NOTES

Effects of Tryprostatin Derivatives on Microtubule Assembly *In Vitro* and *In Situ*

MASUO KONDOH[a,b,†], TAKEO USUI[a], TADANORI MAYUMI[b] and HIROYUKI OSADA[a,*]

[a]Laboratory of Antibiotics, The Institute of Physical and Chemical Research (RIKEN), 2-1 Wako-shi, Saitama 351-0198, Japan
[b]Faculty of Pharmaceutical Science, Osaka University, 1-6 Yamada-oka, Suita, Osaka, 565-0871, Japan

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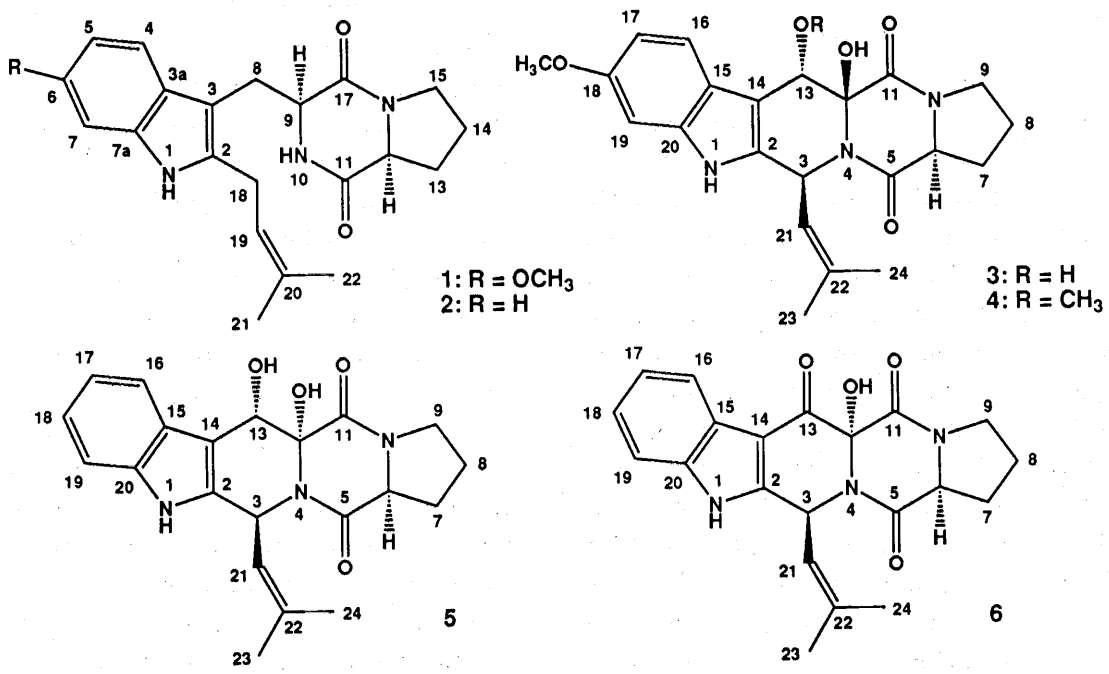
Microtubules are key components of the cytoskeleton of eukaryotic cells and play important roles in many aspects of biological functions. Development of specific inhibitors of microtubule assembly and disassembly is useful for the investigation of the biological function of microtubules and for cancer therapy.[1,2] Antimitotic drugs are classified into two groups based on the mode of action; one is destabilizers of microtubules such as colchicine,[3] *vinca* alkaloids,[4] rhizoxin,[5] and dolastatins,[6] and the other is stabilizers of microtubules such as taxol[7] and epothilones.[8] Each lead compound has led to various naturally occurring or synthesized derivatives intended to improve their biological activity in clinical use.[4,9~11]

We have reported that tryprostatin A (**1**), isolated from *Aspergillus fumigatus* BM 939, is a novel inhibitor of cell cycle progression at M phase and specifically inhibits tau or MAP2-dependent microtubule assembly.[12,13] The producer strain also yielded several derivatives which were classified into three types based on their fundamental structures (Fig. 1); tryprostatins (TPS), cyclotryprostatins (cTPS) and spirotryprostatins (sTPS).[14,15] TPS-A and -B (**1** and **2**) are the main products of the strain: **1** is a 6-methoxy derivative of **2** and the presence of methoxy group reduces the cytotoxicity.[13] Cyclotryprostatin-A, -B, -C, and -D (**3**~**6**) are minor products. All of these compounds inhibited G2/M progression in G2/M synchronous culture of tsFT210 cells,[12,14~16] but little is known about the structure-activity relationships of these compounds. Although the structures of sTPSs are unique, their biological effects were weaker than those of other derivatives.[14] In this note, we report the effects

Fig. 1. Structure of tryprostatin derivatives.



† Present address: Faculty of Pharmaceutical Science, Tokushima Bunri University, 180 Yamashiro-cho, Tokushima, 770-8154, Japan.

Table 1. Effects on cell cycle progression *in situ* and microtubule assembly *in vitro*.

Compound	C-6/C-18	DNA distribution (%)[a]			microtubule assembly
		2C	2-4C	4C	*in vitro* (%)[b]
control	-	67.5	13.1	14.3	-
colchicine	-	11.4	19.5	50.5	43.6 ± 0.7
1	OCH_3	12.0	24.8	55.2	57.9 ± 0.3
2	H		N.D.[c]		33.4 ± 5.0
3	OCH_3	14.4	17.1	49.1	66.4 ± 1.3
4	OCH_3	10.3	19.8	52.0	66.0 ± 0.0
5	H		N.D.		51.1 ± 3.8
6	H		N.D.		137.0 ± 1.4

[a] The exponentially growing 3Y1 cells were treated with 1 μM colchicine or 250 μM compounds. After 24 hours treatment, DNA contents were quantified by flow cytometry.
[b] Percentage activity of *in vitro* microtubule assembly with 6 μM colchicine or 250 μM compounds. Average of two values, with the deviations from the averages presented.
[c] N.D.: Not determined due to the cytotoxicity.

of TPSs and cTPSs on microtubule assembly *in vitro* and *in situ*.

Results and Discussion

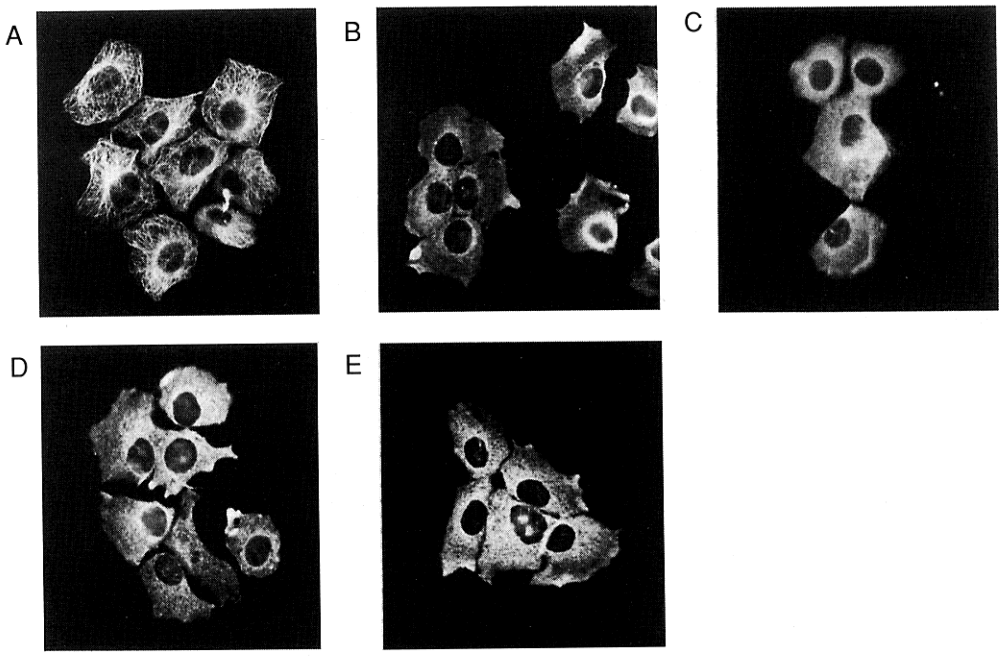
As the primary target of **1** is microtubules,[13] we examined the effect of tryprostatin derivatives on the cell cycle progression and cytoplasmic microtubule network in rat normal fibroblast 3Y1 cells. Colchicine, a potent inhibitor of microtubule assembly, induced M-phase specific inhibition and microtubule disassembly (Table 1, Fig. 2). M-phase specific inhibition and microtubule disassembly were also induced by **1**, **3** and **4** that have a methoxy moiety on the indole ring. In contrast, **2**, **5** and **6**, that lack a methoxy moiety on the indole ring, were highly toxic at 250 μM, and all the cells treated with the demethoxy compounds were rounded up quickly after compound addition (data not shown). Each demethoxy compound was more cytotoxic than its methoxy compound. Compound **2** induced cell round up even at 100-folds lower concentrations than **1** (IC_{50} measured by MTT assay was about 400 μM for **1** and about 4 μM for **2**). These results suggest that the presence of methoxy moiety in the indole ring decreased cytotoxicity but enhanced the specificity against microtubule disruptive activities in TPSs and cTPSs derivatives.

Next, we investigated the effects of tryprostatin derivatives on microtubule assembly *in vitro* by turbidity assay using purified microtubule proteins at a concentration of 2.0 mg/ml. Derivatives **1**~**5** inhibited microtubule assembly in the range of 33.4~66.4% of control at 250 μM (Table 1). These results suggest that methoxy moiety on the indole ring does not affect microtubule assembly *in vitro*. On the contrary, **6** promoted the assembly up to 137.0% of control at the same concentration. It is interesting that **5** and **6** have similar structures except C-13 position (Fig. 1) but showed opposite effects on the *in vitro* microtubule assembly.

We previously reported that **1** inhibited MAPs-dependent microtubule assembly and that its target molecule was thought to be either MAP-2/tau binding site on tubulin or the tubulin binding site on microtubule associated proteins.[13] These results lead us to the hypothesis that TPS derivatives act as antagonists or agonists of tubulin assembly. TPS derivatives might interact with the carboxyl terminal domain of tubulin

Fig. 2. Depolymerization of microtubule network by tryprostatin derivatives *in situ*.

3Y1 cells were incubated with 1 μM colchicine (B) or 250 μM tryprostatin derivatives for 6 hours, and were observed under a fluorescence microscopy. Control cells without compounds (A). TPS-A (C), cTPS-A (D) and cTPS-B (E) treated cells, respectively.



and act as antagonist of MAPs in cases of **2** and **5**, however, **6** might interact with the tubulin and act as an agonist of MAPs. Therefore **6** promoted the microtubule assembly. Although there are many microtubule binders such as colchicine,[3] *vinca* alkaloids,[4] rhizoxin,[5] taxol[7] and epothilones,[8] these compounds show only one directional effect, disassembly or stabilization of microtubule. For example, taxol and its derivatives stabilize microtubule network and do not induce microtubule disassembly. On the contrary, TPS derivatives except for **6** inhibited microtubule polymerization, and **6** promoted microtubule assembly. These properties suggest that TPS derivatives will be a new type of lead compounds for antimitotic and antitumor drugs.

Materials and Methods

All tryprostatin derivatives were purified as described previously[14,15]. Colcemid and other reagents were purchased from Sigma Company. Compounds were dissolved in dimethyl sulfoxide. Rat normal fibroblast 3Y1 cells were grown in Dulbecco's modified MEM medium supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2. Immunofluorescence observation, cell cycle analysis, microtubule preparation and turbidity assay of microtubule assembly were preformed as described previously[13].

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