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ANTITUBULIN ACTIVITIES OF ANSAMITOCINS AND MAYTANSINOIDS

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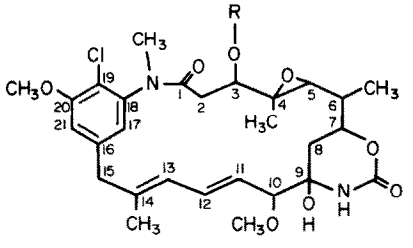
Abstract—Antitubulin activities of ansamitocins, maytansine and four maytansinoids which are structurally related to ansamitocins were studied using three reaction systems; inhibition of polymerization of bovine brain tubulin, depolymerization of the once polymerized tubulin, and immunofluorescent staining of cytoplasmic microtubules in A31 cells.

Ansamitocin P-3, ansamitocin P-4, maytansine, D-maytansine, maytanacine and maytansinol 3-propionate inhibited the polymerization of tubulin and depolymerized the once polymerized tubulin. The concentrations of these compounds causing 50 per cent inhibition of polymerization and 50 per cent depolymerization were around 3.4 and 3.8×10^{-6} M, respectively. Maytansinol also inhibited polymerization of tubulin and depolymerized the once polymerized tubulin. However, maytansinol was about four times less effective in polymerization inhibition and ten times less effective in depolymerization than other compounds.

Except for maytansinol and D-maytansine, these compounds caused a disappearance of fibers of cytoplasmic microtubules in A31 cells at a concentration of $1–6 \times 10^{-8}$ M. The concentration of D-maytansine causing the disappearance of the fibers was about 50 times higher than that of maytansine. Maytansinol did not cause the disappearance of the fibers even at such a high concentration as 4.6×10^{-6} M. These results suggest that the ester moiety at the C-3 position of ansamitocins, maytansine and maytansinoids plays an important role in increasing their permeation into living cells.

Recently, ansamitocins, a group of antibiotics isolated from a fermentation broth of *Nocardia* sp. No. C-15003, were found to have antitumor, antimitotic and antitubulin activities [1, 2]. The chemical structures of ansamitocins are very similar to that of maytansine, an antitumor agent, of plant origin isolated from *Maytenus* species [3–7]. Ansamitocins, maytansine and other four maytansinoids structurally differ from each other only in the ester moiety at the C-3 position (Fig. 1). The antitumor activity of ansamitocins and maytansine is understood to result from the effect on microtubules in cells. On the other hand, Kupchan *et al.* [7] reported recently that maytansinol did not show any *in vivo* antitumor activity, whereas that it showed the polymerization inhibitory activity up to about 30 per cent of that of maytansine. Tanida *et al.* [8] reported that ansamitocin P-3, ansamitocin P-4 and maytansine showed a strong inhibitory activity against cilia regeneration in *Tetrahymena*, but that maytansinol showed only a weak activity. Recently, D-maytansine, obtained in the course of chemical synthesis of maytansine from ansamitocins through maytansinol, was found to show a weak antitumor activity *in vivo* [9, 10].



Maytansinol	R=H
Maytanacine	R=$COCH_3$
Maytansinol 3-propionate	R=$COCH_2CH_3$
Ansamitocin P-3	R=$COCH(CH_3)_2$
Ansamitocin P-4	R=$COCH_2CH(CH_3)_2$
Maytansine	R=$COCH(CH_3)N(CH_3)COCH_3$

Fig. 1. Chemical structures of ansamitocins and maytansinoid.

In order to find the relationship between the structure and biological activity of ansamitocins and other maytansinoids *in vitro* and *in vivo*, we have studied, in detail, the antitubulin activities of ansamitocins, maytansine and other maytansinoids. In the present study, we compared the antitubulin activities of ansamitocin P-3, ansamitocin P-4, maytansine and four maytansinoids using three *in vitro* assay systems, and found that the ester moiety at the C-3 position of ansamitocins and maytansine is essential for antitubulin activity at cellular levels.

MATERIALS AND METHODS

Chemicals. Ansamitocin P-3, ansamitocin P-4, maytansinol, maytanacine and maytansinol 3-propionate were isolated from fermentation broths [11]. Maytansine [(C-3)maytansinol ester of *N*-acetyl-*N*-methyl-L-alanine] and D-maytansine [(C-3)-maytansinol ester of *N*-acetyl-*N*-methyl-D-alanine] were synthesized chemically from ansamitocins [9]. All these chemicals were supplied from the Microbiological Research Laboratories and the Chemical Research Laboratories of our Central Research

Division. These compounds were dissolved in either ethanol or dimethyl sulfoxide, and the solution was diluted with the assay buffer or the culture medium before use. Vincristine sulfate was purchased from Shionogi Pharmaceutical Co., Osaka, Japan.

Cells. A31 cells (a clone of BALB/3T3 cells) were a kind gift from Dr. A. Hakura (Osaka University, Osaka, Japan). The cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10 per cent fetal bovine serum (Flow Laboratories, Inc., Rockville, MD.) and 100 μg of kanamycin (Takeda Chemical Industries, Ltd., Osaka, Japan).

Preparation of tubulin. Tubulin was prepared from bovine and porcine brains by the polymerization–depolymerization technique according to the methods of Shelanski *et al.* [12] and Weingarten *et al.* [13] with a slight modification [2]. Immediately before use, the tubulin was further purified from the stock solution by a repeated cycle of the polymerization–depolymerization, and diluted with the MEG buffer [100 mM 2-*N*-morpholinoethanesulfonic acid (MES), 1 mM ethylene glycol-bis(β-amino-ethyl-ether)-*N*,*N*′-tetraacetic acid (EGTA), 0.5 mM $MgCl_2$ and 1 mM GTP; pH 6.4] to a concentration of 1.25 mg/ml.

Preparation of anti-tubulin IgG. Rabbit anti-porcine tubulin IgG was prepared by the methods as described elsewhere [2].

Immunofluorescent staining. A31 cells (3×10^4) were seeded on a Linbro plastic plate (FB16-24TC) containing a sterilized glass coverslip (14 mm in dia) with MEM supplemented with 10 per cent fetal bovine serum. The culture was maintained at 37° in a humidified incubator under 5 per cent CO_2 in air. After 1 day, the culture medium was changed to a fresh medium containing a compound to be tested, and the cells were incubated at 37° for a further 1 hr. The cells on a cover slip were fixed with 3.7 per cent paraformaldehyde in phosphate buffered saline, pH 7.2 (PBS) and stained with the anti-tubulin IgG and fluorescein-conjugated goat anti-rabbit IgG antibody by the method of Weber *et al.* [14]. The cells were viewed with a Zeiss fluorescence microscope equipped with a vertical fluorescent illuminator and a planapo 40 objective.

Assay of polymerization inhibition. A tubulin solution (400 μl) containing 0.5 mg of bovine brain tubulin was mixed with 100 μl of MEG buffer containing a compound to be tested. The mixture was precooled on ice for 10 min, and then warmed at 37° for 30 min. The degree of polymerization of tubulin was determined by measuring the increase of absorbance at 460 nm [15] in a Hitachi model 101 spectrophotometer.

Assay of depolymerization. A tubulin solution (400 μl) containing 0.5 mg of bovine brain tubulin, which had been polymerized by warming the solution at 37° for 30 min, was mixed with 100 μl of the MEG buffer containing a compound to be tested. The mixture was incubated at 37° for 15 min. The degree of depolymerization of tubulin was determined by measuring the decrease of absorbance at 460 nm as described above.

Protein determination. Concentration of tubulin was determined by the method of Lowry *et al.* [16] with bovine serum albumin as standard.

RESULTS

Inhibition of polymerization of bovine brain tubulin

As shown in Fig. 2, ansamitocin P-3, ansamitocin P-4, maytansine, D-maytansine and maytanacine inhibited polymerization of bovine brain tubulin to almost the same degree, and the degree of inhibition was dependent on their concentrations. Maytansinol also showed a weak but significant polymerization inhibitory activity at higher concentrations. The concentrations of these compounds causing 50 per cent inhibition of the polymerization of tubulin are summarized in Table 1.

In the present study, the purification of tubulin

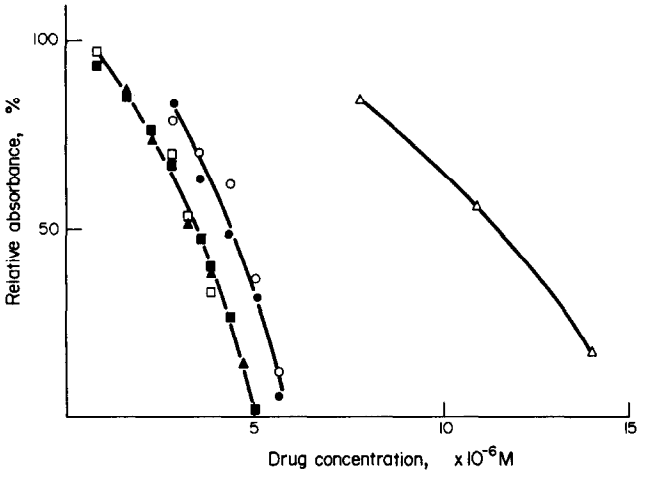


Fig. 2. Effect of ansamitocins and maytansinoids on polymerization of bovine brain tubulin. A solution (400 μl) containing 0.5 mg of bovine brain tubulin was mixed with 100 μl of the assay buffer containing a compound to be tested, and the mixture was incubated at 37° for 30 min. Maytanacine (▲); ansamitocin P-3 (■); ansamitocin P-4 (□); maytansine (●); D-maytansine (○); maytansinol (△).

Table 1. Antitubulin activities of ansamitocins, maytansinoids and vincristine

Compound	Effective concentration* in		
	Polymerization inhibition†	Depolymerization‡	Alteration of microtubules in A31 cells§
Maytansinol	12×10^{-6} M	43×10^{-6} M	$>460 \times 10^{-8}$ M
Maytanacine	3.4	3.8	6.4
Maytansinol 3-propionate	3.4	3.8	1.6
Ansamitocin P-3	3.4	3.8	1.6
Ansamitocin P-4	3.4	3.8	1.6
Maytansine	3.8	3.5	1.4
D-Maytansine	4.2	5.1	72
Vincristine	0.8	—‖	11

* Summary of two to five independent experiments.
† Concentration causing 50 per cent inhibition of bovine brain tubulin polymerization.
‡ Concentration causing 50 per cent depolymerization of the once polymerized tubulin.
§ Concentration causing disappearance of the fibers of cytoplasmic microtubules in A31 cells.
‖ Not determined, because vincristine caused a slight depolymerization followed by an aggregation [2].

was carried out according to the methods of Shelanski *et al.* [12] and Weigarten *et al.* [13]. Their reactivities were consistent with those previously reported by other workers [12, 13]. *Vinca* alkaloids inhibit the polymerization of brain tubulin, but their effective concentrations reported by several workers [6, 17, 18] are considerably different from each other. Bhattacharyya and Wolff [6] reported that the concentration of maytansine to induce 50 per cent inhibition of polymerization of 2.1 mg/ml of rat brain tubulin was 0.35×10^{-6} M. This value seems to be low compared with our present results (3.8×10^{-6} M with 1 mg/ml of bovine brain tubulin, Table 1), but the relative activities of *Vinca* alkaloids to maytansine, presented in this paper, are in good agreement with those reported by Bhattacharyya and Wolff [6]. Furthermore, the relative activities of maytansinol, maytanacine and maytansinol 3-propionate to maytansine obtained in the present paper are consistent with those reported by Kupchan *et al.* [7]. No significant difference in polymerization inhibitory activity of ansamitocin P-3 or maytansine was found even when several tubulin preparations from brains of different sources, such as bovine, rat and guinea pig were used (data not shown). Therefore, the discrepancy of the effective values obtained by different workers may be regarded to be due to the difference in experimental conditions used.

Depolymerization of bovine brain tubulin

As shown in Fig. 3, ansamitocin P-3, ansamitocin P-4, maytansine and maytanacine depolymerized the once polymerized bovine brain tubulin. The concentrations of these compounds causing the depolymerization by 50 per cent were calculated to be around 3.8×10^{-6} M (Table 1). D-Maytansine showed a slightly lower activity than maytansine. The depolymerizing effect of maytansinol was considerably lower than that of ansamitocin P-3 (Fig. 3, Table 1).

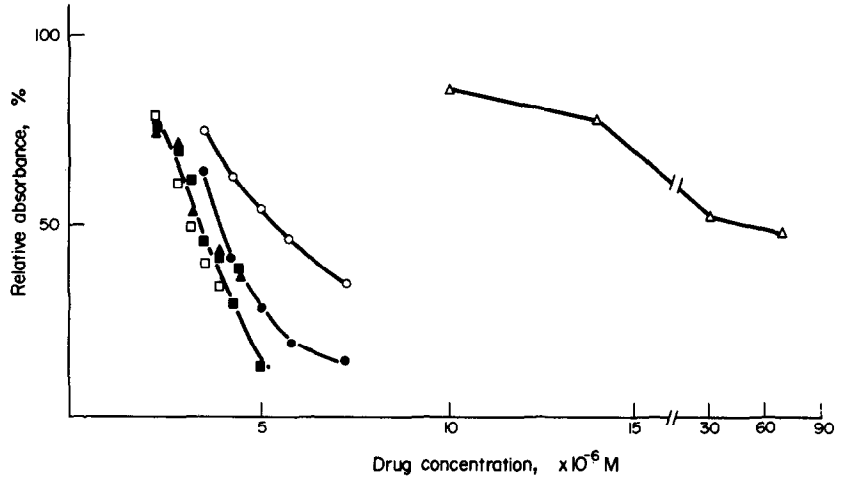


Fig. 3. Effect of ansamitocins and maytansinoids on depolymerization of bovine brain tubulin. A solution (400 μl) containing 0.5 mg of bovine brain tubulin, previously polymerized by incubation at 37° for 30 min, was mixed with 100 μl of the assay buffer containing a compound to be tested, and the mixture was incubated at 37° for 15 min. The symbols were same as those described in legend to Fig. 2.

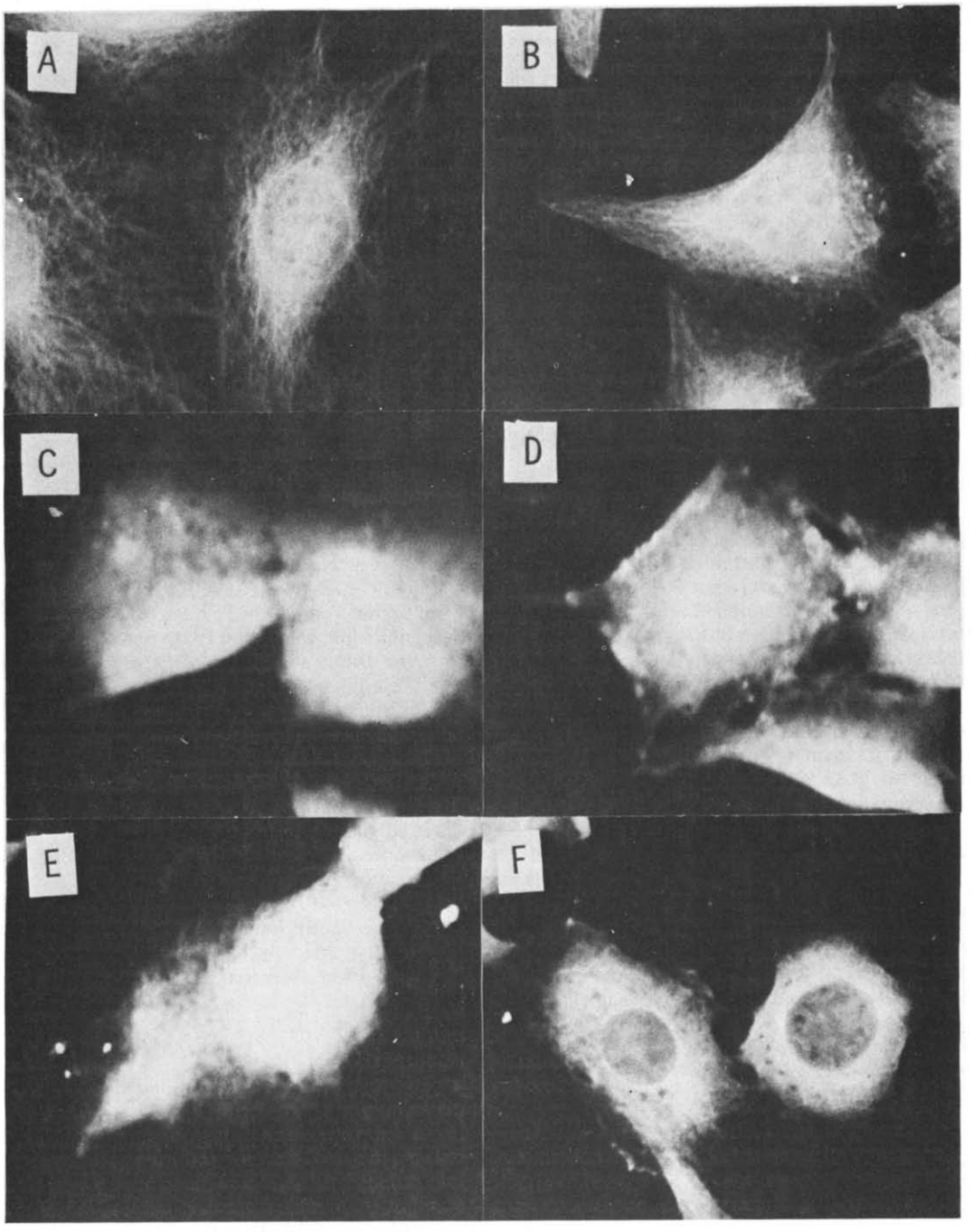


Fig. 4. Effect of ansamitocins, maytansinoids and vincristine on organization of cytoplasmic microtubules in A31 cells. A31 cells grown on a coverslip were incubated with a compound to be tested at 37° for 1 hr, fixed with 3.7 per cent formaldehyde and processed for immunofluorescent staining. (A) Control, (B) 4.6×10^{-6} M maytansinol, (C) 1.6×10^{-8} M ansamitocin P-3, (D) 1.4×10^{-8} M maytansine, (E) 7.2×10^{-7} M D-maytansine, (F) 1.1×10^{-7} M vincristine. Magnification ×600.

Decrease and unclearness of cytoplasmic microtubular fibers in A31 cells

As described above, ansamitocin P-3, ansamitocin P-4, maytansine and maytansinoids clearly inhibited the polymerization of brain tubulin and caused the depolymerization of the once polymerized tubulin *in vitro*. Therefore, we examined the effects of these compounds on the immunofluorescent staining of cytoplasmic microtubules in A31 cells. As shown in Fig. 4(A), finely well-spread fibers were seen in the cytoplasm of the cells by staining with the anti-tubulin antibody and fluorescein-conjugated goat anti-rabbit IgG antibody. These fibers were reduced and obscured by treating the cells either at low temper-

ature (0°) or with colchicine (1 μg/ml, 37° for 1 hr) (data not shown).

On treatment of A31 cells with ansamitocin P-3 [Fig. 4(C)], ansamitocin P-4, maytansine [Fig.4(D)], maytanacine, maytansinol 3-propionate or vincristine [Fig. 4(F)], the fibers of cytoplasmic microtubules in A31 cells were also reduced and obscured. Effective concentrations of these compounds causing disappearance of the fibers were estimated to be $1–6 \times 10^{-8}$ M (summarized in Table 1).

As mentioned above, maytansinol and D-maytansine inhibited the polymerization of tubulin, whereas they showed only a weak antitubulin activity as judged by obscuring the fibers of cytoplasmic microtubules in A31 cells [Fig. 4(B) and (E); Table 1]. Maytansinol did not cause the disappearance even at a high concentration of about 4.6×10^{-6} M.

DISCUSSION

The present study has shown the following lines of new evidence on antitubulin activities of ansamitocins and their related compounds.

(1) Ansamitocin P-3, ansamitocin P-4, maytansine, maytanacine and maytansinol 3-propionate inhibited the polymerization of brain tubulin and depolymerized the once polymerized tubulin. No significant difference in concentration was observed between the polymerization inhibition and the depolymerization for all of these compounds, except for maytansinol (Table 1). Ansamitocin P-3, ansamitocin P-4, maytansine, maytanacine and maytansinol 3-propionate also caused the disappearance of fibers of cytoplasmic microtubules in A31 cells.

(2) The activity of maytansinol was very weak in obscuring the fibers of cytoplasmic microtubules (less than 1 per cent to that of ansamitocin P-3; Table 1). By contrast, the polymerization inhibitory and depolymerizing activities of maytansinol were significant: they were about 30 and 10 per cent of those for ansamitocin P-3, respectively (Table 1). These results indicate that the ester moiety at the C-3 position of these compounds is not important for the manifestation of the antitubulin activity *in vitro* but essential for antitubulin activity at cellular levels.

(3) D-Maytansine showed nearly the same polymerization inhibitory activity as that of maytansine, whereas the antitubulin activity at cellular levels measured by the degree of obscuring the fibers of cytoplasmic microtubules was markedly low (Table 1). These results strongly suggest that the ester moiety at C-3 position of maytansine is necessary for the permeation of these compounds through the cell membrane.

We estimated that ansamitocins and maytansine might act by interfering with the microtubule assembly system to result in an inhibition of the formation of mitotic spindle fiber and, ultimately, in cytokilling [2]. D-Maytansine and maytansinol inhibited the polymerization of tubulin *in vitro* (Table 1), whereas they showed a weak antitumor activity *in vivo* [9, 10]. Although Kupchan *et al.* [7] reported that maytansinol had a weak inhibitory activity against the cleavage of sea urchin eggs, they did not refer to the direct effect of maytansinol on the microtubular system in cells. In this study, we showed that D-maytansine and maytansinol required high concentrations for obscuring the fibers of cytoplasmic microtubules in A31 cells. Therefore, these results suggest that the weak antitumor activity of D-maytansine and maytansinol is due to their low permeation into living cells. Thus, the present study established the necessity of the ester moiety at the C-3 position of ansamitocins and maytansine. Antitubulin activities of other maytansinoids are now under study in order to reveal the binding site of ansamitocins and maytansine to tubulin.

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