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Microtubule Effects of Welwistatin, a Cyanobacterial Indolinone that Circumvents Multiple Drug Resistance

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SUMMARY

Welwitindolinones are a family of novel alkaloids recently isolated from the blue-green alga *Hapalosiphon welwitschii.* We demonstrate that incubation of SK-OV-3 human ovarian carcinoma cells and A-10 vascular smooth muscle cells with welwitindolinone C isothiocyanate, now termed welwistatin, results in dose-dependent inhibition of cell proliferation, which is correlated with increases in the percentage of cells in mitosis. Treatment of A-10 cells with welwistatin resulted in reversible depletion of cellular microtubules but did not affect microfilaments. Pretreatment of A-10 cells with paclitaxel prevented microtubule depolymerization in response to welwistatin. Welwistatin inhibited the polymerization of purified tubulin *in vitro* but did not alter the ability of tubulin to bind [^{3}H]colchicine or to hydrolyze GTP. Also, welwistatin did not induce the formation of topoisomerase/DNA complexes. Results of the present study indicate that welwistatin is a new antimicrotubule compound that circumvents multiple drug resistance and so may be useful in the treatment of drug-resistant tumors.

Microtubules have a number of functional and structural activities in cells, including organelle transport and formation of the mitotic spindle and the cytoplasmic cytoskeleton (1). These processes are dependent on the continuous addition and loss of tubulin dimers to microtubules, and a number of compounds have been demonstrated to alter the dynamic equilibrium of microtubules. For example, vinblastine (2), colchicine (3), and paclitaxel (4) are well known antimicrotubule drugs that inhibit the dynamic equilibrium of microtubule assembly and disassembly (5, 6). Because of the role of microtubules in the dynamic process of mitotic spindle formation and chromosome motion during cell division, these compounds can arrest the cell cycle in the mitotic phase. Consequently, antimicrotubule agents are often used as anticancer drugs (7–10).

Unfortunately, most natural-product antimicrotubule agents are excellent substrates for the drug-efflux pump P-glycoprotein, which is thought to be a significant factor in both intrinsic and acquired drug resistance (11–13). Therefore, chronic therapy with these compounds is generally unsuccessful (14, 15). One approach to overcoming the problem of drug resistance has been to combine natural product drugs with P-glycoprotein antagonists. For example, combinations of estramustine, which has been shown to interact with P-glycoprotein *in vitro* (16), and vinblastine or paclitaxel have shown promise in phase I and II clinical trials (17–19). In an alternate approach, we recently identified a family of natural products, called cryptophycins, that potently induce microtubule depolymerization but are equally cytotoxic toward parental and P-glycoprotein-overexpressing cells (20). Similarly, incubation of tumor cells with welwistatin, a novel cyanobacterial indolinone, has been shown to inhibit cell proliferation (21, 22). The present study demonstrates that this compound disrupts microtubule structure in several cultured cell lines. Because P-glycoprotein-overexpressing cells show virtually no resistance to welwistatin (22), this new compound may be useful in the chemotherapy of drug-resistant tumors.

Experimental Procedures

Materials. Welwistatin (Fig. 1), originally named welwitindolinone C isothiocyanate, was isolated from extracts of the blue-green alga *Hapalosiphon welwitschii* (21). Paclitaxel was obtained from Calbiochem (San Diego, CA), and vinblastine, TRITC-phalloidin, sulforhodamine B, and antibodies against β-tubulin (T-4026) were obtained from Sigma Chemical Co. (St. Louis, MO). α-minimum essential culture medium and fetal bovine serum were obtained from GIBCO-BRL (Grand Island, NY). [^{3}H]Colchicine (67 Ci/mmol), [γ-^{32}P]GTP (30 Ci/mmol), and [^{3}H]thymidine (20 Ci/mmol) were purchased from DuPont-NEN (Boston, MA).

Cellular assays. For cytotoxicity assays with A-10 (American Type Culture Collection CRL 1476) and SK-OV-3 (American Type Culture Collection HTB 77) cells, we used the sulforhodamine binding assay (23) as previously described (16, 22). The mitotic index was determined using SK-OV-3 cells treated with welwistatin for 24 hr. Cells were fixed with methanol and stained with 10% Giemsa in

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ABBREVIATIONS: TRITC, tetramethylrhodamine B isothiocyanate.

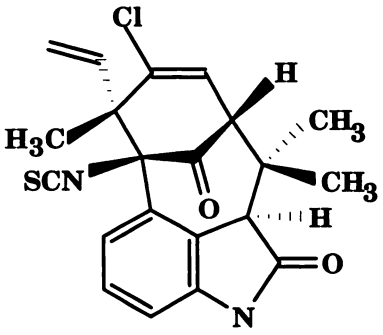

Fig. 1. Structure of welwistatin.

phosphate-buffered saline. At least 400 cells/treatment were scored for the presence of mitotic figures, and the percentage of cells with mitotic figures was calculated. For cell cycle analyses, cells were treated with welwistatin for 24 hr, fixed with ethanol, and stained with propidium iodide. DNA levels were measured using a Beckton Dickinson FACScan flow cytometer and analyzed using the program MacCycle. The effects of compounds on the structure of microtubules and microfilaments in A-10 cells were determined by immunofluorescent staining with anti-β-tubulin antibodies and by binding of TRITC-conjugated phalloidin, respectively, as previously described (20).

Microtubule assembly assays. Bovine brain microtubule protein was purified by two cycles of temperature-dependent assembly and disassembly according to the procedure of Tiwari and Suprenant (24). Tubulin was purified by chromatography on DEAE-Sepharose as described by Vallee (25). For studies on the effects of welwistatin on tubulin assembly, samples containing 20 μM tubulin in 1 M glutamate, pH 6.6, were incubated with welwistatin, vinblastine, or colchicine at 4° for 15 min or 25° for 120 min. GTP (0.4 mM) was then added to the samples, and they were transferred to an Ultrospec III spectrophotometer (Pharmacia Biotech, Piscataway, NJ) equipped with a thermostatically regulated liquid circulator and rapidly warmed to 35°. The absorbance at 350 nm was continuously monitored for ≥20 min.

[^{3}H]Colchicine binding assay. Duplicate samples of microtubule protein (0.2 mg/ml, ~1.6 μM tubulin) were incubated with 5 μM [^{3}H]colchicine (0.1 μCi/sample) and varying concentrations of welwistatin, colchicine, or podophyllotoxin in 1 M glutamate, pH 6.6, containing 1 mM $MgCl_2$, 1 mM GTP, 100 mM glucose-1-phosphate, and bovine serum albumin (0.5 mg/ml) for 1 hr at 37° (26). Samples were filtered through three layers of DEAE-cellulose paper (Whatman DE-81) to capture tubulin and were washed three times with ice-cold incubation buffer. The amounts of bound [^{3}H]colchicine were determined by scintillation counting. Nonspecific binding was determined by including a 100-fold excess of unlabeled colchicine with some samples.

Tubulin GTPase activity assay. Similar to the method of Heusele and Carlier (27), purified tubulin (10 μM) was incubated with varying concentrations of welwistatin or colchicine in 1 M glutamate, pH 6.6, containing 5% dimethylsulfoxide. Reactions were initiated by the addition of 1 mM $MgCl_2$ and 100 μM [γ-^{32}P]GTP (0.1 μCi/sample). After 5 min at 37°, GTPase reactions were stopped by the addition of 1 ml of 300 mM perchloric acid containing 5 mg of activated charcoal (28). Samples were then incubated at room temperature for 10 min to allow adsorption of the nucleotide onto the charcoal, followed by centrifugation at 3000 × *g* for 5 min. An aliquot (0.25 ml) of the supernatant was removed and analyzed by liquid scintillation counting to determine the amount of released $^{32}PO_4$. Blank samples were stopped immediately after the addition of [γ-^{32}P]GTP, and their counts were subtracted from the test samples.

Cleavable complex formation assay. The K^+/sodium dodecyl sulfate precipitation assay was used to test the effects of welwistatin on topoisomerases (29). Briefly, SK-OV-3 cells were grown for 24 hr in the presence of [^{3}H]thymidine (1 μCi/ml) in 24-well tissue culture plates. At that time, the medium was replaced with unlabeled α-minimum essential medium containing 10% fetal bovine serum, and cells were exposed to multiple doses of welwistatin, or to camptothecin or etoposide as positive controls, for 45 min. The cells were then lysed with 1.25% sodium dodecyl sulfate, and protein-complexed DNA was precipitated by the addition of 325 mM KCl. Pellets were washed twice, and the amount of precipitated [^{3}H] was determined by liquid scintillation counting.

Results

Effects of welwistatin on cell proliferation and cycling. SK-OV-3 and A-10 cell lines were incubated for the duration of two cell cycles with welwistatin over a broad range of concentrations. The concentrations of welwistatin that inhibited the proliferation of SK-OV-3 and A-10 cells by 50% (IC_{50}) were 72 ± 8 and 900 ± 300 nM, respectively. Previous studies have demonstrated an IC_{50} value of 130 nM for both MCF-7 and P-glycoprotein-overexpressing MCF-7/ADR cells (22). As demonstrated in Fig. 2, the dose-dependency for welwistatin-induced inhibition of SK-OV-3 proliferation correlated well with the accumulation of these cells in mitosis. Cell cycle arrest was confirmed by flow cytometric analyses of propidium iodide-stained cells that indicated that treatment of SK-OV-3 cells with 3 μM welwistatin resulted in accumulation of virtually 100% of the cells in the G2/M phase (data not shown).

Effects of welwistatin on cytoskeletal structure. Aortic smooth muscle (A-10) cells were grown onto glass coverslips and treated with various concentrations of welwistatin for 24 hr. The microtubule network was then visualized by indirect immunofluorescence, and the microfilament network was stained with TRITC/phalloidin. The morphological effects of each concentration were examined and imaged using confocal microscopy. Untreated cells displayed extensive microtubule systems with perinuclear organizing centers (Fig. 3B), whereas microfilament bundles were predominantly

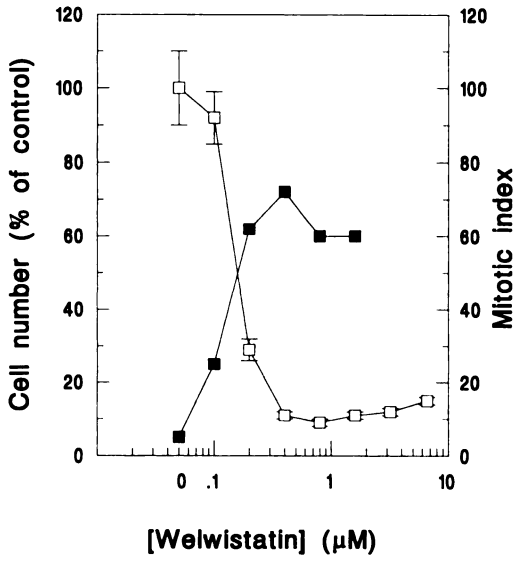

Fig. 2. Effects of welwistatin on SK-OV-3 proliferation and mitotic index. Exponentially growing cultures of SK-OV-3 cells were incubated with the indicated concentrations of welwistatin. The effects of this compound on cell proliferation (□) and the percentage of cells with condensed chromosomes (■) were determined as indicated in Experimental Procedures. *Values*, mean ± standard deviation for six samples in one of three similar experiments.

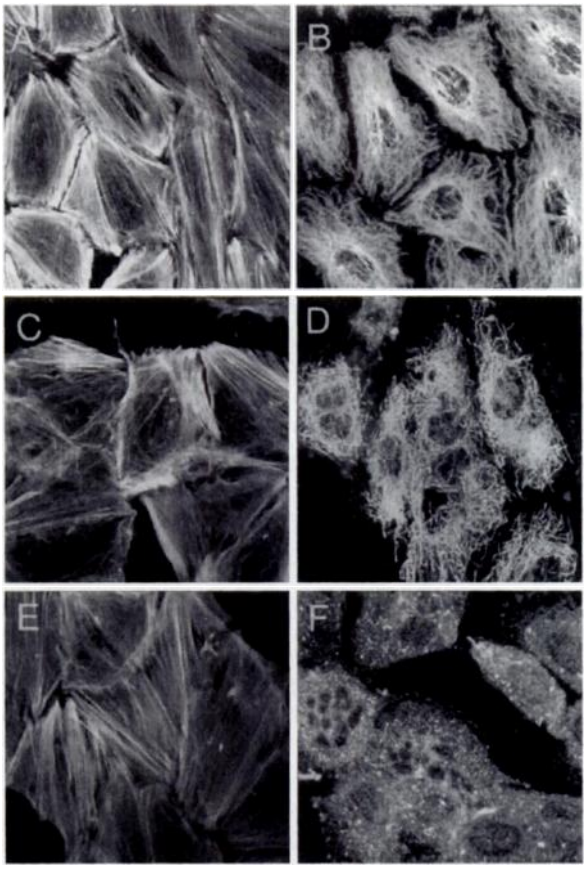

Fig. 3. Effects of welwistatin on cytoskeletal structure. A-10 smooth muscle cells were grown on coverslips and treated with EtOH (A and B), 0.8 μM (C and D), or 3.3 μM (E and F) welwistatin for 24 hr. The cells were then fixed and stained for microfilaments (*left*) or microtubules (*right*) and imaged by confocal microscopy as described in Experimental Procedures. Images are from a representative experiment.

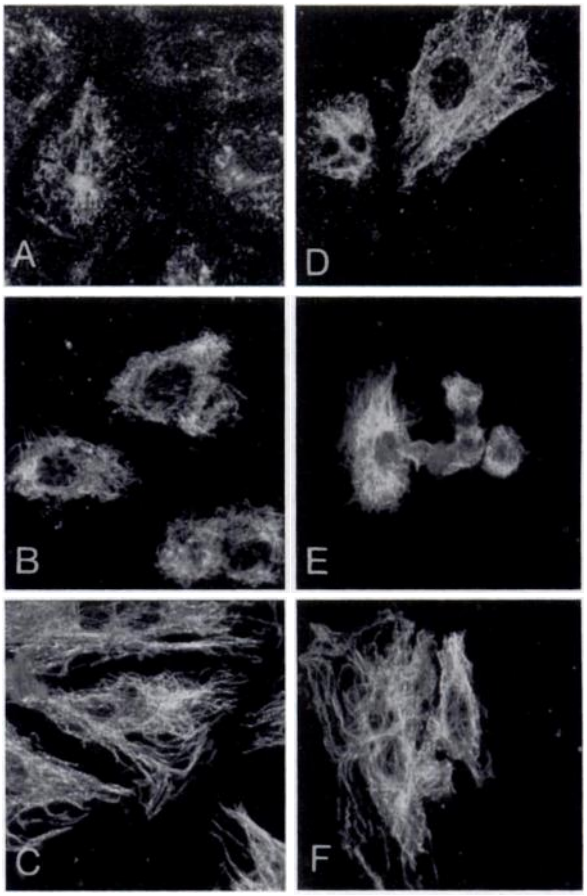

Fig. 4. Reversibility of microtubule depolymerization induced by welwistatin and vinblastine. A-10 cells were treated with 3.3 μM welwistatin (A–C) or 100 nM vinblastine (D–F) for 24 hr. The cells were then washed and incubated with drug-free medium for periods of 1 hr (A and D), 4 hr (B and E), or 24 hr (C and F). Microtubules were stained and imaged as described in Experimental Procedures. Images are from a representative experiment.

concentrated along the major axis of the cell (Fig. 3A). When cells were treated with the approximate IC_{50} of welwistatin, microtubules became less organized, and numerous binucleated cells were observed (Fig. 3D). Treatment of the cells with higher doses of the compound (3.3 μM) resulted in the complete loss of microtubules, with tubulin staining diffusely distributed throughout the cytoplasm (Fig. 3F). Many cells in these cultures exhibited aberrant nuclei. In contrast with these marked alterations in microtubule structure, cells treated with either dose of welwistatin maintained extensive microfilament organization (Fig. 3, C and E).

To test the reversibility of these microtubule effects, we treated A-10 cells with equitoxic doses of welwistatin or vinblastine for 24 hr, followed by removal of the drug. At set time points, the cells were fixed and microtubules were visualized by immunofluorescence. Within 1 hr of welwistatin removal, microtubules began to reassemble, especially near the nucleus, most likely at the microtubule organizing center. By 4 hr, significant reassembly had occurred; it approached completion by 24 hr (Fig. 4, A–C). The reassembly of microtubules after removal of vinblastine was slightly faster than recovery from welwistatin (Fig. 4, D–F).

Because paclitaxel protects microtubules from drug- and cold-induced depolymerization (30, 31), we examined the effects of pretreatment with this compound on microtubule response to welwistatin. As previously noted (20, 31), paclitaxel induced reorganization of microtubules, with extensive bundling in the cell polar regions. Pretreatment of the cells with 0.1 μM (Fig. 5, A and C) or 10 μM (Fig. 5, B and D) paclitaxel for 3 hr prevented microtubule depolymerization in response to doses of welwistatin that induced complete loss of microtubules (Fig. 3). The addition of paclitaxel to cells in which microtubules had been depleted by treatment with 3.3 μM welwistatin resulted in the reassembly of tubulin into microtubules (data not shown).

Effects of welwistatin on microtubule assembly and structure. The effects of welwistatin, vinblastine, and colchicine on the *in vitro* assembly of purified tubulin are shown in Fig. 6. With brief times of pretreatment (15 min at 4°), vinblastine and colchicine markedly suppressed tubulin assembly, measured as increased light scattering, whereas welwistatin was without effect under these conditions. This was true to ≥25 μM welwistatin, which was in stoichiometric excess to tubulin and nearly 20-fold higher than doses that resulted in microtubule depolymerization in intact cells. In contrast, prolonged incubation (120 min at 25°) with welwistatin or benzylthiocyanate, a compound previously shown to slowly bind to tubulin (32), inhibited the assembly of purified tubulin (Fig. 6B).

In some experiments, samples of microtubules polymerized

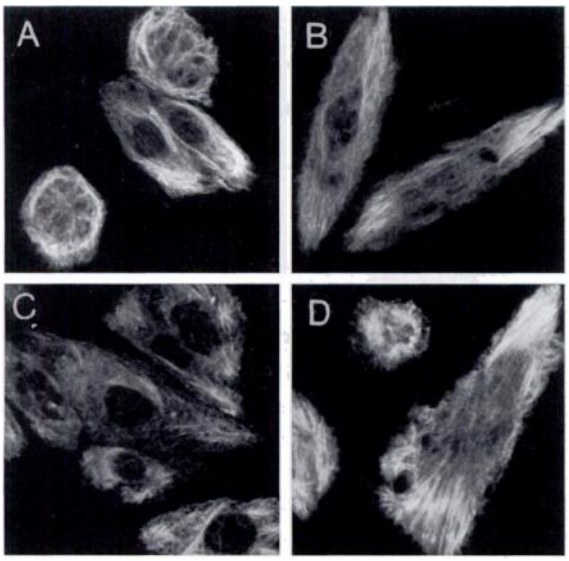

Fig. 5. Effects of paclitaxel on welwistatin-induced microtubule depolymerization. A-10 cells were treated with 0.1 μM (A and C) or 10 μM (B and D) paclitaxel for 3 hr before the addition of EtOH (A and B) and 3.3 μM welwistatin (C and D). After an additional 24 hr, microtubules were stained and imaged as described in Experimental Procedures. Images are from a representative experiment.

in the presence of solvent, welwistatin, colchicine, or vinblastine were fixed with glutaraldehyde, stained with uranyl acetate, and analyzed by transmission electron microscopy. Microtubules assembled in the absence of drug were long, homogeneous in protofilament structure and unbranched. Assembly in the presence of vinblastine or colchicine resulted in the formation of short, densely staining spirals and elongated twisted ribbons, respectively. Assembly in the presence of welwistatin was characterized by the production of shorter microtubules that appeared to have fractured at multiple sites during the drying of the samples onto the carbon grid (data not shown).

Effects of welwistatin on tubulin and topoisomerase. Possible interactions of welwistatin with tubulin were examined by measuring its ability to alter binding of [^{3}H]colchicine and hydrolysis of [γ-^{32}P]GTP by microtubule protein and chromatographically purified tubulin. Concentrations of welwistatin up to ≥25 μM did not alter the binding of [^{3}H]colchicine to microtubule protein (Fig. 7A). Although colchicine promoted dose-dependent increases in GTP hydrolysis by purified tubulin, concentrations of welwistatin up to ≥25 μM did not alter tubulin GTPase activity (Fig. 7B).

Because compounds that inhibit topoisomerase I or II often demonstrate antimitotic effects in cell culture (10, 33), we tested the ability of welwistatin to promote the formation of protein/DNA covalent complexes in SK-OV-3 cells. Treatment of [^{3}H]thymidine-labeled cells with either 5 μM camptothecin or 50 μM etoposide caused 4–5-fold increases in the amount of ^{3}H-labeled DNA that was precipitated by K^+/SDS (Fig. 8), consistent with their known abilities to stabilize the "cleavable complex" of DNA and topoisomerase I or II, respectively. Concurrent experiments with welwistatin indicated that this compound, at concentrations up to at least 25 μM, did not cause similar stabilization of topoisomerase/DNA interactions.

Discussion

Welwistatin was first characterized as a novel cyanobacterial natural product that weakly reverses P-glycoprotein-mediated multiple drug resistance (22). An *N*-methylated analogue was more effective at antagonizing P-glycoprotein than was welwistatin; however, the intrinsic cytotoxicity of welwistatin was ~25-fold greater than that of the methylated compound. Although welwistatin is less potent than many cytotoxins, its ability to block the proliferation of cells that overexpress P-glycoprotein, and so are resistant to natural product anticancer drugs, made desirable the further characterization of welwistatin. During our initial studies, it was noted that welwistatin causes cells to become round, similar to responses observed with agents that disrupt the cytoskeleton. To identify the mechanism of action of welwistatin, we have examined its effects on cytoskeletal structures in epithelial and smooth muscle cells.

Immunofluorescence studies of tubulin organization clearly indicate that welwistatin causes dose-dependent disruption of microtubules in intact cells (Figs. 3–5). This occurs in the absence of alteration of microfilament structure, demonstrating target specificity and suggesting a molecular mechanism similar to that of *Vinca* alkaloids and other tubulin-binding agents. It is unlikely that the antimitotic effects of welwistatin are mediated by inhibition of topoisomerases because it did not increase the amount of K^+/SDS-precipitable DNA in [^{3}H]thymidine-labeled cells. Disruption of microtubule dynamics is highly likely to be the molecular mechanism of welwistatin-induced cytotoxicity in view of (i) the specificity of the effects of welwistatin, i.e., toward microtubules but not microfilaments; (ii) the reversibility of the microtubule depletion removal of welwistatin; (iii) the ability of paclitaxel to prevent microtubule loss in response to welwistatin; (iv) the ability of paclitaxel to promote microtubule reassembly in cells treated with welwistatin; and (v) the ability of welwistatin to inhibit tubulin polymerization *in vitro*.

The reversibility of the microtubule effects indicates that welwistatin does not cause permanent loss of tubulin function, e.g., by proteolysis or denaturation. Immunoblotting experiments with antibodies against both α-tubulin and β-tubulin indicated that the cellular levels of these proteins were not altered by treatment of the cells with welwistatin (data not shown). Additional evidence against nonspecific loss of tubulin function in response to welwistatin is provided by experiments that showed that paclitaxel could still promote microtubule formation in welwistatin-treated cells. This also indicates that the microtubule domains required for binding paclitaxel are not blocked by welwistatin.

Since 1968, when tubulin was identified as the intracellular binding site of colchicine (34), a number of natural and synthetic compounds that alter microtubule structure and function have been characterized. Natural-product compounds that act as antimitotic agents due to depletion of cellular microtubules are often classified according to their abilities to bind to either the colchicine site or the vinblastine site of tubulin (35, 36). Binding is generally reflected by the ability of these compounds to inhibit the assembly of microtubules *in vitro*. In initial experiments, stoichiometric doses of welwistatin did not alter either the kinetics or extent of microtubule assembly *in vitro*, measured as increased light

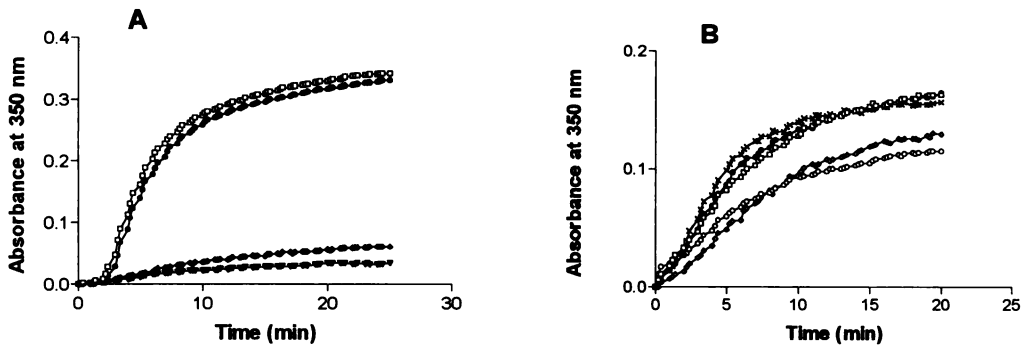

Fig. 6. Effects of antimitotic drugs on tubulin polymerization *in vitro*. A, Purified tubulin (20 μM) was incubated at 4° with dimethylsulfoxide (solvent control) (□), 25 μM welwistatin (●), 10 μM vinblastine (▼), or 10 μM colchicine (♦) for 15 min before the addition of GTP and warming to 37° as described in Experimental Procedures. Polymerization of microtubules was monitored as light scattering (A_{350}) at 20-sec intervals. All experiments were conducted at least three times, and the assembly kinetics are shown for a representative experiment. B, Purified tubulin (20 μM) was incubated at 25° with dimethylsulfoxide (solvent control) (□), 25 μM welwistatin (●), 50 μM welwistatin (○), 20 μM benzylthiocyanate (×), or 50 μM benzylthiocyanate (◇) for 120 min before the addition of GTP, warming to 37°, and analysis of polymerization as indicated above. All experiments were conducted at least three times, and the assembly kinetics are shown for a representative experiment.

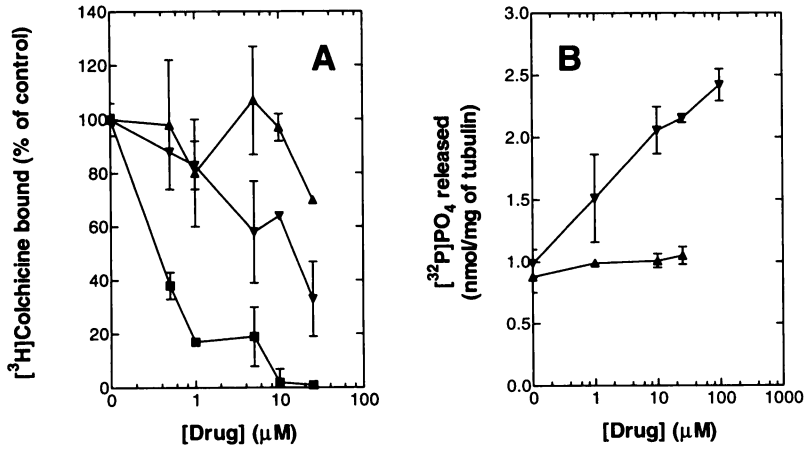

Fig. 7. Effects of welwistatin on colchicine binding and GTP hydrolysis by tubulin. A, Microtubule protein was incubated with the indicated concentrations of colchicine (▼), podophyllotoxin (■), or welwistatin (▲) before the binding of [^{3}H]colchicine was determined as indicated in Experimental Procedures. B, Purified tubulin was incubated with the indicated concentrations of colchicine (▼) or welwistatin (▲) before the hydrolysis of [γ-^{32}P]GTP was determined as indicated in Experimental Procedures. Values represent the mean ± standard deviation of triplicate samples in one of three similar experiments.

scattering with the use of solutions of bovine brain microtubule protein or chromatographically purified tubulin. Further experiments following conditions defined by Abraham *et al.* for compounds that have slow kinetics of binding to tubulin (32) demonstrated that welwistatin can inhibit the polymerization of purified tubulin.

The limitations of the use of *in vitro* microtubule polymerization assays for identifying tubulin-binding agents have been discussed by Hamel *et al.* (37). The incubation time of the polymerization assay is limited by the instability of unpolymerized tubulin, resulting in minimization of the extent of inhibition by slowly binding compounds. Consequently, effects on microtubules in intact cells are both more easily observed and more indicative of the mechanism of cytotoxicity. In the present case, welwistatin and benzyl thiocyanate demonstrated approximately equal abilities to inhibit microtubule polymerization *in vitro*, whereas welwistatin was ≥20-fold more potent for depleting cellular microtubules. It is notable that benzyl thiocyanate covalently interacts with tubulin sulfhydryls (38); however, the lack of radiolabeled welwistatin prevents a similar analysis of this compound.

Welwistatin did not alter assembly-stimulated GTP hydrolysis or [^{3}H]colchicine binding by tubulin, suggesting that the compound does not interfere with either of these ligand domains. Microtubules assembled in the presence of vinblastine or colchicine have characteristic morphologies, i.e., dense spirals (39) and twisted ribbons (40), respectively. In contrast, microtubules assembled in the presence of welwistatin appeared normal. Furthermore, microtubules assembled in the absence or presence of welwistatin demonstrated similar sensitivities to depolymerization by Ca^{2+} or low temperature (data not shown).

Methylation of the indolinone nitrogen atom of welwistatin, to yield *N*-methylwelwitindolinone C isothiocyanate (22), greatly reduced its cytotoxicity and caused a corresponding decrease in the antimicrotubule activity of the compound. An analogue in which the isothiocyanate moiety of welwistatin is replaced by an isonitrile group (22) had antimicrotubule activity at doses very similar to those of welwistatin; however, quantities of this compound were inadequate to characterize its properties *in vitro*. Benzyl thiocyanate is ~125-fold less cytotoxic than welwistatin, suggesting that the ring systems of the later compound are important in directing its interaction with tubulin.

Like other drugs that disrupt microtubules (6, 20), the dose-dependencies for inhibition of cell proliferation by welwistatin and accumulation of cells in mitosis were closely correlated. Welwistatin-induced depletion of microtubules was observed in both actively growing and quiescent cultures, indicating that microtubule disruption is likely to be the cause, not a consequence, of mitotic arrest. Importantly, welwistatin arrested both MCF-7/ADR and MCF-7 cells in

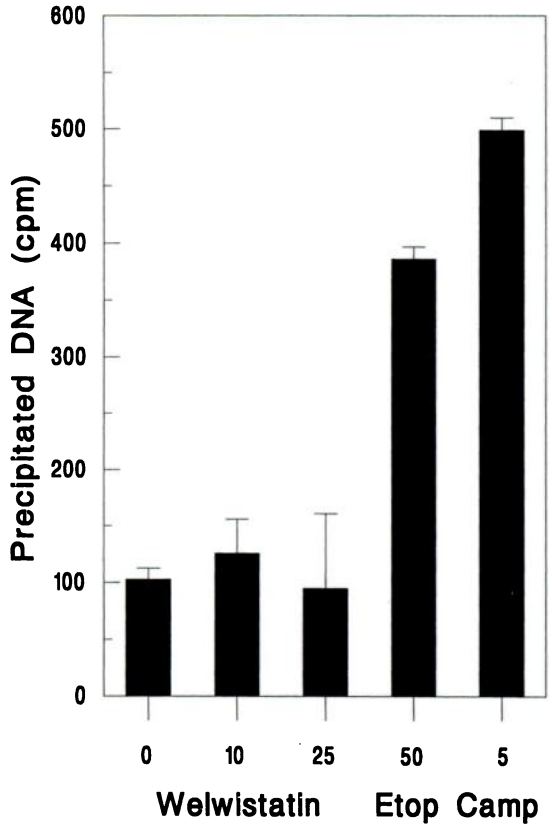

Fig. 8. Effect of welwistatin on protein-DNA cross-linking in SK-OV-3 cells. SK-OV-3 cells were labeled with[^{3}H]thymidine for 24 hr and then incubated with the indicated concentrations of welwistatin (μM), 50 μM etoposide (*Etop*), or 5 μM camptothecin (*Camp*) for 45 min. The samples were then lysed with sodium dodecyl sulfate, and the amount of ^{3}H precipitated by the addition of KCl was determined as indicated in Experimental Procedures. *Values*, mean ± standard deviation for triplicate samples in one of two similar experiments.

G2/M, which is in agreement with our previous report that overexpression of P-glycoprotein does not confer resistance to this compound (22). This property may allow welwistatin to serve as an effective cytotoxic and/or radiosensitizing agent (41) toward both drug-sensitive and drug-resistant tumor cells.

Acknowledgments

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References

1. Mandelkow, E., and E. M. Mandelkow. Microtubule structure. *Curr. Opin. Struct. Biol.* **4:**171–179 (1994).
2. Gerzon, K. Dimeric *Catharanthus* alkloids, in *Anticancer Agents Based on Natural Product Models* (J. M. Cassady and J. D. Douros, eds.). Academic Press, New York, 271–331 (1980).
3. Taylor, E. W. The mechanism of colchicine inhibition of mitosis. *J. Cell. Biol.* **25:**145–160 (1965).
4. Schiff, P. B., and S. B. Horwitz. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. USA* **77:**1561–1565 (1980).
5. Toso, R. J., M. A. Jordan, K. W. Farrell, B. Matsumoto, and L. Wilson. Kinetic stabilization of microtubule dynamic instability *in vitro* by vinblastine. *Biochemistry* **32:**1285–1293 (1993).
6. M. A. Jordan, R. J. Toso, D. Thrower, and L. Wilson. Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. *Proc. Natl. Acad. Sci. USA* **90:**9552–9556 (1993).
7. Rowinsky, E. K., and R. C. Donehower. The clinical pharmacology and use of antimicrotubule agents in cancer chemotherapeutics. *Pharmacol. Ther.* **52:**35–84 (1991).
8. Deconti, R. C., and W. A. Creasey. Clinical aspects of the dimeric *Catharanthus* alkaloids, in *The Catharanthus Alkaloids: Botany, Chemistry, Pharmacology and Clinical Use* (W. I. Taylor and N. R. Farnsworth, eds.). Marcel Dekker, New York, 237–278 (1975).
9. Runowicz, C. D., P. H. Wiernik, A. I. Einzig, G. L. Goldberg, and S. B. Horwitz. Taxol in ovarian cancer. *Cancer (Phila.)* **71:**1591–1596 (1993).
10. Calabresi, P., J. Clark, A. R. Hanauske, and M. C. Wiemann. Pharmacology of antineoplastic agents, in *Medical Oncology* (P. Calabresi and P. S. Schein, eds.). 2nd ed. McGraw-Hill, New York, 263–321 (1993).
11. Endicott, J. A., and V. Ling. The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu. Rev. Biochem.* **58:**137–171(1989).
12. Gottesman, M. M., and I. Pastan. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* **62:**385–427 (1993).
13. Moscow, J. A., and K. H. Cowan. Multidrug resistance. *J. Natl. Cancer Inst.* **80:**14–20 (1988).
14. Ford, J. M., and W. N. Hait. Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol. Rev.* **42:**155–199 (1990).
15. Raderer, M., and W. Scheithauer. Clinical trials of agents that reverse multidrug resistance: a literature review. *Cancer (Phila.)* **72:**3553–3563 (1993).
16. Speicher, L. A., L. R. Barone, A. E. Chapman, G. R. Hudes, N. Laing, C. D. Smith, and K. D. Tew. P-glycoprotein binding and modulation of the multidrug-resistant phenotype by estramustine. *J. Natl. Cancer Inst.* **86:** 688–694 (1994).
17. Hudes, G. R., R. Greenberg, R. L. Krigel, S. Fox, R. Scher, S. Litwin, P. Watts, L. Speicher, K. D. Tew, and R. Comis. Phase II study of estramustine and vinblastine, two microtubule inhibitors, in hormone-refractory prostate cancer. *J. Clin. Oncol.* **10:**1754–1761 (1992).
18. Seidman, A., H. I. Scher, D. Petrylak, D. D. Dershaw, and T. Curley. Estramustine and vinblastine: use of prostate specific antigen as a clinical trial end point for hormone-refractory prostate cancer. *J. Urol.* **147:**931–934 (1992).
19. Hudes, G. R., C. Obasaju, A. Chapman, J. Gallo, C. McAleer, and R. Greenberg. Phase I study of paclitaxel and estramustine: preliminary activity in hormone-refractory prostate cancer. *Semin. Oncol.* **22:** 6–11 (1995).
20. Smith, C. D, X. Zhang, S. L. Mooberry, G. M. L. Patterson, and R. E. Moore. Cryptophycin: a new antimicrotubule agent active against drug-resistant cells. *Cancer Res.* **54:**3779–3784 (1994).
21. Stratmann, K., R. E. Moore, R. Bonjouklian, J. B. Deeter, G. M. L. Patterson, S. Shaffer, C. D. Smith, and T. A. Smitka. Welwitindolinones, unusual alkaloids from the blue-green algae *Hapalosiphon welwitschii* and *Westiella intricata*: relationship to fischerindoles and hapalindoles. *J. Am. Chem. Soc.* **116:**9935–9942 (1994).
22. Smith, C. D., J. T. Zilfou, K. Stratmann, G. M. L. Patterson, and R. E. Moore. Welwitindolinone analogues that reverse P-glycoprotein-mediated multiple drug resistance. *Mol. Pharmacol.* **47:**241–247 (1995).
23. Skehan, P., R. Stoneng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, and M. R. Boyd. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **82:**1107–1112 (1990).
24. Tiwari, S. C., and K. A. Suprenant. A pH- and temperature-dependent cycling method that doubles the yield of microtubule protein. *Analyt. Biochem.* **251:**96–103 (1993).
25. Vallee, R. B. Purification of brain microtubules and microtubule-associated protein 1 using taxol. *Methods Enzymol.* **134**:89–104 (1986).
26. Borisy, G. G. A rapid method for quantitative determination of microtubule protein using DEAE-cellulose filters. *Analyt. Biochem.* **13:**373–385 (1972).
27. Heusele, C., and M.-F. Carlier. GTPase activity of the tubulin-colchicine in relation with tubulin-tubulin interactions. *Biochem. Biophys. Res. Commun.* **103:**332–338 (1981).
28. Smith, C. D., and W. W. Wells. Solubilization and reconstitution of a nuclear envelope associated ATPase: synergistic activation by RNA and polyphosphoinositides. *J. Biol. Chem.* **259:**11890–11894 (1984).
29. van der Graaf, W. T. A., E. G. E. de Vries, H. Timmer-Bosscha, G. J. Meersma, G. Mesander, E. Vellenga, and N. H. Mulder. Effects of amiodarone, cyclosporin A, and PSC 833 on the cytotoxicity of mitoxanthrone, doxorubicin, and vincristine in non-P-glycoprotein human small cell lung cancer cell lines. *Cancer Res.* **54:**5368–5373 (1994).
30. Manfredi, J. J., and S. B. Horwitz. Taxol: an antimitotic agent with a new mechanism of action. *Pharmacol. Ther.* **25:**83–125 (1984).
31. Smith, C. D., S. L. Mooberry, X. Zhang, and A. M. Helt. A sensitive assay for taxol and other microtubule-stabilizing agents. *Cancer Lett.* **79:**213–219 (1994).
32. Abraham, I., R. L. Dion, C. Duanmu, M. M. Gottesman and E. Hamel. 2:4-Dichlorobenzyl thiocyanate, an antimitotic agent that alters microtubule morphology. *Proc. Natl. Acad. Sci. USA* **83:**6839–6843 (1986).
33. Pratt, W. B., R. W. Ruddon, W. D. Ensminger, and J. Maybaum. Inhibitors

of chromatin function, in *The Anticancer Drugs.* 2nd ed. Oxford University Press, New York, 183–198 (1994).
34. Weisenberg, R. C., G. G. Borisy, and E. W. Taylor. The colchicine-binding protein of mammalian brain and its relation to microtubules. *Biochemistry* **7:**4466–4478 (1968).
35. Hamel, E. Interactions of tubulin with small ligands, in *Microtubule Proteins* (J. Avila, ed.). CRC Press, Boca Raton, FL, 89–191 (1990).
36. Correia, J. J. Effects of antimitotic agents on tubulin-nucleotide interactions. *Pharmacol. Ther.* **52:**127–147 (1991).
37. Hamel, E., A. V. Blokhin, D. G. Nagle, H.-D. Yoo, and W. H. Gerwick. Limitations in the use of tubulin polymerization assays as a screen for the identification of new antimitotic agents: the potent marine natural product curacin A as an example. *Drug Dev. Res.* **34:**110–120 (1995).
38. Bai, R., C. Duanmu, and E. Hamel. Mechanism of action of the antimitotic drug 2,4-dichlorobenzyl thiocyanate: alkylation of sulfhydryl group(s) of β-tubulin. *Biochim. Biophys. Acta* **994:**12–20 (1989).
39. Na, G. C., and S. N. Timasheff. Stoichiometry of the vinblastine-induced self-association of calf brain tubulin. *Biochemistry* **19:**1347–1354 (1980).
40. Andreu, J. M., and S. N. Timasheff. Tubulin bound to colchicine forms polymers different from microtubules. *Proc. Natl. Acad. Sci. USA* **79:**6753–6756 (1982).
41. Sinclair, W. K. Cyclic x-ray responses in mammalian cells *in vitro*. *Radiat. Res.* **33:**620–643 (1968).

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