Chemistry & Biology, Vol. 11, 799–806, June, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.chembiol.2004.03.028

The Anticancer Natural Product Pironetin Selectively Targets Lys352 of α-Tubulin

Takeo Usui,[1] Hiroyuki Watanabe,[3,6] Hiroshi Nakayama,[2] Yukio Tada,[4] Naoki Kanoh,[1] Masuo Kondoh,[1,7] Tetsuji Asao,[4] Koji Takio,[2,8] Hidenori Watanabe,[3] Kiyohiro Nishikawa,[5] Takeshi Kitahara,[3] and Hiroyuki Osada[1,*]

[1]Antibiotics Laboratory
[2]Biomolecular Characterization Team
RIKEN Discovery Research Institute
2-1 Hirosawa
Wako-shi, Saitama 351-0198
Japan
[3]Department of Applied Biological Chemistry
Graduate School of Agricultural and Life Science
The University of Tokyo
1-1-1 Yayoi
Bunkyo-ku, Tokyo 113-8657
Japan
[4]Chemistry Laboratory
Hanno Research Center
Taiho Pharmaceutical Co., Ltd.
1-27 Misugidai
Hanno, Saitama 357-8527
Japan
[5]Research and Development Division
Nippon Kayaku Co., Ltd.
3-31-12 Shimo
Kita-ku, Tokyo 115-8588
Japan

Summary

Pironetin is a potent inhibitor of tubulin assembly and arrests cell cycle progression in M phase. Analyses of its structure-activity relationships suggested that pironetin covalently binds tubulin. To determine the binding site of pironetin, we synthesized biotinylated pironetin, which inhibited tubulin assembly both in vitro and in situ. The biotinylated pironetin selectively and covalently bound with tubulin. Partial digestion of biotinylated pironetin-treated tubulin by several proteases revealed that the binding site is the C-terminal portion of α-tubulin. By systematic alanine scanning, the pironetin binding site was determined to be Lys352 of α-tubulin. Lys352 is located at the entrance of a small pocket of α-tubulin, and this pocket faces the β-tubulin of the next dimer. This is the first compound that covalently binds to the α subunit of tubulin and Lys352 of α-tubulin and inhibits the interaction of tubulin heterodimers.

Introduction

Drugs that interact with microtubules are expected to be useful not only as antitumor agents, but also as tools for understanding a wide variety of the cellular functions of microtubules, such as mitosis, cell signaling, and motility in eukaryotes. Microtubule inhibitors displaying great structural diversity have been identified and well characterized because of their extensive application in medicinal and basic research. Paclitaxel and vinblastine are clinically used as anticancer drugs. They bind to different sites of β-tubulin and show opposite effects in vitro: paclitaxel induces microtubule bundling and vinblastine induces microtubule disassembly [1, 2]. Epothilones bind the paclitaxel binding site on β-tubulin and stabilize microtubule filaments [3, 4], and clinical trials using several derivatives are undergoing [5]. The other microtubule inhibitors, for example, TZT-1027, 2-methoxyestradiol, and taxane derivatives, are in clinical development [6, 7]. We have reported that tryprostatin A inhibited microtubule-associated protein-dependent tubulin assembly by binding to tubulin at binding sites different from those used by colchicine and vinblastine [8–10]. However, in most of the cases, microtubule-directed drugs bound to β-tubulin with a few exceptions (dinitroaniline herbicides and B-ring of colchicines) [11–13].

Some bioactive chemicals containing α,β-unsaturated lactone are known to bind to proteins. For example, cyclopentenone prostaglandins bind to Cys179 of IKKβ and inhibit inflammatory reactions by decreasing IKK complex kinase activity [14]. In addition to endogenous compounds, several chemicals covalently bind to their own target proteins by Michael addition. Leptomycin B, a nuclear export inhibitor, binds to Cys529 of CRM1/exportin 1 and inhibits protein export from nuclei [15–19]. It is suggested that the PP2A-specific inhibitor fostriecin binds to Cys269 of PP2A and inhibits its phosphatase activity [20, 21]. Recently, we also reported that isoavenaciolide binds to the catalytic center cysteine of dual-specificity phosphatase VHR and inhibits its phosphatase activity [22, 23]. Michael addition is a reaction that is likely to be nonspecific to biological nucleophiles such as cysteine, but it has been shown that most of the chemicals listed above are site/protein specific. Their high specificity is thought to be due to structural elements in addition to the lactone moiety.

Previously, we reported that pironetin (**1a** in Figure 1) and its derivatives are M phase inhibitors and exhibit antitumor activity by apoptosis induction via microtubule disassembly [24, 25]. Pironetin inhibits tubulin binding of radiolabeled vinblastine, and the affinity of pironetin to tubulin is stronger than that of vinblastine. Furthermore, pironetin has a unique structure containing only one pyran residue and an alkyl chain, which is

*Correspondence: hisyo@riken.jp
[6]Present address: Technical Research Center, T. Hasegawa Co., Ltd., 335 Kariyado, Nakahara-ku, Kawasaki-shi, Kanagawa 211-0022, Japan.
[7]Present address: Department of Pharmaceutics and Biopharmaceutics, Showa Pharmaceutical University, 3-3165 Machida, Tokyo 194-8543, Japan.
[8]Present address: Large Scale Protein Production Team, RIKEN Harima Institute, 1-1-1 Kouto, Mikazuki, Sayo, Hyogo 679-5148, Japan.

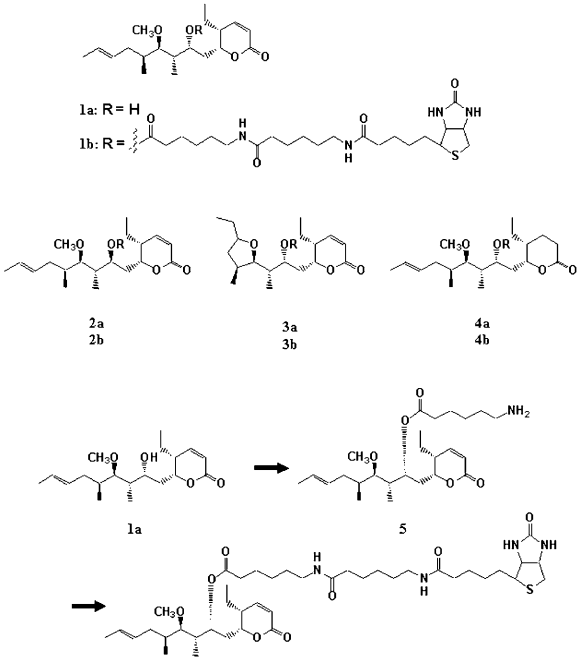

Figure 1. Structure of Pironetin and Its Derivatives

Shown are pironetin (**1a**) and the derivatives **2-4a, b,** and **5**.

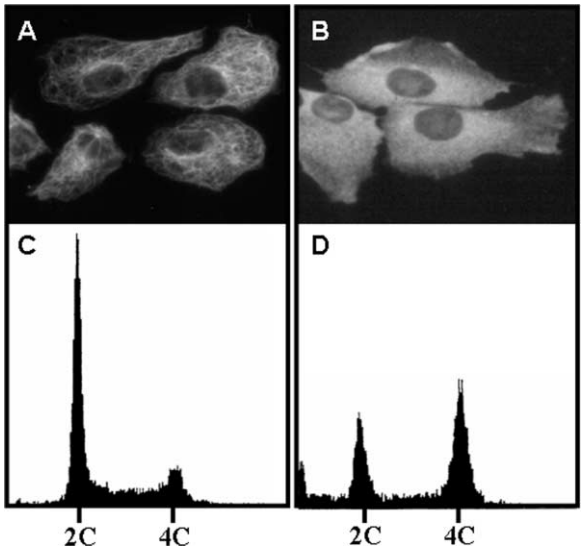

Figure 2. The Effects of Biotinylated Pironetin on the Microtubule Network and Cell Cycle Progression in 3Y1 Cells

(A and B) Microtubule network in 3Y1 cells treated with (B) or without (A) 1 μg/ml biotinylated pironetin for 18 hr. Microtubules were observed under a fluorescence microscopy.

(C and D) Distribution of DNA content in asynchronous culture of 3Y1 cells treated with (D) or without (C) 1 μg/ml biotinylated pironetin for 18 hr.

simpler than the structure of other M phase inhibitors. These features of pironetin, which are distinct from known tubulin binding agents, suggest that it is possible to create a new drug useful for cancer therapy from pironetin as a lead compound. From the analyses of structure-activity relationships, it was revealed that α,β-unsaturated lactone is important for microtubule inhibition [26]. This characteristic strongly suggests that pironetin covalently binds to tubulin by Michael addition.

In this paper, we determined the binding site of pironetin to be the Lys352 of α-tubulin by using biotinylated analogs as probes. Pironetin is the first compound shown to bind to α-tubulin directly. The binding site is located on the surface of α-tubulin facing the β-tubulin of the next heterodimer, which corresponds to the vinblastine binding site.

Results

Pironetin Covalently Binds to Tubulin In Vitro and In Situ

We previously reported that a 2,3-dihydro derivative of pironetin, with a saturated lactone moiety, was dramatically weaker in microtubule disassembly activity and cell cycle arrest in M phase. These results suggested that pironetin inhibits tubulin assembly through covalent binding. To investigate whether or not pironetin binds covalently, we synthesized biotinylated pironetin (**1b** in Figure 1). Biotinylated pironetin induced microtubule disassembly in proliferating 3Y1 cells and cell cycle arrest in M phase at the concentration of 1 μM (Figure 2). The effects of biotinylated pironetin and natural pironetin were indistinguishable and no other effect was observed in biotinylated pironetin-treated cells, although the concentrations were higher than that of natural pironetin (20 nM). The differences of effective concentrations between pironetin and biotinylated pironetin was not observed in vitro microtubule polymerization assay (data not shown), suggesting that the permeability of biotinylated pironetin is much lower than that of pironetin.

We next investigated whether or not biotinylated pironetin covalently modifies tubulins in situ. 3Y1 cells were cultured with 1 μM biotinylated pironetin for 4 hr, the biotin-containing complexes were isolated by streptavidin-conjugated agarose beads, and the bound proteins were analyzed by SDS-PAGE followed by Western blotting (Figure 3). Western blotting using streptavidin-horseradish peroxidase showed a number of biotinylated proteins in both the control cells and biotinylated pironetin-treated cells. However, the sample containing biotinylated pironetin revealed one additional 50 kDa protein (Figure 3A, lane 1, arrow). This band was not detected when the sample was treated with inactive biotinylated pironetin derivatives or biotin alone (Figure 1, **2-4b**, and Figure 3A, lanes 2–4, data not shown). The binding was also blocked when 20 nM of pironetin was added as a competitor to the cell culture 2 hr before the biotinylated pironetin addition (Figure 3B, lane 2). But the binding was not blocked when the cell culture was treated with inactive derivatives **2a** (Figure 3B, lane 3). These results indicate that pironetin specifically and covalently binds to the 50 kDa cellular protein.

Pironetin is a potent microtubule inhibitor and inhibits polymerization of purified tubulin in vitro. Since tubulins consist of heterodimers of α- and β-tubulins with ~50 kDa molecular weight, the 50 kDa protein detected by treatment with biotinylated pironetin was thought to be

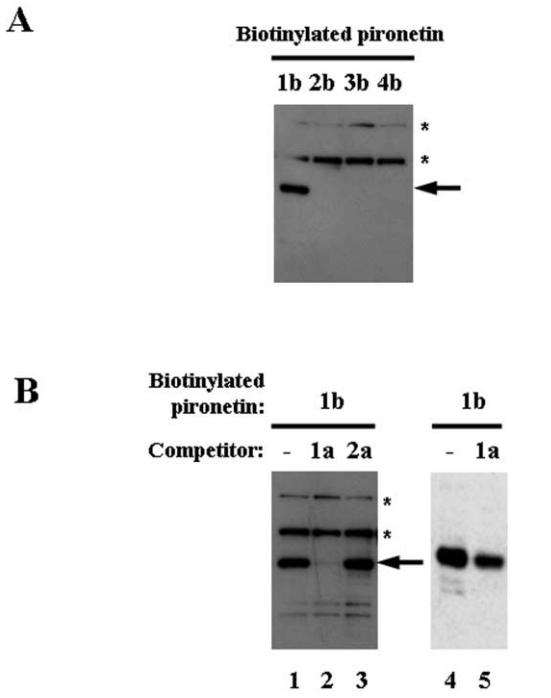

Figure 3. Pironetin Covalently Binds to Tubulin In Situ and In Vitro

(A) Rat normal fibroblast 3Y1 cells were treated with biotinylated pironetin (**1b**) and inactive biotinylated derivatives (**2-4b**). A single 50 kDa protein (corresponding to tubulin; arrow) emerged by treatment with biotinylated pironetin but not biotinylated inactive pironetin. Asterisks indicate intrinsic biotin binding proteins.

(B) Protein binding of biotinylated pironetin in the presence of pironetin competitor (**1a**), but not inactive pironetin (**2a**) in situ (left) and in vitro (right).

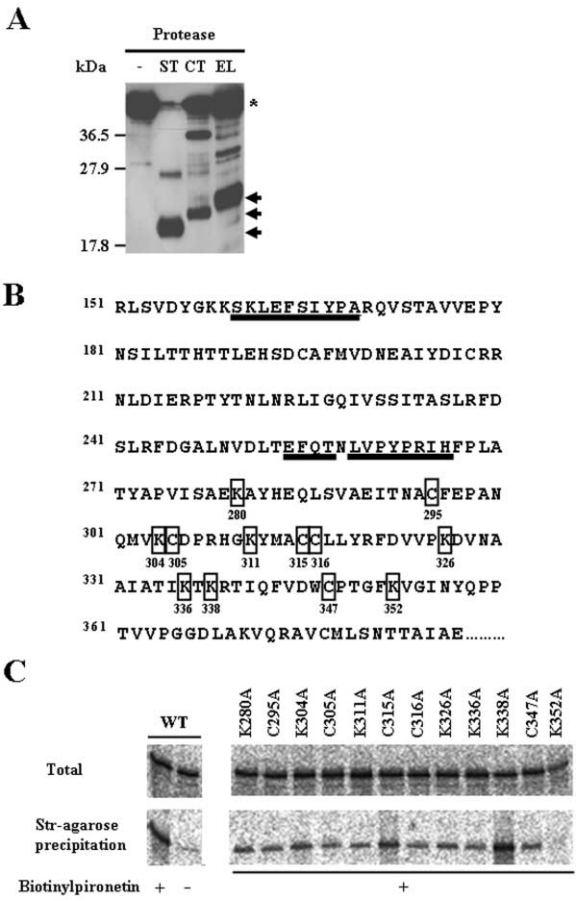

Figure 4. Determination of Pironetin Binding Site

(A) Biotinylated tubulin and partially digested peptides are indicated by an asterisk and arrows, respectively. ST, subtilisin-BPN; CT, chymotrypsin; ET, elastase.

(B) The alignments of biotinylated pironetin bound peptides. The obtained sequences were aligned with chicken α-tubulin (underlined). Putative pironetin binding residues, i.e., all cysteines and lysines between the 270th to 360th residues, are shown as squares.

(C) Pironetin failed to bind Lys352Ala mutated tubulin. All cysteine and lysine residues between the 270th and 370th residues of α-tubulin were changed to alanine and translated in vitro using reticulocyte lysate. After incubation of ^{35}S-labeled translated mutant tubulins with biotinylated pironetin, biotinylated tubulins were precipitated by the immobilized streptavidin matrix. Bar show mean ± deviation obtained from two independent experiments.

α- or β-tubulin. To determine whether or not the biotinylated pironetin was bound to tubulin, we tested the in vitro binding using purified bovine tubulin. Purified tubulin protein was incubated with biotinylated pironetin in combination with or without competitor. Biotinylated pironetin covalently bound to purified tubulin, and its binding was retarded by addition of competitor (Figure 3B, lanes 4 and 5). Together with the previous biological results that pironetin inhibits tubulin assembly in situ and in vitro [25], this finding led us to conclude that pironetin binds to tubulin heterodimers covalently.

Pironetin Binds to Lys352 of α-Tubulin

During the course of the binding assay, we noticed that the binding of pironetin to tubulin was labile under acidic and basic pH conditions (pH $<$ 4.5 and $>$ 8.0, data not shown). These results suggest that retro-Michael addition readily occurs under nonphysiological pH conditions. Furthermore, this characteristic made it difficult to determine the binding site by LC-MS, because an acidic condition is usually used to separate the protease-digested peptides. Therefore, we first used partial protease digestion to determine the region of the binding site. Biotinylated pironetin-treated tubulin was partially digested with several proteases, and the biotinylated pironetin bound peptides were separated by Tris/Tricine SDS-PAGE followed by Western blotting with Streptavidin-HRP (Figure 4A). There were several biotinylated peptides, and the N-terminal amino acid sequences of these biotinylated peptides—19 kDa (subtilisin-BPN), 22 kDa (chymotrypsin), 24 kDa (elastase), and 17 kDa (Lys-C)—were determined. These sequence corresponded to L_{259}VPYPRIHFPLATY$_{272}$ (subtilisin-BPN), L_{259}VPYPRIH$_{266}$ (chymotrypsin), E_{254}FQT$_{257}$ (elastase), and S_{160}KLEFSIYPA$_{169}$ (Lys-C) of chick α-tubulin (Figure 4B). According to the size of Lys-C-treated peptide fragment, the pironetin binding site was suggested from the 270th and 370th residue of α-tubulin.

As the sulfhydryl group of cysteine residues or the ε-amino group of lysine residues are expected as a binding site of pironetin, we used alanine scanning of both cysteine and lysine to determine the pironetin binding

site. All cysteine and lysine residues between the 270th and 360th residue of human α-tubulin were changed to alanine by PCR mutagenesis. All mutants functioned in situ as judged from the incorporation of GFP-fusion mutant proteins into the microtubule network by transfection to HeLa cells (data not shown). Next, we used an in vitro translation system with rabbit reticulocyte lysate to determine the binding site. ^{35}S-labeled mutant tubulins were synthesized by in vitro transcription/translation and tested for their ability to bind biotinylated pironetin. As shown in Figure 4C, ^{35}S-labeled wild-type tubulin was precipitated with the immobilized streptavidin matrix in a biotinylated pironetin-dependent manner (Figure 4C, left). All cysteine mutants were precipitated with biotinylated pironetin, but it took overnight incubation to precipitate the Cys315Ala and Cys316Ala mutants (see Discussion). In contrast, one of the lysine mutants, Lys352Ala, failed to precipitate with drug irrespective of the incubation time (Figure 4C, right). These results strongly suggest that pironetin binds to Lys352 of α-tubulin by Michael addition. The observation that pironetin binds to α-tubulin is unexpected, because in most of the cases in which the binding site has been determined, the known tubulin binding compounds bind to β-tubulin.

Pironetin Binding Model

To confirm that the pironetin binding site was Lys352 of α-tubulin, we simulated a binding model of tubulin with pironetin using the tubulin structure data obtained from electron crystallography [27, 28]. Lys352 of α-tubulin is on the strand of β sheet 9 and located in the entrance of a small cavity. Cys315 and Cys316 are located on neighboring β sheet 8 and are not on the surface of α-tubulin but just behind Lys352, meaning these residues are not pironetin binding sites.

The 3D structure of the complex of tubulin and pironetin was estimated through energy minimization and dynamics simulation. The initial structure was generated by bonding carbon atom at the 3-position of pironetin to the ε-amino group of Lys352 of α-tubulin manually based on the putative binding site and the interaction energy in a vacuum using the computer program. The 3D structure of the tubulin molecule based on electron-ray crystallography was used as the initial structure. The results are shown in Figures 5A and 5B. There are three hydrogen bonds: an oxygen atom of the 9-methoxy moiety of pironetin forms both intramolecular and intermolecular hydrogen bonds with the hydrogen atom of the 7-hydroxy moiety of pironetin (1.87 Å) and the amine moiety of Asn258 (2.05 Å), respectively. The methyl and methoxy moiety of pironetin is fitted quite well into the cavity formed with α helixes 8/10 and β sheets 8/9, and 7-hydroxyl moiety of pironetin forms both intramolecular and intermolecular hydrogen bonds with the pyran ring and Asn258 of α-tubulin, respectively. Asn258 locates on the α helix 8, the same helix Glu254 locates on, and it is speculated that Asn258 forms a hydrogen bond network with Glu254 via Lys352. Glu254, a residue conserved in essentially all α-tubulins, is in an ideal position to be involved in the hydrolysis of GTP of the E site in β-tubulin [29]. This binding model suggests that pironetin binding with Lys352 and Asn258 disrupts the hydrogen network among Glu254, Lys352, and Asn258 and then influences the GTP hydrolysis activity of Glu254.

To confirm the binding model of pironetin and α-tubulin, we made an α-tubulin mutant, Asn258Ala, and tested the binding efficiency by in vitro translation/binding assay. As expected, the binding efficiency of Lys352Ala and Asn258Ala were decreased to 20% and 75%, respectively (Figure 5C). These results reinforce the binding model and strongly suggest that pironetin covalently binds Lys352 on α-tubulin.

Discussion

We previously reported that pironetin is a potent inhibitor of cell cycle progression at the M phase and shows antitumor activity against a murine tumor cell line, P388 leukemia, transplanted in mice [24]. Pironetin has a unique structure containing only one pyran residue and an alkyl chain, which is simpler than the structures of other M phase inhibitors. To create a new drug useful for cancer therapy from pironetin as a lead compound, we here investigated the pironetin binding site on tubulin. According to the structure-activity relationship analyses, we speculated that pironetin covalently binds tubulin by Michael addition via its α,β-unsaturated δ-lactone [26]. To determine whether pironetin binds tubulin, we synthesized biotinylated pironetin (Figure 1, **1b**). The biotinylated pironetin (**1b**), which inhibited tubulin assembly both in vitro and in situ, covalently bound with tubulin, and its binding was inhibited by natural pironetin (**1a**) (Figure 3). We could not exclude the possibility that biotinylated pironetin binds the other protein with undetectable level. The finding that inactive pironetin derivatives failed to bind tubulin in situ suggested that covalent binding is important for pironetin to inhibit tubulin polymerization.

Although it is known that the compounds that bind to their target proteins by Michael addition bind to the sulfhydryl group of cysteine residues or ε-amino group of lysine residues [30], most compounds bind to the sulfhydryl group of cysteine residues. However, we assumed that pironetin binds to the ε-amino group of lysine, because tubulin binding by pironetin is labile under acidic and basic pH conditions. To determine the binding site of pironetin, we used an in vitro translation system with rabbit reticulocyte lysates, because it was reported that this in vitro system contains a chaperonine system needed for correct folding of tubulin [31, 32]. Partial proteolytic analyses of biotinylated pironetin bound peptides, followed by systematic alanine scanning of both cysteine and lysine residues, strongly suggested that Lys352 of α-tubulin is the pironetin binding site (Figures 4 and 5). This is surprising, because the reactivity of the ε-amino group of lysine is quite low compared with that of the sulfhydryl group of cysteine. The in silico analysis of the pironetin-tubulin complex suggested that α helixes 8/10 and β sheet 8 form a cavity that fits pironetin well (Figure 5B). This binding model is supported by the following results.

First, pironetin binding to the Cys315Ala- and Cys316Ala-mutated tubulins was much slower than the

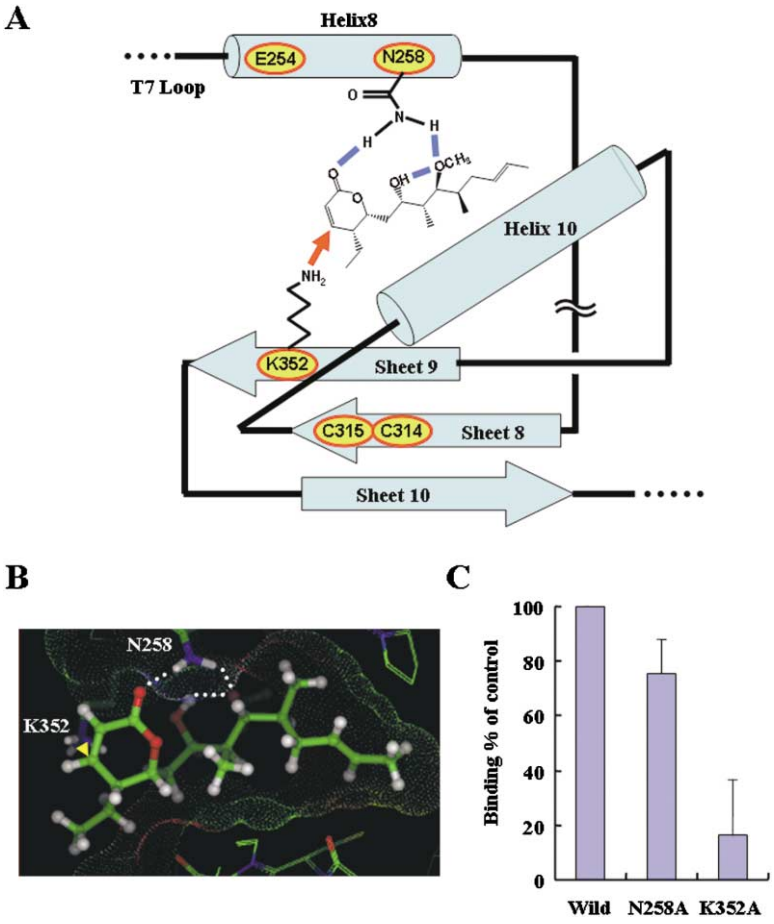

Figure 5. Proposed Binding Model of α-Tubulin-Pironetin Complex

(A) Secondary structure around the pironetin binding site on α-tubulin and binding model. (B) The white dashed lines show possible hydrogen bonds, and the yellow arrowhead shows the ϵ-amino moiety of the Lys352 attack site of pironetin.

(C) Pironetin did not bind to N258A and K352A mutant α-tubulins efficiently.

binding to the wild-type and the other mutants (Figure 4, data not shown). Since these residues locate just behind Lys352, we speculate that the mutations fail to position Lys352 correctly.

Second, pironetin binding to Asn258Ala-mutated α-tubulin was decreased to 75% compared with that to wild-type α-tubulin. Although Asn258 seems to be the key residue forming hydrogen bonds with pironetin in this model, the effects on the binding were low compared with those by the binding site mutation Lys352Ala. This is because pironetin fits the cavity so well that the contribution of the water repulsive force is much higher than that of the hydrogen bond formation on the decrease enthalpy.

Third, yeast cells harboring *tub1-K353A* (corresponding to Lys352Ala α-tubulin) showed pironetin resistance (see Supplemental Data at http://www.chembiol.com/cgi/content/full/11/6/799/DC1). These results strongly suggest that the binding site of pironetin on α-tubulin is Lys352.

We previously reported that pironetin decreased the tubulin binding of [^{3}H]vinblastine and slightly increased the binding of [^{3}H]colchicine, so we speculated that pironetin binds the *vinca* alkaloid binding site on β-tubulin [25]. However, we have to correct this conclusion here, because we obtained evidence that Lys352 of α-tubulin is the pironetin binding site. Although pironetin binds α-tubulin, how does pironetin inhibit tubulin binding of vinblastine? Lys352 is located on the entrance of a small pocket of α-tubulin, and this pocket is on the interdimer surface facing the GTP binding domain of the β-tubulin of the next dimer (Figure 6, Lys352 is enclosed with red circle). On the other hand, the *vinca* alkaloid binding site is speculated to locate to residues 177–215 in β-tubulin [33], the site near the GTP binding site, and this region is on the interdimer interface of the β-tubulin side (Figure 6, yellow circle) [1, 2]. So, the pironetin binding site on α-tubulin gets closer to the vinblastine binding site on β-tubulin when two tubulin heterodimers polymerize (Figure 6). These structural data prompt us to hypothesize that pironetin bound α-tubulin can bind to microtubules and cover the *vinca* alkaloid binding site on β-tubulin irrespective of direct or indirect interaction with pironetin, since vinblastine would be accessible to the *vinca* alkaloid binding site on β-tubulin if the pironetin bound α-tubulin lost binding activity. Otherwise, both pironetin and *vinca* alkaloid binding sites might be formed when tubulin heterodimers polymerize. The reports that Lys350 of β-tubulin, at analogous positions on different subunits, is involved in colchicines binding [34] and that colchicine B ring interacts with α-tubulin in addition to A/C ring interacts with β-tubulin [13] raise the possibility that the colchicine and pironetin/*vinca* alkaloid binding sites might be similar sites but at different subunit interfaces. Because colchicine stimulated [^{3}H]vinblastine binding and pironetin/vinblastine stim-

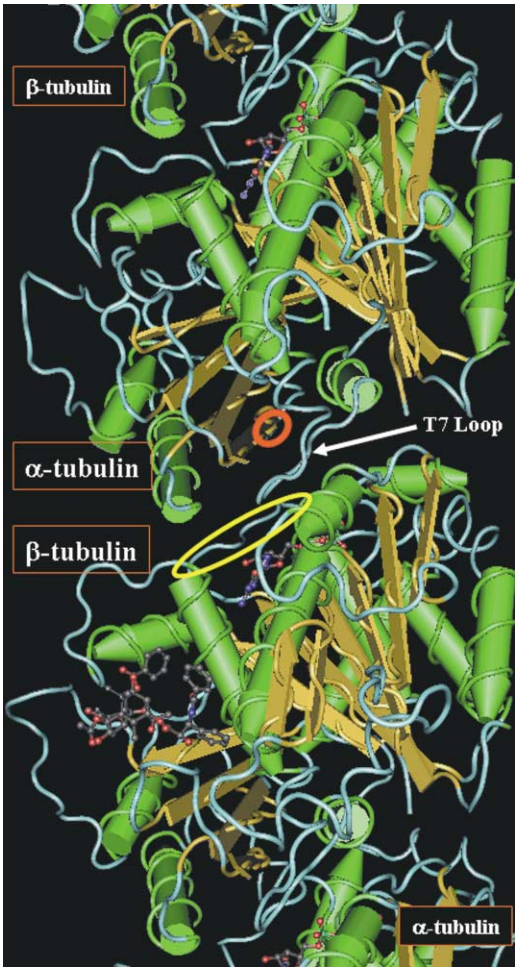

Figure 6. The Pironetin Binding Site on α-Tubulin Comes in Close Proximity to the *vinca* Alkaloid Binding Site and the GTPase Site on β-Tubulin by Interdimer Polymerization

The interdimer interfaces of both α- and β-tubulin are shown. Red circle: the pironetin binding site on α-tubulin. Yellow circle: the *vinca* alkaloid binding site on β-tubulin.

ulated [^{3}H]colchicines binding on tubulin [25], these compounds might induce the similar conformational changes on binding and neighboring subunit. These possibilities should be elucidated by several methods.

How does pironetin inhibit microtubule polymerization? Despite the fact that the binding subunit is different from that of vinblastine, the effects of pironetin on vinblastine and colchicine binding to tubulin were similar to that of vinblastine. These results might suggest that pironetin induces conformational changes of β-tubulin by interdimer or intradimer interaction and that pironetin and vinblastine inhibit microtubule polymerization by the same mechanism, such as by changing GTPase activity on the E site. Indeed, Lys352 locates close to Glu254, which is a residue conserved in essentially all α-tubulins, and is in an ideal position to be involved in the hydrolysis of GTP of the E site in β-tubulin. Furthermore, the *tub1-K353A* in yeast did not complement the deletion of α-tubulin like *tub1-E255A* (corresponding to Glu254 of mammalian α-tubulin) (Supplemental Data) [29]. The influences of pironetin binding upon both GTPase activity on the E site and interdimer interactions remain to be determined.

In conclusion, we determined the pironetin binding site to be Lys352 on α-tubulin. Furthermore, the data demonstrate that the natural product pironetin possesses the novel property of highly site-selective binding to the ε-amino moiety of lysine by Michael addition. These data have important implications for the design of molecularly targeted small molecules.

Significance

Many inhibitors for microtubule functions have been reported, for example, *vinca* alkaloid, paclitaxel, epothilones, and so on. As far as we know, all the natural compounds inhibiting tubulin polymerization/depolymerization act on β-tubulin. In this paper, we found that pironetin, a potent inhibitor of tubulin assembly, covalently binds α-tubulin. This is the first compound that covalently binds to α-tubulin. We also identified that pironetin binding site is Lys352 of α-tubulin. Since it is known that most of the compounds containing α,β-unsaturated lactone covalently bind to sulfhydryl group of cysteine residue, pironetin is a unique compound, which binds specific lysine residue on α-tubulin. These features of pironetin, which are distinct from known tubulin binding agents, suggest that it is possible to create a new drug useful for cancer therapy from pironetin as a lead compound.

Experimental Procedures

Synthesis of Biotinylated Pironetin and Its Derivatives

Pironetin (**1a** in Figure 1) was purified from *Streptomyces* sp. NK10958 [35]. Inactive pironetin derivatives (**2-4a**) were synthesized as previously reported [26]. Biotinylated pironetin (**1b**) was synthesized by two-step reaction from **1a**. A mixture of **1a** (20.0 mg, 0.062 mmol), *N*-Boc-ε-aminocapronic acid (21.4 mg, 0.093 mmol), 4-(dimethylamino)pyridine (0.5 mg), *n*-hexane (5 ml), and *N.N*-dicyclohexylcarbodiimide (19.1 mg, 0.093 mmol) was stirred at room temperature for 12 hr. The mixture was filtered through a celite pad and then concentrated in vacuo. The residue was chromatographed on silica gel (10 g) eluted with *n*-hexane/ethylacetate (~5:1–3:1) to give **5** (19.6 mg). To a solution of **5** (3.5 mg, 0.0065 mmol) in dichloromethane (1 ml) was added trifluoroacetic acid (0.5 ml). After being stirred for 1 hr, the mixture was concentrated in vacuo and the residue was dissolved in dimethylformamide (1 ml). After adding triethylamine (30 μl, 0.22 mmol) and succinimidyl-5-(biotinamido) hexanoate (EZ-Link NHS-LC-Biotin; Pierce, Rockford, IL), the mixture was stirred at 0°C for 1 hr and concentrated in vacuo. The residue was dissolved in chloroform (1 ml) and methanol (20 μl), stirred for 10 min, concentrated in vacuo, and purified by reverse-phase preparative thin layer chromatography (H_2O/methanol = 1:4) to give biotinylated pironetin (**1b**, 4.8 mg). The other biotinylated derivatives of inactive pironetin (**2-4b**) were synthesized by the same scheme. The structures of the compounds were confirmed by ^{1}H NMR using a BRUKER AC300 spectrometer (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany).

Cell Culture, Flow Cytometry, Immunofluorescence Procedure, and Transfection

3Y1 cells (rat normal fibroblasts) and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2. Flow cytometry and an immunofluorescence procedure were then performed as described previously [9]. GFP-fused α-tubulins (wild-type and Ala-scanned mutants created as described below) were con-

structed with pEGFP-C1 (CLONTEC Laboratories, Inc., Palo Alto, CA). The gene was transfected into exponentially growing HeLa cells with FuGENE 6 (Promega Corporation, Madison, WI), and GFP signals were observed after 48 hr incubation with a cooled charge-coupled device (CCD) camera (Olympus PROVIS AX70; Olympus, Tokyo, Japan).

Preparation of Microtubules and Tubulin Protein

Microtubule proteins were prepared from calf brain as described previously [9]. Tubulin was further purified to remove microtubule-associated proteins by phosphocellulose (P11; Whatman, Kent, UK) column chromatography of microtubule proteins.

Pironetin Binding Assay

To investigate in situ binding, 3Y1 cells (2×10^7 cells) in culture were pretreated with 0.1% DMSO (control) or 100 nM of competitors for 30 min and then incubated with 1 μg of biotinylated pironetin for 2 hr. After the cells had been lysed with RB (100 mM MES, 1 mM EGTA, 0.5 mM $MgCl_2$ [pH 6.7]) containing 1 mM PMSF and 0.1% NP-40, the supernatants were prepared by centrifugation. Twenty microliters of streptavidin-agarose (EMD Biosciences, Inc., Darmstadt, Germany) were added to the lysates prepared from the cells treated with biotinylated pironetin, and the mixtures were incubated for 30 min at 4°C. The bound proteins were washed thoroughly and boiled in 50 μl of SDS sample buffer. Each sample was subjected to SDS-PAGE [12.5% (w/v) gel], and the proteins were transferred to a poly(vinylidene fluoride) (PVDF) membrane. The transferred proteins covalently bound with biotinylated pironetin were subjected to immunoblotting using horseradish peroxidase-conjugated Neutravidin (Pierce). The protein bands were visualized using the SuperSignal Substrate (Pierce).

For the test of in vitro binding, microtubule proteins or tubulin were treated with biotinylated pironetin at 4°C. After 2 hr incubation, streptavidin-agarose was added to the reaction mixture.

Partial Digestion of Biotinylated Pironetin-Treated Tubulin and Determination of N-Terminal Amino Acid Sequence

Partial digestion of biotinylated pironetin-treated tubulin was performed as described below. After treatment of 1 mg of tubulin with 10 μg of biotinylated pironetin, subtilisin-BPN, chymotrypsin, elastase, or Lys-C were added to the mixture, which was then incubated for 20 min at 37°C. Protease digestion was terminated by adding a 1/3 volume of 4× SDS sample buffer. Digested peptides were separated with 20% Tris-Tricine SDS-PAGE and were transferred to a PVDF membrane. N-terminal amino acid sequences of the proteolytic peptides containing biotinylated pironetin were determined using a protein sequencer (ABI model 477A; Applied Biosystems, Foster City, CA).

Alanine Scanning of Cys and Lys Residues

All of the Cys and Lys residues in α-tubulin aa 269–360 were mutagenized to Ala by PCR. After confirmation of mutation sites by DNA sequencing, mutagenized fragments were ligated in GFP-fused α-tubulin (CLONTECH). The functionality of mutant α-tubulin was investigated by transfection to HeLa cells with FuGENE6. An in vitro biotinylated pironetin binding assay was performed with a TNT T7-transcription/translation coupled system of reticulocyte lysate (Invitrogen, Carlsbad, CA) as described below. Template DNA for in vitro transcription/translation was amplified with a T7 promoter containing primers 5′-TAATACGACTCACTATAGGGAGACCACCA TGCGTGAGTGCATCTCCATC-3′ (sense) and 5′-GGGCTCGAGCA GATCTCCTCAGCTAGCTTAGTATTCCTCTCCTTCTTC-3′ (antisense). α-tubulin was synthesized by a TNT transcription/translation kit with an amplified DNA template and [^{35}S]methionine (3000 Ci/mmol; Amersham Biosciences) (final 25 μl) and diluted to 1 ml with RB buffer. The mixture was incubated with 1 μl of biotinylated pironetin (10 mg/ml) overnight at 4°C, then supplemented with 10 μl of streptavidin-agarose and further incubated for 30 min. After being washed with 500 μl of ice-cold RB buffer three times, agarose beads were incubated with 1 ml of Ca^{2+} buffer (100 mM MES [pH 6.8], 5 mM $CaCl_2$) for 30 min at 37°C. The supernatant was removed, and the beads were washed with 500 μl of ice-cold RB buffer, 1× SDS sample buffer. The binding activities of mutant α-tubulin were determined by autoradiography with BAS2500 (Fuji Film, Tokyo, Japan).

Binding Model of the Tubulin-Pironetin Complex

The 3D structure of the complex of tubulin and pironetin molecules was estimated through energy minimization. The initial structure was generated by docking a pironetin molecule to an α-tubulin molecule manually based on the putative binding site and the interaction energy in vacuo using the computer program QUANTA (Accelrys Inc., San Diego, CA). The 3D structure of the α-tubulin molecule based on electron crystallographic findings has already been reported and was used in the present study as the initial structure. The extended structure was used as the initial structure of the pironetin molecule. We used the program CHARMm 23.2 (Accelrys) for energy minimization in vacuo under α-carbon atom constraints using the adopted-based Newton-Raphson method.

Supplemental Data

A supplemental figure, showing the effects of pironetin, is available online at http://www.chembiol.com/cgi/content/full/11/6/799/DC1.

Acknowledgments

This study was supported by Grants of Basic Research (Bioarchitect Project and Chemical Biology Project) in RIKEN and a grant from the Ministry of Education, Culture, Sports, Science, and Technology.

Received: January 23, 2004
Revised: March 1, 2004
Accepted: March 24, 2004
Published: June 25, 2004

References

1. Nogales, E. (2000). Structural insights into microtubule function. Annu. Rev. Biochem. *69*, 277–302.
2. Downing, K.H. (2000). Structural basis for the interaction with proteins and drugs that affect microtubule dynamics. Annu. Rev. Cell Dev. Biol. *16*, 89–111.
3. Bollag, D.M., McQueney, P.A., Zhu, J., Hensens, O., Koupal, L., Liesch, J., Goetz, M., Lazarides, E., and Woods, C.M. (1995). Epothilones, a new class of microtubule-stabilizing agents with a taxol-like mechanism of action. Cancer Res. *55*, 2325–2333.
4. Kowalski, R.J., Giannakakou, P., and Hamel, E. (1997). Activities of the microtubule-stabilizing agents epothilones A and B with purified tubulin and in cells resistant to paclitaxel (Taxol). J. Biol. Chem. *272*, 2534–2541.
5. Smaletz, O., Galsky, M., Scher, H.I., DeLaCruz, A., Slovin, S.F., Morris, M.J., Solit, D.B., Davar, U., Schwartz, L., and Kelly, W.K. (2003). Pilot study of epothilone B analog (BMS-247550) and estramustine phosphate in patients with progressive metastatic prostate cancer following castration. Ann. Oncol. *14*, 1518–1524.
6. Mooberry, S.L. (2003). New insights into 2-methoxyestradiol, a promising antiangiogenic and antitumor agent. Curr. Opin. Oncol. *15*, 425–430.
7. Natsume, T., Watanabe, J., Koh, Y., Fujio, N., Ohe, Y., Horiuchi, T., Saijo, N., Nishio, K., and Kobayashi, M. (2003). Antitumor activity of TZT-1027 (Soblidotin) against vascular endothelial growth factor-secreting human lung cancer in vivo. Cancer Sci. *94*, 826–833.
8. Cui, C.B., Kakeya, H., Okada, G., Onose, R., Ubukata, M., Takahashi, I., Isono, K., and Osada, H. (1995). Tryprostatins A and B, novel mammalian cell cycle inhibitors produced by *Aspergillus fumigatus*. J. Antibiot. *48*, 1382–1384.
9. Usui, T., Kondoh, M., Cui, C.B., Mayumi, T., and Osada, H. (1998). Tryprostatin A, a specific and novel inhibitor of microtubule assembly. Biochem. J. *333*, 543–548.
10. Osada, H. (2003). Development and application of bioprobes for mammalian cell cycle analyses. Curr. Med. Chem. *10*, 727–732.
11. Anthony, R.G., Waldin, T.R., Ray, J.A., Bright, S.W.J., and Hussey, P.J. (1998). Herbicide resistance caused by spontaneous

mutation of the cytoskeletal protein tubulin. Nature *393*, 260–263.

12. Anthony, R.G., and Hussey, P.J. (1999). Double mutation in *Eleusine indica* α-tubulin increases the resistance of transgenic maize calli to dinitroaniline and phosphorothioamidate herbicides. Plant J. *18*, 669–674.
13. Chaudhuri, A.R., Seetharamalu, P., Schwarz, P.M., Hausheer, F.H., and Luduena, R.F. (2000). The interaction of the B-ring of colchicine with α-tubulin: a novel footprinting approach. J. Mol. Biol. *303*, 679–692.
14. Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., and Santoro, M. (2000). Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IκB kinase. Nature *403*, 103–108.
15. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I.W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell *90*, 1051–1060.
16. Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature *390*, 308–311.
17. Ossareh-Nazari, B., Bachelerie, F., and Dargemont, C. (1997). Evidence for a role of CRM1 in signal-mediated nuclear protein export. Science *278*, 141–144.
18. Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E.P., Yoneda, Y., Yanagida, M., Horinouchi, S., and Yoshida, M. (1998). Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. Exp. Cell Res. *242*, 540–547.
19. Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E.P., Wolff, B., Yoshida, M., and Horinouchi, S. (1999). Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. Proc. Natl. Acad. Sci. USA *96*, 9112–9117.
20. Walsh, A.H., Cheng, A., and Honkanen, R.E. (1997). Fostriecin, an antitumor antibiotic with inhibitory activity against serine/threonine protein phosphatase types 1 (PP1) and 2A (PP2A), is highly selective for PP2A. FEBS Lett. *416*, 230–234.
21. Evans, D.R.T., and Simon, J.A. (2001). The predicted β12-β13 loop is important for inhibition of PP2Acα by the antitumor drug fostriecin. FEBS Lett. *498*, 110–115.
22. Hamaguchi, T., Sudo, T., and Osada, H. (1995). RK-682, a potent inhibitor of tyrosine phosphatase, arrested the mammalian cell cycle progression at G1 phase. FEBS Lett. *372*, 54–58.
23. Ueda, K., Usui, T., Nakayama, H., Ueki, M., Takio, K., Ubukata, M., and Osada, H. (2002). 4-isoavenaciolide covalently binds and inhibits VHR, a dual-specificity phosphatase. FEBS Lett. *525*, 48–52.
24. Kondoh, M., Usui, T., Kobayashi, S., Tsuchiya, K., Nishikawa, K., Nishikiori, T., Mayumi, T., and Osada, H. (1998). Cell cycle arrest and antitumor activity of pironetin and its derivatives. Cancer Lett. *126*, 29–32.
25. Kondoh, M., Usui, T., Nishikiori, T., Mayumi, T., and Osada, H. (1999). Apoptosis induction via microtubule disassembly by an antitumour compound, pironetin. Biochem. J. *340*, 411–416.
26. Watanabe, H., Watanabe, H., Usui, T., Kondoh, M., Osada, H., and Kitahara, T. (2000). Synthesis of pironetin and related analogs: studies on structure-activity relationships as tubulin assembly inhibitors. J. Antibiot. *53*, 540–545.
27. Nogales, E., Wolf, S.G., and Downing, K.H. (1998). Structure of the αβ tubulin dimer by electron crystallography. Nature *391*, 199–203.
28. Lowe, J., Li, H., Downing, K.H., and Nogales, E. (2001). Refined structure of αβ-tubulin at 3.5 Å resolution. J. Mol. Biol. *313*, 1045–1057.
29. Anders, K.R., and Botstein, D. (2001). Dominant-lethal α-tubulin mutants defective in microtubule depolymerization in yeast. Mol. Biol. Cell *12*, 3973–3986.
30. Perlmutter, P. (1992). Conjugate Addition Reactions in Organic Synthesis, Volume 9 (Oxford, UK: Pergamon Press).
31. Yaffe, M.B., Farr, G.W., Miklos, D., Horwich, A.L., Sternlicht, M.L., and Sternlicht, H. (1992). TCP1 complex is a molecular chaperone in tubulin biogenesis. Nature *358*, 245–248.
32. Sternlicht, H., Farr, G.W., Sternlicht, M.L., Driscoll, J.K., Willison, K., and Yaffe, M.B. (1993). The t-complex polypeptide 1 complex is a chaperonin for tubulin and actin in vivo. Proc. Natl. Acad. Sci. USA *90*, 9422–9426.
33. Rai, S.S., and Wolff, J. (1996). Localization of the vinblastine-binding site on β-tubulin. J. Biol. Chem. *271*, 14707–14711.
34. Lee, V.D., and Huang, B. (1990). Missense mutations at lysine 350 in β2-tubulin confer altered sensitivity to microtubule inhibitors in *Chlamydomonas*. Plant Cell *2*, 1051–1057.
35. Kobayashi, S., Tsuchiya, K., Harada, T., Nishide, M., Kurokawa, T., Nakagawa, T., Shimada, N., and Kobayashi, K. (1994). Pironetin, a novel plant growth regulator produced by *Streptomyces* sp. NK10958. I. Taxonomy, production, isolation and preliminary characterization. J. Antibiot. *47*, 697–702.