Relationship between the Structures of Taxane Derivatives and Their Microtubule Polymerization Activity

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Paclitaxel (Taxol), one of the most potent anticancer drugs, is a microtubule-stabilizing compound that inhibits microtubule depolymerization within the cell. The structure of paclitaxel is composed of two key elements, a taxane ring and an N-benzoylphenylisoserine side chain at C-13. A number of natural and artificial compounds with taxane skeletons have been isolated, but almost none of their bioactivities have been evaluated. In this study, we focused on compounds having a taxane skeleton structure and examined their effects on tubulin dynamics. Although none of these compounds had an N-benzoylphenylisoserine side chain, three were found to promote tubulin assembly. On the other hand, one compound inhibited tubulin assembly in a way similar to nocodazole. These compounds exhibited novel structure-activity relationships of taxane compounds.

Key words: taxane; paclitaxel; microtubule; polymerization; cryo-electron microscopy (cryo-EM)

Microtubules are part of the cell’s apparatus for dividing and replicating itself. They result from the head-to-tail longitudinal self-assembly of tubulin protein,11 and exhibit distinct assembly-disassembly dynamics called dynamic instability, an essential property of microtubules.2, The compounds used in cancer chemotherapy affect microtubule dynamics: inhibition of these structural dynamics ultimately results in cell death.3 Paclitaxel (Fig. 1A, 1) binds stoichiometrically and specifically to the β-tubulin subunit in microtubules (Supplemental Fig. S1A and B, see Biosci. Biotechnol. Biochem. Web site),4,5 and promotes microtubule assembly by stabilizing microtubule structure within cells. Hence, paclitaxel is one of the most potent anticancer drug.3–7 Nocodazole (Fig. 1B) binds to a distinct site from that of paclitaxel (Supplemental Fig. S1A), and exerts its effect on cells by interfering with tubulin association.8,9

Structure-activity relationship studies have provided insight into structural determinants that are important for the activity of paclitaxel.10,11 The N-benzoylphenylisoserine side chain at C-13 and the C-2 benzoyl group are essential for the stabilization of microtubules.12,13 Chatterjee et al. compared the dynamic properties of paclitaxel and those of one of its “inactive” analogs, baccatin III (Fig. 1C, 2), and concluded that they behave similarly in their interactions with tubulin.14 Andreev and Barasoaia also asserted the importance of the baccatin III core in the binding process.15 They estimated that C-2 and C-4 substitutions in the core structure account for approximately 75% of the free energy change during the paclitaxel-binding process.

Various taxane compounds have been reported from the studies on total organic synthesis of paclitaxel, but the effects of only a few of these compounds on tubulin assembly have been evaluated. On the other hand, a number of natural compounds with a taxane skeleton have been isolated to date.10 In this study, we performed a fluorescence-based assay to discover novel candidates for the development of an antimicrotubule drug from various taxane compound libraries, natural taxanes, and their synthetic derivatives. Three compounds were found to promote tubulin assembly, while one acted as an inhibitor.

Materials and Methods

Taxane analogs for bioassay. Taxanes 1 (Taxinine), 2 (taxusin), 3 (taxinine M),4,5 4, 5 (Shi and Kiyota, manuscripts in preparation), and 6 were isolated from the Japanese yew Taxus cuspidata. Taxanes 7–10,11,12 were prepared by our methods. The synthesis of 13–18 is described in Supporting Information.

Protein preparation. Tubulin proteins were prepared from bovine brain by 3 cycles of temperature-dependent polymerization and depolymerization, as described by Castoldi and Popov.23 As described in our previous report, SDS-PAGE and Western blot analysis ensured
the purity of the tubulin, which was not contaminated with proteins such as tau. The concentration of tubulin was determined with a BCA protein assay kit (Pierce, Rockford, IL) standardized with bovine serum albumin.

Tubulin polymerization assay. Evaluation of the compounds was performed by Barrons’ method, with some modifications as follows: In initial experiments to determine whether the compounds show an assembly or a disassembly effect, tubulin assembly was performed in 96-well black plates (Corning, NY) and was observed by monitored fluorescence with Infinite F200 (Tecan, Durham, NC). The excitation and emission wavelengths were set at 360 and 465 nm respectively. A 96-well plate was placed in a holder pre-warmed at 37 °C. A solution (100 μL) containing 12 μM 4,6-diamidino-2-phenylindole (DAPI), 15 μM tubulin, and 200 μg/mL of taxane compounds dissolved in dimethyl sulfoxide in PM buffer (100 mM Pipes pH 6.9, 5 mM ethylene glycol tetraacetic acid, 2 mM MgCl₂, and 1 mM dithiothreitol). In this assay method, acceleration of tubulin assembly is observed in the presence of paclitaxel (<0.1 μM, supplemental Fig. S2A). Four compounds (observed to have >15% difference from the control) were further evaluated in dose-response experiments.

Tubulin depolymerization assay. Tubulin assembly was initiated as described in the section just above. Taxanes 14 and 15 (final concentration 400 μM), nocodazole (1 μM), or CaCl₂ (10 mM) were added 1,200 s after initiation of assembly to evaluate the depolymerization pattern.

Dark-field microscopy and cryo-electron microscopy for microtubule polymerization analysis. Microtubule polymerization was observed using a dark-field microscope (BX51, Olympus, Tokyo) equipped with a high-pressure mercury lamp. The images were recorded using a charged-coupled device camera (DR-328G, Andor, Belfast, North Ireland). The room temperature was maintained at 28 ± 3 °C to favor microtubule polymerization in the presence of the compounds (200 μg/mL of each compound). Microtubules polymerized in the presence of the taxane compounds were also examined by cryo-electron microscopy (cryo-EM), as described previously.

Results

Initial assay of taxane compound

We adapted 18 taxane compounds to the fluorescence-based assay (Supplemental Fig. S3). None of the compounds showed fluorescence under these assay conditions. Four compounds showed a significant effect (>15% difference from control) in tubulin assembly (Fig. 2A), and three of them, designated taxane 3 (taxinine M), 6, and 7, had promotional effect, taxane 14 inhibited tubulin assembly. Further evaluation of taxanes 3, 6, and 7 revealed that they promoted tubulin assembly in a dose-dependent manner (Fig. 2B–D).

Dark-field microscopy and cryo-EM

Figure 3A shows the structures of the microtubules polymerized in the absence and the presence of taxane compounds in dark-field microscopy. Filamentous structures were formed in the absence of the taxane compounds. The number of microtubules formed in the presence of taxanes 3, 6, and 7 was higher. On the other hand, no filamentous structures were observed in the tubulin containing taxane 14. Next, we used cryo-EM to analyze microtubule structure at high resolution (Fig. 3B). No structural differences were apparent between the microtubule polymerized in the absence of taxane compounds and that polymerized in the presence of taxane compounds. On the other hand, granular precipitation was observed in the microtubule polymerized in taxane 14.

Inhibition of taxane 14

Taxane 14 (Fig. 4A) inhibited tubulin assembly in a dose-dependent manner (Fig. 4B). The half-maximal inhibitory concentration (IC₅₀) against tubulin assembly (Δfluorescence intensity per min) was 95 μM. The inhibition mechanism of taxane 14 was compared with those of other inhibitors of tubulin assembly by detection of the depolymerization pattern after the addition of the compound during the polymerization phase (Fig. 4C). In this evaluation method, the addition of paclitaxel accelerates tubulin assembly (supplemental Fig. S2B). Nocodazole and CaCl₂ inhibit tubulin assembly by distinct mechanisms; nocodazole inhibits tubulin association, while CaCl₂ induces tubulin depolymerization. Taxane 14 stalled tubulin assembly without eliminating fluorescence. This fluorescence pattern was similar to that of nocodazole. The concentration of taxane 14 used in this experiment was high (400 μM), and hence one should take into consideration the possibility that non-specific interaction between taxane 14 and tubulin affects tubulin assembly. However, we concluded that thaxane 14 specifically inhibited tubulin assembly, because taxane 15, with a structure similar to structure that of 14, showed no effect on tubulin assembly (Fig. 4) even at a high concentration (400 μM).

Discussion

Promotion activity for tubulin assembly without the C-2 and C-13 side chains

Paclitaxel has a hydrophobic benzoyl group at its C-2 and C-13 side chains (Fig. 1), and these side chains are
Fig. 2. Time Course of the Tubulin Assembly, Monitored by DAPI Fluorescence ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 465$ nm). Polymerization contained 15 $\mu$M tubulin, 12 $\mu$M DAPI, and 200 $\mu$g/mL of taxane compounds in PM buffer. The temperature was 37°C. (A) Evaluation of the taxane compounds. The results for 10 of the 18 screened compound are shown. Dose-dependent enhancement of the polymerization activities of taxane 3 (B), 6 (C), and 7 (D).

Fig. 3. Images of the Tubulin Assembly in the Absence and Presence of the Taxane Compounds (final concentration 200 $\mu$g/mL of each). (A) Dark-field microscopy. Scale bars, 10 $\mu$m. (B) Cryo-EM images. Scale bars, 0.5 $\mu$m.

Fig. 4. Inhibition of Taxane 14. (A) Structures of taxanes 14, 15, and 16. (B) Dose-dependent inhibition of taxane 14 in tubulin assembly. The reactions were performed in the presence of 0, 40, 100, 200, 300, and 400 $\mu$M taxane 14. (C) Effects of the compounds on tubulin assembly. Taxanes 14 and 15 (final concentration 400 $\mu$M), nocodazole (1 $\mu$M), or CaCl$_2$ (10 mM) were added at the indicated times.
thought to be essential for stabilization of the microtubules.\textsuperscript{12,13} All 18 taxane compounds used in this study lack such hydrophobic substitutions (Supplemental Fig. S3). Three taxane compounds, 3, 6, and 7, were found to promote tubulin assembly (Figs. 2 and 3). These findings strongly support the hypothesis of Chatterjee and coworkers that the C-13 side chain is not an absolute requirement for the taxane molecule.\textsuperscript{14} Furthermore, our findings on taxanes 3, 6, and 7 also suggest that the C-2 benzoyl group is not indispensable for a promotional effect on tubulin assembly.

**Taxane 14 inhibited assembly of the tubulin dimer**

Fluorescence-based screening assay detected taxane 14 as an inhibitor of tubulin assembly, whereas the other taxane compounds showed promotional effects (Fig. 2). The depolymerization pattern after the addition of taxane 14 revealed that it inhibited tubulin assembly (Fig. 4C), indicating that the binding of taxane 14 to tubulin interrupts the tubulin-tubulin interaction, as in nocodazole. Microscopy analysis revealed that no filamentous structure formed in the presence of taxane 14 (Fig. 3). Instead, a particle structure appeared, confirming that taxane 14 bound to the tubulin dimer to inhibit head-to-tail interaction.

Nocodazole and paclitaxel bind to different sites in the microtubule (Supplemental Fig. S1A). The structural similarity of taxane 14 and paclitaxel (Figs. 1A and 4A) led us to expect that taxane 14 binds to tubulin in a form similar to paclitaxel. Indeed, when taxane 14 was placed on the paclitaxel-binding site of the B-tubulin structure, the acetyl group at the C-13 side chain and the acetal ring at the C-2 position did not constitute a steric hindrance to tubulin (Supplemental Fig. S1C). Nocodazole and taxane 14 caused similar inhibition in tubulin assembly, although they bind to distinct sites in the microtubule.

**Structure-activity relationship of taxane 14**

Of the 18 compounds used in this study, taxanes 15 and 16 possessed structures that are similar to that of taxane 14, but 15 and 16 showed no effect on tubulin assembly (Figs. 2 and 4). Compared to the structure of taxane 14, the C-13 side chain of taxane 15 was deacetylated, and the acetal ring at the C-2 side chain of 16 was cleaved. These differences indicate that both acetyl groups at the C-13 side chain and the acetal ring are required for the inhibitory effect of taxane 14.

**Conclusion**

Using a fluorescence-based assay system with some modifications, we obtained compounds that can regulate tubulin association. These compounds have not been evaluated, because they do not have a C-13 side chain that are thought to be indispensable for biological activity.\textsuperscript{12,13} Certainly, the compounds of taxanes 3, 6, 7, and 14 might not be potent for practical use, but the novel function of 14 might have the potential to reveal novel structure-activity relationships.

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**References**