol, 0.1 M Tris-HCl pH 8.5).

Polymerization of free-tubulin *in vitro*

Four quartz cuvettes containing 96 μL of tubulin solution (5 mg/mL pure bovine tubulin, purchased from Cytoskeleton, USA) in glycerol buffer (5% glycerol in 80 mM Na-PIPES, pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA) were placed in ice. To each cuvette, 4 μL of DMSO (control) or 4 μL of SJ compounds in DMSO was added and mixed. The cuvettes were placed in ice until the initiation of polymerization assay. At the initiation of polymerization reaction, 2 μL of GTP (100 mM) was added to each cuvette and the cuvettes were incubated at 35°C. Tubulin polymerization was observed by measuring the absorbance of the solution (340 nm) over time.

RESULTS

Microtubule depolymerizing effects of SJ8002 on various human cell lines

To examine the effect of SJ8002 on microtubules, six human cell lines were treated with various concentrations of SJ8002 and fixed. The fixed cells were stained with mouse monoclonal anti-α-tubulin antibody and TRITC-conjugated rabbit anti-mouse IgG antibody. The distribution of microtubule was examined using a fluorescence microscope. In control cells, an organized array of microtubules emanating from a centrosome to the cells periphery was observed (data not shown; see Fig. 2 for microtubules in HeLa cells). Addition of SJ8002 at a high concentration (2 g/mL) completely depolymerized microtubules of all examined cell lines within 2 h (data not shown, see Fig. 2 and 3 for disruption of microtubules). To examine the effect of SJ8002 in detail, SJ8002 was added to HeLa cells and the cells were fixed 10, 30, and 60 min after the drug treatment then stained with antibodies as above (Fig. 2). In control cell's, an organized array of microtubules was clearly visible. In these cells, microtubules were emanated from a common center near the nucleus (centrosome) and elongated to the cells periphery. In the cells periphery, it was possible to trace each microtubule. SJ8002 disrupted the organized microtubules rapidly. The disruption of microtubule was already evident in cells that were fixed at 10

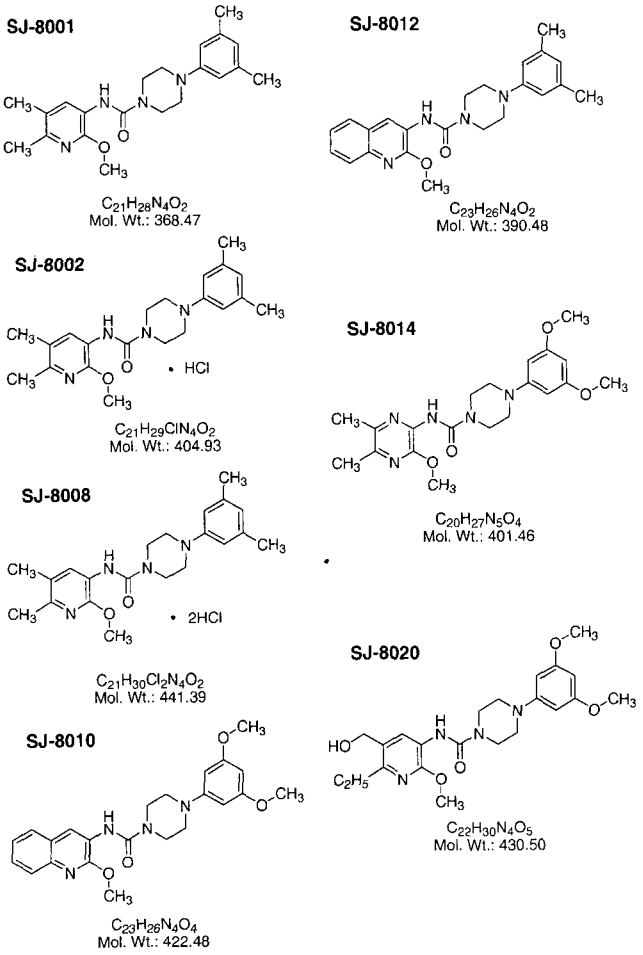

Fig. 1. The structures and molecular weights of SJ compounds

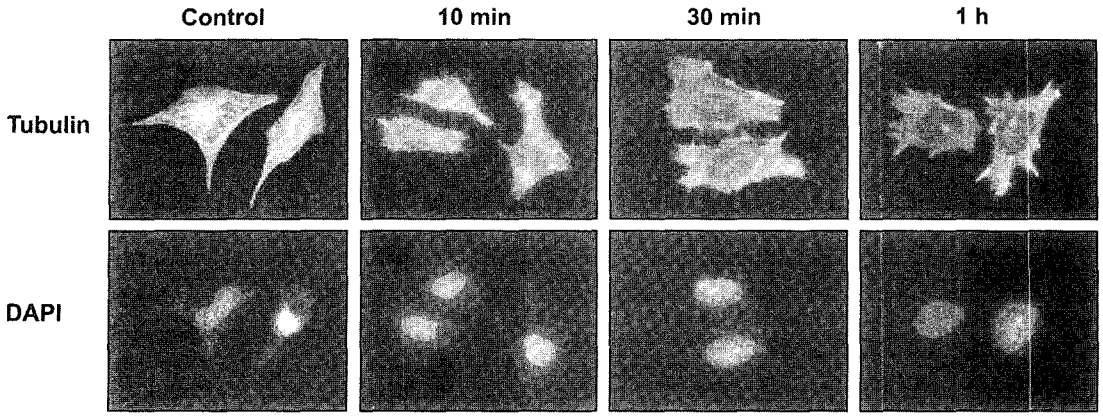

Fig. 2. The effects of SJ8002 on HeLa cell microtubules. SJ8002 was added to growing HeLa cells to a final concentration of 2 μg/mL. The cells were fixed 10, 30, and 60 min after the drug treatment. The effect of SJ8002 on the microtubules of HeLa cells was visualized by immunostaining with mouse monoclonal anti-α-tubulin antibody and TRITC-rabbit anti-mouse-IgG antibody. Tubulin; distribution of microtubules. DAPI; DAPI staining for nucleus.

min after the drug addition (Fig. 2). These cells still had microtubules but these microtubules were short and disorganized significantly. At 30 and 60 min after the drug treatment, cells had no organized array of microtubules. This disruption of microtubule organization by SJ8002 also caused noticeable changes in the cells' shape (Fig. 2). We obtained almost identical results with 293 cells (data not shown). These data showed SJ8002 is a potent novel antimicrotubule toxin.

To further examine the effectiveness of SJ8002 as an antimicrotubule toxin, we add SJ8002 at low concentrations (0.05, 0.1, 0.5, and 1.0 μg/mL) on 4 human cancer cell lines (one prostate cancer cell line, DU145; and three hepatoma cell lines, Huh7, HepG2, and Hep3B) and examined the organization of microtubules at 10 and 30 min after the drug treatment (Table I). When cells were treated with 0.05 μg/mL SJ8002, disruption of microtubule was not noticeable in the 4 examined cell lines. When cells were treated with 0.1 μg/mL SJ8002, the 4 cell types showed varying sensitivities to the drug. In HepG2 cells, partial disruption of microtubules in the periphery of the cells was observed in 20 and 56% of the cells at 10 and 30 min, respectively (Table I). In Hep3B cells, about 40% of cells had partially disrupted microtubules in the periphery at 10 min. When the cells were examined at 30 min, most of the cells had fragmented microtubules (Table I). However, microtubule depolymerization was not clearly evident in Huh7 cells and DU145 cells (Table I). Addition of 0.5 μg/mL SJ8002 caused microtubule depolymerization in HepG2, Hep3b, and DU145 cells (Table I). At 10 min after the drug addition, disruption of microtubule was evident in most of the three cell lines. Even though SJ8002 caused the disruption of microtubules in the three cell lines, the effect of SJ8002 was different depending on cell lines. In Hep3B, fragmentation of microtubule was clearly visible at 10 min and, at 30 min, microtubules were observed only around the nucleus. In HepG2 cells, disruption of microtubules was observed most of the cells. However, the microtubule disruption was evident only in the cells' peripheries as observed in cells treated with 0.1 μg/mL of the drug. At 30 min after the drug treatment, these cells still had microtubules in the middle of the cells. In DU145 cells, SJ8002 treatment not only caused disruption of microtubules but also induced accumulation of

Table I. The effect of SJ8002 on microtubules of four human cancer cell lines.

Cell lines	0.05 μg/mL	0.1 μg/mL	0.5 μg/mL	1.0 μg/mL
Huh7				
10 min	-	-	-	-/+
30 min	-	-	-	-/+
DU145				
10 min	-	-	+	
30 min	-	-	++	
HepG2				
10 min	-	-/+	++	
30 min	-	-/+	+++	
Hep3B				
10 min	-	-/+	++	
30 min	-	+	+++	

To each cell culture, SJ8002 was added to a final concentration of 0.05, 0.1, 0.5, or 1.0 μg/mL. The cells were fixed at 10 or 30 min after the drug treatment and fixed. The fixed cells were immunostained with mouse monoclonal anti-α-tubulin antibody and TRITC-rabbit anti-mouse-IgG antibody. The microtubule depolymerizing effects of SJ8002 on microtubules of these cell lines were examined under fluorescent microscope.

+: Empirically determined degree of microtubule disruption in affected cells.

tubulin into spots at the cells' periphery. Different from the three cell lines, Huh7 cells showed no visible microtubule depolymerization under this condition. Disruption of microtubule in Huh7 cells was first observed when the cells were treated with 1.0 μg/mL of SJ8002. Addition of 1.0 μg/ml of SJ8002 resulted in almost complete disruption of microtubules in other cell types (Table I, data not shown).

Microtubule depolymerizing effect of other SJ compounds

Since SJ8002 showed potent antimicrotubule activity on microtubules in various cell types, we have tested whether other SJ compounds also have similar antimicrotubule activities. HeLa cells were treated for 1 h with various SJ compounds (8001, 8008, 8010, 8012, 8014, and 8020, final conc. 1 μg/mL, Fig. 1) and fixed. Fixed cells were stained with anti-α-tubulin antibody and TRITC-conjugated secondary antibody as above (Fig. 3). Among those tested compounds, SJ8014 exhibited a similar antimicrotubule activity to that of SJ8002. SJ8001, 8008, 8012, and 8020 were less effective than SJ8002 in disrupting HeLa cell microtubules. SJ8010 only caused partial disruption of microtubule. Besides the general depolymerization of cytoplasmic microtubules, these compounds had different effects on the organization of HeLa cell microtubules. In cells treated with SJ8001, the centrosomes were very strongly stained with the tubulin antibody. In cells treated with SJ8014 and 8020, tubulin was concentrated in nuclear region. When the HeLa cells treated with different SJ compounds were stained with FITC-phalloidin to examine a possible effect of these compounds on actin filament, significant increase in the number of stress fiber was observed in cells treated with SJ8001 or 8002 (data not shown).

Inhibition of tubulin polymerization by SJ8002 *in vitro*

Our data showed that addition of SJ compounds disrupted microtubules. To study on the mechanism of SJ compounds, we first examined the effect of SJ8002 on the polymerization of free tubulin into microtubules *in vitro* (Fig. 4). Pure tubulin (5 mg/mL) was incubated with various concentrations of SJ8002 and the polymerization of tubulin was estimated based on the increase in optical density at 340 nm (Materials and Methods). Without SJ8002, the polymerization of free tubulin was completed within 5 min. The OD value was at the maximum at 5 min after the initiation of polymerization and then decreased slowly. At 40 min, the OD value decreased to 87% of the peak value. This rapid polymerization of free-tubulin was also observed in many other systems (Barbier *et al.*, 2001; Combeau *et al.*, 2000). Addition of SJ8002 inhibited polymerization of free-tubulin in a concentration dependent way. In the presence of low concentration of SJ8002 (0.5 μg/mLl), the *in vitro* tubulin polymerization was not inhibited significantly. The OD value increased rapidly reaching the peak value at 5 min then decreased slowly as observed in the control. However, the OD values were about 80% of the control at respective stages (86% at 5 min and 73% at 40 min). Addition of 5 μg/mL of SJ8002 at the initiation of

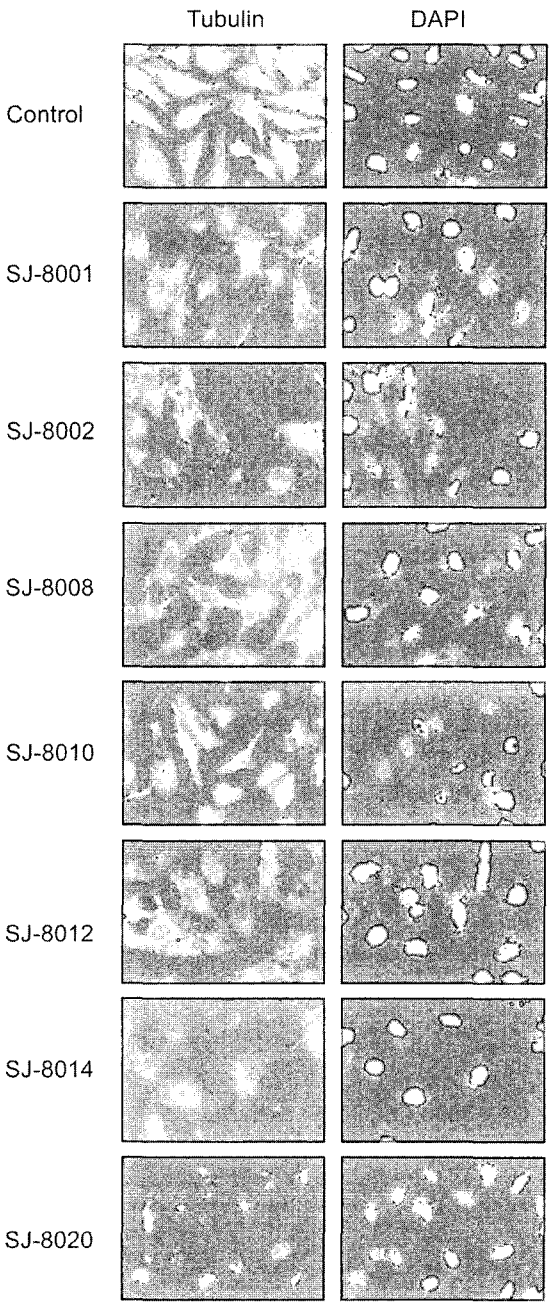

Fig. 3. Microtubule depolymerizing activity of various SJ compounds. Different SJ compound was added to growing HeLa cells to a final concentration of 1.0 μg/mL. One hour after the drug treatment, the cells were fixed and immunostained as in Fig. 2. Tubulin: Immunostaing with anti-α-tubulin antibody. DAPI: DAPI staining for nucleus.

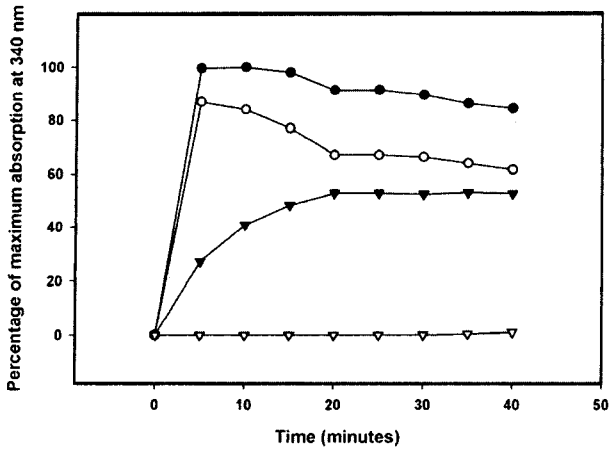

Fig. 4. SJ8002 inhibits *in vitro* polymerization of tubulin. Tubulin polymerization *in vitro* was carried out as described in Materials and Methods. Each point represents the average of two separate experiments after correcting for background (absorbance at 0 min for each reaction). The absorbance is plotted as percentage of the maximum absorption in controls (OD 340 at 5 min; 1.46 for experiment 1 and 1.50 for experiment 2). Symbols: °, control; , 0.5 μg/mL; °¤, 5 μg/mL; °„, 10 μg/mL.

polymerization significantly inhibited the polymerization. At 5 min after the initiation, the OD value increased to 27% of the control value. The maximum polymerization was observed at 20 min after the initiation. At that time, the OD value was 57% of that of the control. Addition of 50 μg/mL SJ8002 at the initiation of polymerization completely inhibited the polymerization reaction.

DISCUSSION

Addition of SJ8002 to six human cell lines resulted in disruption of microtubules of the cells. At a high concentration (2 μg/mL), SJ8002 depolymerized microtubules almost completely in the six tested cell lines within 2 h. Even at lower concentrations (0.5 μg/mL), disruption of microtubules was evident at 30 min after the drug treatment in HepG2, Hep3B, and DU145 cells. However, the microtubules of Huh7 cells were not depolymerized noticeably in these conditions. These data showed that SJ8002 is a potent novel antimicrotubule toxin, which efficiently disrupts microtubules of different cell types, and that different cell types had different sensitivities to SJ8002. Other SJ compounds, except for 8010, were also effective in disrupting HeLa cell microtubules. However, each compound had different effect on the organization of HeLa cell microtubules (Fig. 3). The nature of the cell type-dependent differences in the sensitivity to SJ8002 and the different effects on the organization of HeLa cell microtubules by different SJ compounds are not clear. It might be because either each SJ compound has different toxic effect on different tubulin isotypes or each compound acts on a common site in tubulin isotypes with different activities (Murphy, 1991; Raff, 1994; Nogales, 2001). It is also possible that different cell types might have different activity in transporting these compounds across the cell membrane.

Most known antimicrotubule toxins exhibit their toxic effect by disrupting dynamic nature of microtubules either by inhibiting microtubule formation or by stabilizing microtubules (Jordan *et al.*, 1998; Nogales, 2001). Colchicine and vinblastine are two well-known examples of the former group (Jordan and Wilson, 1998). Cholchicine binds to β-tubulin of a tubulin dimer and inhibits the incorporation of this dimer into microtubules hence induce microtubule depolymerization. Vinblastine, at low concentrations, binds to tubulins in microtubules and stabilizes the microtubule at plus end. However, at high concentrations, vinblastine binds to free-tubulin dimer and disrupts microtubules. Taxol is one example of the later group. It stabilizes the formed microtubules hence inhibits the dynamic nature of microtubules (Jordan and Wilson, 1998). Several other agents inhibit tubulin polymerization by alkylating sulfhydlyl group of tubulin (Jordan *et al.*, 1998). Besides these agents that act on tubulin, a few kinesin inhibitors are also known to disrupt mitotic spindle formation (Sakowicz *et al.*, 1998; Mayer *et al.*, 1999). The inhibition of *in vitro* polymerization of pure tubulin by SJ8002 suggested that SJ8002 acted on free tubulin and prevented the tubulin from polymerizing into microtubules. Even though this inhibition required high concentration of SJ8002 (5 and 50 g/mL), it is not a unique property of SJ8002. The requirement of high concentration of microtubule toxins for inhibition of *in vitro* polymerization has been observed in other systems (Kruczynski and Hill, 2001; Smith and Xhang, 1996) but the nature of this phenomenon is not known yet.

Due to the essential role of microtubules in eukaryotic cell division, tubulin has been studied extensively as a target for anticancer drug and hundreds of microtubule toxins are now known, but only a few of them are currently used to treat cancer. This limitation in the number of therapeutic antimicrotubule drugs and the development of resistance to these drugs (Shan *et al.*, 1999; Gottsman *et al.*, 1996, 2002) necessitate a continuous search for new compounds. Our data show that SJ8002 and related compounds are potent antimicrotubule toxins with anticancer activity (Cho *et al.*, 2002). Further studies on the mechanism of antimicrotubule activity of SJ compounds would potentiate the possible development of a new anticancer drug based on SJ compounds.

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REFERENCES

Barbier, P., Gregoire, C., Devred, F., Sarrazin, M., and Peyrot, V., *In vitro* effect of cryptophycin 52 on microtubule assembly and tubulin: Molecular modeling of the mechanism of action of a new antimitotic drug. *Biochemisry*, 40, 13510-13519 (2001).

Cho, E.-H., Chung, S. G., Lee, S. H., Kwon, H. S., Kang, D. W., Joo, J. H., and Oh, J. W., 9-Anminoacridine derivatives and process for the preparation Thereof. PCT/KR02/00392, March, 2002.

Choudhury, G. G., Maity, S., Bhattacharyya, B., and Biswas, B. B., B-ring of colchicines and its role in taxol-induced tubulin polymerization. *FEBS Lett.*, 197, 31-34 (1986).

Combeau, C., Provost, J., Lancerin, F., Tournoux, Y., Prodhomme, F., Herman, F., Lavelle, F., Leboul, J., and Vuilhorgne, M., RPR112378 and RPR115781: Two representatives of a new family of microtubule assembly inhibitors. *Mol. Pharm.*, 57, 553-563 (2000).

Gottsman, M. M., Fojo, T., and Bates, S. E., Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat. Rev. Cancer*, 2, 48-58 (2002).

Gottsman, M. M., Pastan, I., and Ambudkar, S. V., P-glycoprotein and multidrug resistance. *Curr. Op. Genet. Dev.*, 6, 610-617 (1996).

Hartley-Asp, B. and Gunnarsson, P. O., Growth and cell survival following treatment with estramucin, nor-nitrogen mustard, esteadiol and testosterone of a human prostate cancer cell line (DU145). *J. Urol.*, 127, 818-822 (1982).

Jordan, A., Hadfield, J. A., Lawrence, N. J., and McGown, A. T., Tubulin as a target for anticancer drugs: Agents which interact with the mitotic spindle. *Med. Res. Rev.*, 18, 259-296 (1998).

Jordan, M. A. and Wilson, L., Microtubules and actin filaments: dynamic targets for cancer chemotherapy. *Curr. Op. Cell Biol.*, 10, 123-130 (1998).

Kruczynski, A. and Hill, B. T., Vinflunine, the latest Vinca alkaloid in clinical development. A review of its preclinical anticancer properties. *Crit. Rev. Oncol. Hematol.*, 40, 159-173 (2001).

Meyer, T. U., Kapoor, T. M., Haggarty, S. J., King, R. W., Schreiber, S. L., and Mitchison, T. J., Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. *Science*, 286, 913-914 (1999).

Murphy, D. B., Function of tubulin isoforms. *Curr. Op. Cell Biol.*, 3, 43-51 (1991).

Nogales, E., Structral insights into microtubule function. *Annu. Rev. Biophys. Biomol. Struct.*, 30, 397-420 (2001).

Raff, E. C., The role of multiple tubulin isoforms in cellular microtubule function. Microtubules, pp 85-109, Modern Cell Biology Vol. 13. Hyams J. S. and Lloyd C. W. (Ed). Wiley-Liss, Inc. New York (1994).

Sakowicz, R., Berdelis, M. S., Ray, K., Blackburn, C. L., Hopmann, C., Faulkner, D. J., and Goldstein, L. S. B., A marine natural product inhibitor of kinesin motors. *Science*, 280, 292-295 (1998).

Shan, B., Medina, J. C., Santha, E., Frankmoelle, W. P., Chou, T.-C., Learned, R. M., Narbut, M. R., Stott, D., Wu, P., Jaen, J. C., Rosen, T., Timmermans, P. B. M. W. M., and Beckmann, H., Selective, covalent modification of -tubulin residue Cys-239 by T138067, an antitumor agent with *in vivo* efficacy against multidrug-resistant tumors. *Proc. Natl. Acad. Sci. U.S.A.*, 96, 5686-5691 (1999).

Smith, C. D. and Zhang, X., Mechanism of action of cryptophycin. *J. Biol. Chem.*, 271, 6192-6198 (1996).