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(−)-Phenylahistin Arrests Cells in Mitosis by Inhibiting Tubulin Polymerization

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(−)-Phenylahistin, a fungal diketopiperazine metabolite composed of phenylalanine and isoprenylated dehydrohistidine, arrested cells in mitosis and inhibited the proliferation of A549 cells. The microtubule network in A549 cells was disrupted by (−)-phenylahistin, which also inhibited the polymerization of both microtubule protein from bovine brain and phosphocellulose-purified tubulin *in vitro*. Competitive binding studies indicated that (−)-phenylahistin interacted with the colchicine binding site on tubulin but not with the vinblastine binding site.

Tubulin consists of two 50 kDa subunits (α- and β-tubulin) and is the major constituent of microtubules[1]. Microtubules are the main components of spindles in the mitotic apparatus of eucaryotic cells, and also involved in several essential cell functions, such as axonal transport, cell motility and determination of cell morphology. Therefore, inhibitors of microtubule function should have broad biological activity, and be applicable to medicinal and agrochemical purposes.

While screening for new cell cycle inhibitors, (−)-phenylahistin [(−)-PLH][2], a diketopiperazine composed of L-phenylalanine and isoprenylated dehydrohistidine (Fig. 1) isolated from the agar-culture medium of *Aspergillus ustus* NSC-F038, showed cytotoxic and cell cycle inhibitory activities during the G2/M phase in P388 cells. (−)-PLH was evaluated according to the drug evaluation program of the Japanese Foundation for Cancer Research, a system based on the growth inhibition of 38 human tumor cell lines[3]. The results showed that the inhibition profile of (−)-PLH was similar to that of vinca alkaloid or paclitaxel, suggesting action on microtubules. However, the precise mechanisms underlying the cytotoxic and cell cycle inhibitory activities of (−)-PLH are unknown.

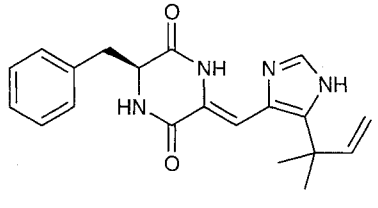
In the present study, we investigated the mechanism of (−)-PLH actions, with a particular emphasis on its effects on microtubule function. Specifically, we examined the effects of (−)-PLH on proliferation, mitosis and microtubule structure of A549 cells (human lung carcinoma), and its inhibitory effects on the *in vitro* polymerization of bovine brain microtubule protein and purified tubulin. In addition, we also investigated (−)-PLH binding sites on tubulin by competitive binding experiments using colchicine (CLC) and vinblastine (VLB), two typical antimitotic agents that bind to individual binding sites on tubulin.

Materials and Methods

Materials

(−)-PLH was isolated from the agar-culture medium of *A. ustus* NSC-F038[2]. Briefly, the ethyl acetate extract of the cultured medium was chromatographed on a silica gel column, then (−)-PLH and (+)-PLH were precipitated as a scalemic mixture in ethyl acetate. (−)-PLH was finally purified by chiral HPLC to >99.8%. CLC was obtained from Sigma (St. Louis, MO)

Fig. 1. Structure of (−)-phenylahistin ((−)-PLH).



and VLB was from Wako Pure Chemicals (Tokyo, Japan). [^{3}H]CLC was purchased from Du Pont/New England Nuclear (Boston, MA) and [^{3}H]VLB was from Amersham (Buckinghamshire, UK). A549 cell line (human lung carcinoma) was obtained from American Type Culture Collection (Rockville, MD). A549 cells were cultured in EMEM medium (phenol red free, Nissui Pharmaceuticals, Tokyo, Japan) supplemented with MEM non-essential amino acids (Sigma) and 10% fetal bovine serum (JRH Biosciences, Lenaxa, KS).

Alamar Blue™ Assay

Exponentially growing A549 cells were seeded into 96-well tissue culture plates (2×10^3 cells/100 μl/well) and cultured for 16 hours. (−)-PLH or CLC was then added to each well at various concentrations, and the cells were cultured for an additional 48 hours. Live cells were counted using Alamar Blue™ (BioSource International, Camarillo, CA)[4].

Mitotic Index

Exponentially growing A549 cells were seeded into 24-well tissue culture plates (5×10^3 cells/500 μl/well) and cultured for 16 hours. (−)-PLH or CLC was then added to each well at various concentrations, and the plates were incubated for an additional 24 hours. We counted the number of cells in mitosis (round cells) and total cells in eight randomly selected fields under a phase-contrast microscope. In a series of preliminary studies, we confirmed that the round cells in this condition were cells in mitosis by using flow cytometry and Hoechst 33258 staining.

Immunocytochemistry

Immunocytochemical staining was performed using the method described previously[5]. For this purpose, A549 cells were cultured on glass coverslips and incubated with the test drug for 6 hours. The cells were then fixed with 3.7% formaldehyde for 30 minutes, permeabilized with 0.2% Triton X-100 for 5 minutes, and incubated with a mouse monoclonal antibody against α-tubulin (Calbiochem®, Oncogene Research Products, Cambridge, MA), followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cosmo Bio Co., Tokyo, Japan). Finally, the cells were examined under an immunofluorescent microscope.

Microtubule Protein and Tubulin Preparation

Microtubule protein was prepared from bovine brain tissue by two cycles of assembly and disassembly[6]. Tubulin was purified from microtubule protein by phosphocellulose chromatography[7], and its purity was evaluated by polyacrylamide gel electrophoresis[8]. Essentially, no microtubule-associated proteins were detected in this preparation. Protein concentrations were determined using the Coomassie® Protein Assay Reagent (Pierce, Rockfold, IL).

Polymerization Assay

Polymerization of microtubule protein was monitored by an increase in turbidity at 37°C in microtubule assembly buffer containing 100 mM MES, 0.5 mM $MgCl_2$, 1 mM EGTA and 1 mM GTP[9]. Polymerization of purified tubulin was measured using the same method except the microtubule assembly buffer contained 4 M glycerol[10]. Microtubule protein and tubulin polymerization was initiated by a temperature shift from 0°C to 37°C. Turbidity was measured on a thermo-controlled spectrophotometer (Beckman DU-20, Fullerton, CA) at 360 nm. Drugs were dissolved in dimethyl sulfoxide (DMSO), which was used in all experiments at a final concentration of 2% (v/v).

Electron Microscopy

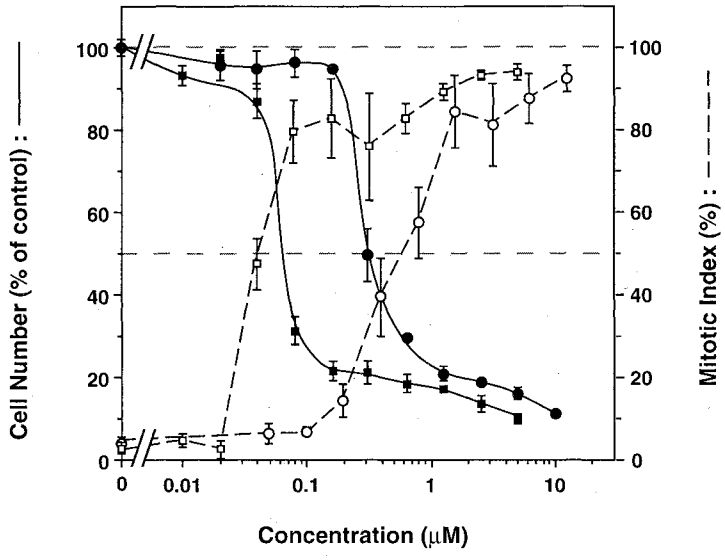
Microtubule protein (1.5 mg/ml) was polymerized at 37°C for 20 minutes in the presence of 100 μM (−)-PLH or vehicle (DMSO). A portion of each sample was diluted 5-fold with 1% glutaraldehyde in microtubule assembly buffer. Samples were then placed on formvar- and carbon-coated grids, stained with 2% uranyl acetate, and examined using a JEOL JEM-1200 EXII electron microscope.

Competition Assay

[^{3}H]CLC binding to tubulin was evaluated by the ultrafiltration method[11] with slight modifications. For this purpose, bovine brain tubulin (0.2 mg/ml) was incubated in the microtubule assembly buffer with various concentrations of [^{3}H]CLC for 20 minutes at 37°C. Each sample (200 μl) was applied to the reservoir of the ultrafiltration unit (UFC3 LTK00, Nihon Millipore Ltd., Yonezawa, Japan) and centrifuged at $1,500 \times g$ for 4 minutes at room temperature to obtain approximately 60 μl of the filtrate. The concentration of unbound [^{3}H]CLC in the filtrates was determined using a liquid scintillator (EcoLite™(+), ICN Pharmaceuticals Inc., Costa Mesa, CA). Specific bound [^{3}H]CLC concentrations were determined by adding an excess (× 100) of unlabeled CLC to the reaction mixture.

For the competition assay, bovine brain tubulin

Fig. 2. Effects of (−)-PLH and CLC on proliferation and mitotic index of A549 cells.



To measure the antiproliferative activity of (−)-PLH, cells were incubated for 48 hours with (−)-PLH (closed circles) or CLC (closed squares) at the indicated concentrations, and the cell number was determined by Alamar Blue™ assay. Data represent the mean ± SD of four experiments. To measure the mitotic index, cells were incubated with (−)-PLH (open circles) or CLC (open squares) for 24 hours, then mitotic and total cells were counted under a phase-contrast microscope. Data represent the mean ± SD of eight observations.

(0.2 mg/ml) was incubated in the microtubule assembly buffer with 0.5 μM [3H]CLC and various concentrations of competitors for 20 minutes at 37°C. [^{3}H]CLC binding was measured as described above. DMSO was used as a co-solvent at a concentration that did not affect drug binding to tubulin (final 2% v/v). Measurement of [^{3}H]VLB binding was followed by the DEAE-cellulose filter method[12], and the competition assay was carried out as described for CLC.

Results

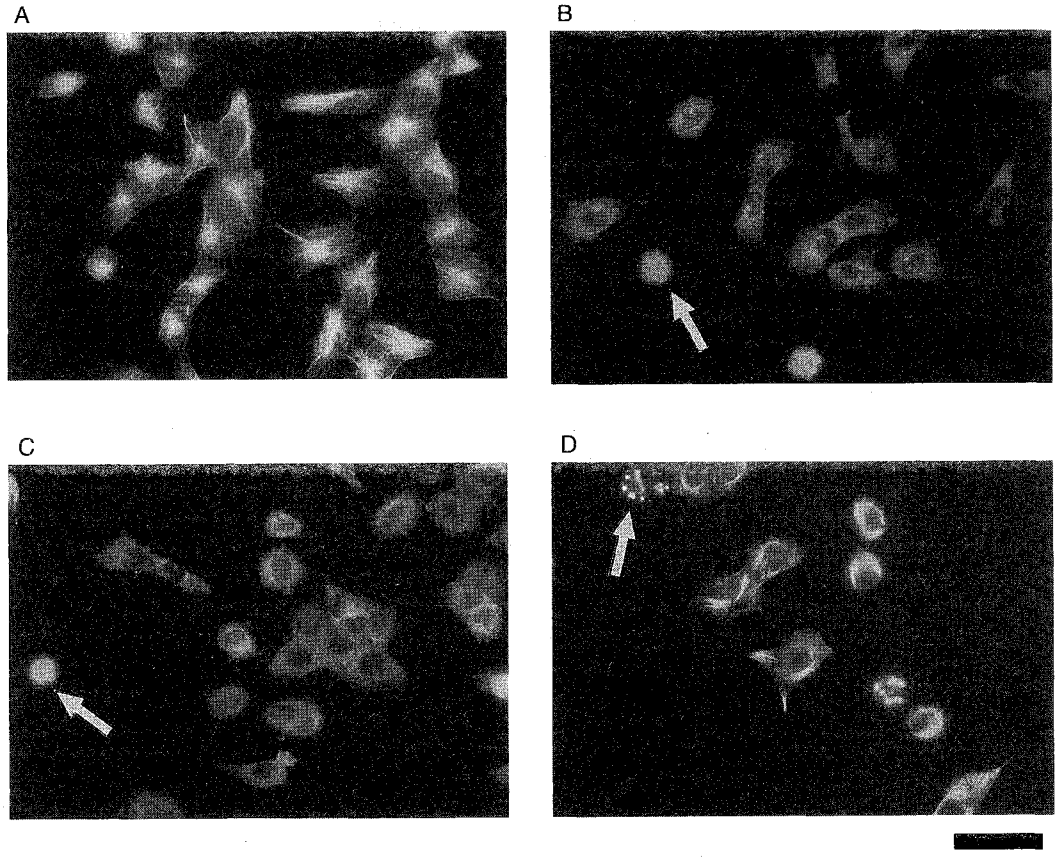
Inhibitory Effects of (−)-PLH on Proliferation and Mitosis of A549 Cells

We first examined the effects of (−)-PLH on the proliferation and mitosis of A549 cells. As shown in Fig. 2, (−)-PLH inhibited the proliferation of A549 cells in a dose-dependent manner with a 50% inhibitory concentration (IC_{50}) value of 0.3 μM, indicating that (−)-PLH was 5-fold less potent than CLC under our assay conditions. On the other hand, the mitotic index increased, which correlated with decreased cell proliferation (Fig. 2). These results indicated that (−)-PLH arrested the cell cycle during mitosis, which subsequently reduced cell proliferation, similar to other mitotic inhibitors like CLC or VLB[13]. The anti-mitotic effect of (−)-PLH seemed to be reversible, because cells that arrested in M phase by (−)-PLH returned to proliferate again following washing of (−)-PLH and replacement with a fresh medium (data not shown).

Immunofluorescence Staining of Microtubules in A549 Cells

In the next step, we investigated the effects of (−)-PLH on microtubule structure in A549 cells using an anti α-tubulin antibody and a secondary antibody conjugated with FITC. These studies aimed to determine whether (−)-PLH can inhibit the microtubule assembly like CLC or hyper-stabilize microtubules in a manner similar to that of paclitaxel (taxol®). In control cells (Fig. 3A), a network of cytoskeletal microtubules was clearly visible, and mitotic spindles could be observed in mitotic cells (not on focus in Fig. 3A). The addition of (−)-PLH resulted in the disappearance of the microtubule network (Fig. 3B), and the entire mitotic cell (round cells; indicated by arrow) was uniformly stained with α-tubulin antibody, whereas mitotic spindles were never seen. Cells incubated with CLC exhibited a staining profile similar to that of (−)-PLH-treated cells (Fig. 3C). Paclitaxel, a potent

Fig. 3. Immunofluorescence staining of A549 cells with anti-α-tubulin antibody.



Control cells [**A**]. Cells were incubated with 3 μM (−)-PLH [**B**], 0.4 μM CLC [**C**] or 0.2 μM paclitaxel [**D**] for 8 hours, fixed and stained with anti-α-tubulin antibody and a secondary antibody conjugated with FITC. (bar: 50 μm). Arrows in B and C: round cells showing a homogeneous staining pattern. Arrow in D: aster formation.

microtubule-stabilizer, produced thick microtubule bundles and multiple bright fluorescent aster formation (arrow, Fig. 3D)[14]. These results suggested that (−)-PLH inhibited microtubule assembly in A549 cells, but did not stabilize microtubules.

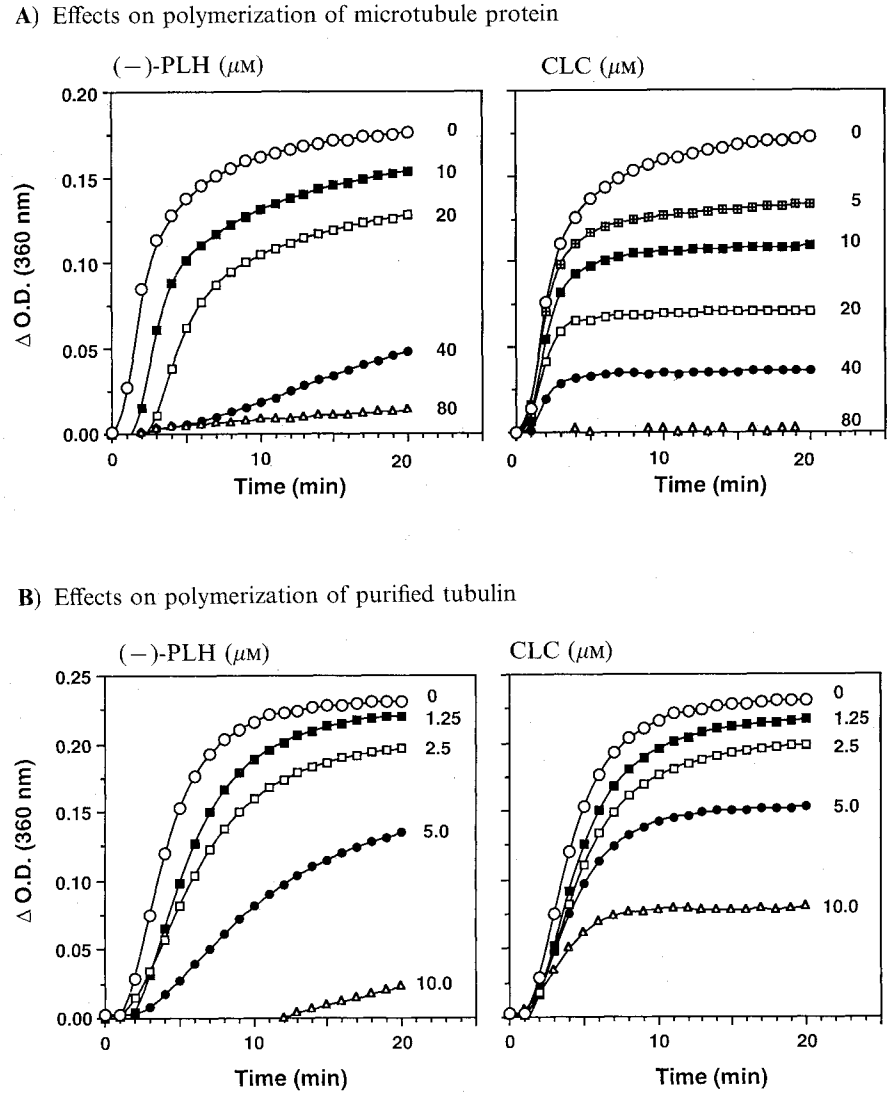
Effects of (−)-PLH on Polymerization of Microtubule Protein and Purified Tubulin

Since the above immunofluorescence staining studies suggested that (−)-PLH inhibits microtubule assembly in A549 cells, we examined the effect of (−)-PLH on the polymerization of microtubule protein obtained from bovine brain. Figure 4A shows the time course of the turbidity change in the presence of various concentrations of (−)-PLH and CLC. (−)-PLH inhibited the *in vitro* polymerization of microtubule protein in a concentration-dependent manner, 80 μM of (−)-PLH completely inhibited the polymerization of microtubule protein. We also examined the effect of (+)-PLH on microtubule assembly, (+)-PLH did not affect it up to a concentration of 200 μM.

It is reported that VLB[15] or dolastin 10[16] increases the turbidity in microtubule protein polymerization at concentrations higher than those required for inhibition. However, (−)-PLH did not increase the turbidity at concentrations up to 200 μM.

To determine whether (−)-PLH acts on tubulin directly or on associated proteins, we performed further experiments on the assembly using phosphocellulose-purified tubulin, which was free of microtubule associated proteins. Like microtubule protein, (−)-PLH inhibited tubulin polymerization in a concentration-dependent manner (Fig. 4B). The IC_{50} value of (−)-PLH determined from turbidity at 20 minutes was 4.9 ± 1.3 μM (mean ± standard deviation of three independent experiments), while the IC_{50} value of CLC was 6.6 ± 1.7 μM under the same experimental conditions. These results indicated that (−)-PLH is as effective as CLC in in-

Fig. 4. Effects of (−)-PLH and CLC on *in vitro* polymerization of microtubule proteins and purified tubulin.



Various concentrations of drugs were mixed with microtubule proteins (1.5 mg/ml) [**A**] or purified tubulin (1.5 mg/ml) [**B**] at 0°C and incubated at 37°C. Changes in turbidity were monitored at 360 nm. Typical result of three individual experiments is revealed.

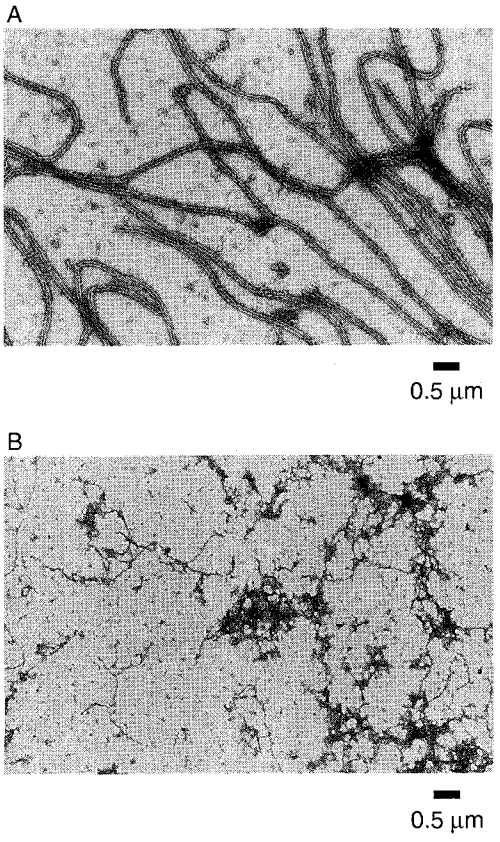
hibiting tubulin polymerization, and that (−)-PLH acts on tubulin directly rather than on associated proteins.

We further examined the effect of (−)-PLH on polymerization of the microtubule protein by electron microscopy (Fig. 5). The control sample contained singular microtubules with a normal cylindrical structure (Fig. 5A), whereas a sample treated with 100 μM (−)-PLH did not contain such structures (Fig. 5B). These findings confirmed the turbidity measurements and indicated that (−)-PLH inhibited the polymerization of microtubule protein.

(−)-PLH Inhibited CLC Binding to Tubulin

To investigate the binding site of (−)-PLH on tubulin, we conducted competitive binding studies using [^{3}H]-CLC and [^{3}H]VLB, which are typical antimitotic agents that bind to CLC and VLB binding sites, respectively. [^{3}H]CLC binding was measured by the ultrafiltration method as described above. Under our experimental conditions, the *Kd* value of CLC to tubulin was 5.3×10^{-7} M, which is in good agreement with the previously reported value[17]. As shown in Fig. 6, (−)-PLH dose-dependently inhibited CLC binding to tubulin. The inhibition manner of (−)-PLH was analyzed by Dixon

Fig. 5. Electron micrographs of microtubule proteins.



Microtubule proteins (1.5 mg/ml) in microtubule assembly buffer were incubated at 37°C for 30 minutes in the absence [**A**], or presence of 100 μM (−)-PLH [**B**]. Note the presence of several singular tubular structures in [A] and their absence in [B].

plot (Fig. 6B), indicating that (−)-PLH competitively inhibited the CLC binding to tubulin with the *Ki* value of 7.4×10^{-6} M. VLB slightly enhanced CLC binding to tubulin, which was probably caused by the stabilization effect of VLB on the CLC binding activity of tubulin[18]. On the other hand, the binding of [^{3}H]VLB was not inhibited by (−)-PLH as shown in Figure 6B. CLC enhanced and (−)-PLH slightly enhanced [^{3}H]VLB binding to tubulin. These results suggested that (−)-PLH binds to the CLC binding site (CLC-site) on tubulin or to a site overlapping the CLC-site.

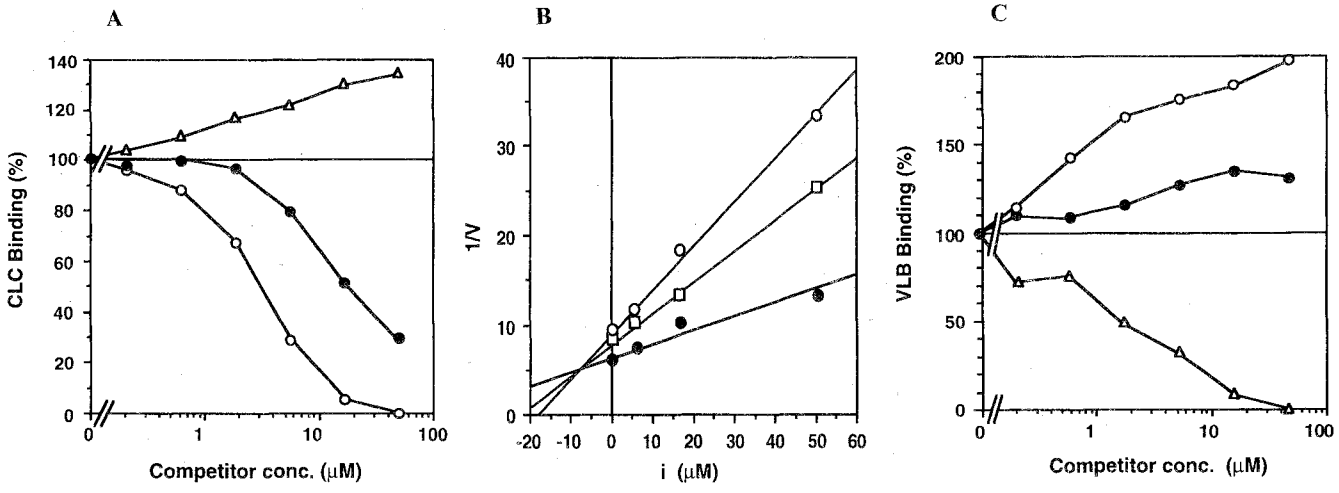
Discussion

We described in this study the antimitotic activity and mechanism of action of (−)-PLH, a new diketopiperazine metabolite produced by *A. ustus*. We demonstrated that (−)-PLH dose-dependently increased the mitotic index in parallel with inhibition of proliferation of A549 cells (Fig. 2), indicating that it prevents cell proliferation by arresting the cell cycle in M phase. Most antimitotic agents such as CLC and VLB are known to exert anti-microtubule activity, including disruption of the process of mitotic spindle formation, resulting in cell arrest in mitosis. (−)-PLH also exhibited an anti-microtubule activity, as evident by depolymerization of cytoskeletal microtubule in A549 cells treated with (−)-PLH (Fig. 3). To investigate the mechanism underlying the anti-microtubule activity of this compound, we performed an *in vitro* polymerization assay using a microtubule protein and phosphocellulose-purified tubulin from bovine brain. As shown in Fig. 4, (−)-PLH inhibited polymerization of both microtubule protein and purified tubulin, suggesting that (−)-PLH directly acted on tubulin, rather than interacting with microtubule associated proteins. Although (−)-PLH was as effective as CLC in inhibiting the polymerization of purified tubulin, it was 5-fold less potent than CLC in inhibiting the proliferation of A549 cells. These differences may be due to a low permeability of the cell membrane to (−)-PLH compared to CLC. Alternatively, the difference may be due to intracellular transformation of (−)-PLH to an inactive form. The competitive binding assay using radiolabeled CLC and VLB showed that (−)-PLH inhibits the binding of CLC to tubulin, indicating that (−)-PLH is a competitive inhibitor of CLC binding to tubulin and its binding site may be similar or very close to that of CLC.

A number of natural and synthetic antimitotic agents have been reported to inhibit mitosis by binding to the CLC-site on tubulin[19,20]. It seems likely that these CLC-site ligands such as CLC, steganacin[21], podophyllotoxin[22] and combretastatins[23], interact at two hydrophobic sites on tubulin with biaryl groups located at appropriate distances and angles, whereas curacin A[24] probably interacts in a different manner. In the case of (−)-PLH, the spatial arrangement of two aryl groups, the phenyl group and imidazole moiety, is probably important for binding to tubulin, because the enantiomer (+)-PLH showed little effect on the polymerization of microtubule protein up to the concentration of 200 μM (data not shown).

Recently, Usui and colleagues[25,26] reported that tryprostatin A and its related compounds, which are diketopiperazines consisting of isoprenylated tryptophan and proline, affect the microtubule assembly. They also showed that tryprostatin A inhibited microtubule polymerization by interacting with MAP2/tau-binding site

Fig. 6. Effects of (−)-PLH on binding of [^{3}H]CLC and [^{3}H]VLB to tubulin.



Phosphocellulose-purified tubulin (0.2 mg/ml) was incubated in the assembly buffer (100 mM MES, 0.5 mM $MgCl_2$, 1 mM EGTA and 0.5 mM GTP) with 0.5 μM of [^{3}H]CLC [**A**] or 0.5 μM of [^{3}H]VLB [**C**], and the indicated concentrations of unlabeled competitors (open circles, CLC; open triangles, VLB; closed circles, (−)-PLH) for 20 minutes at 37°C. Data are the average of two samples in a single experiment. [**B**]: Dixon analysis of the inhibitory effect of (−)-PLH on [^{3}H]CLC binding to tubulin. Reaction mixtures contained the (−)-PLH concentrations indicated on the abscissa (i), 0.2 mg/ml tubulin, and [^{3}H]CLC as follows: closed circle, 3 μM; open square, 5 μM; open circle, 6 μM. The ordinate (1/V) units are (mg/ml tubulin)/(μmol CLC bound).

rather than with CLC binding site, at relatively higher concentrations[25]. Although tryprostatin A and (−)-PLH have a similar structural motif, the diketopiperazine ring composed of isoprenylated heterocyclic amino acids, the binding site and effective concentration against microtubule polymerization are different. It is interesting that the two diketopiperazine compounds, which have different constituent amino acids, bind to distinct sites of tubulin molecule and exert a similar biological activity. Combinatorial chemical studies using a diketopiperazines consisting of heterocyclic amino acid as a scaffold may produce new anti-tubulin agents, which are useful probes for the elucidation of the mechanism of microtubule/tubulin polymerization.

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