

# Ceratamines, Structurally Simple Microtubule-Stabilizing Antimitotic Agents with Unusual Cellular Effects

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## Abstract

**Ceratamine A and ceratamine B are heterocyclic alkaloids recently identified in a screen for compounds that arrest cells in mitosis. Treatment of breast carcinoma MCF-7 cells causes a concentration-dependent block of cell cycle progression exclusively at mitosis. *In vitro* studies with purified tubulin indicate that the ceratamines directly stimulate microtubule polymerization in the absence of microtubule-associated proteins. Cells treated with ceratamines show a dense perinuclear microtubule network in interphase and multiple pillar-like tubulin structures in mitotic cells. The ceratamines do not compete with paclitaxel for binding to microtubules *in vitro*. Unlike other microtubule-stabilizing agents, the ceratamines have simple structures with no chiral centers, making them attractive drug leads.** (Cancer Res 2005; 65(8): 3040-3)

## Introduction

The clinical utility of the taxanes and *Vinca* alkaloids in cancer therapy has stimulated an intensive search for new microtubule-targeting antimitotic agents (1). A large number of structurally distinct agents have been described that, like the *Vinca* alkaloids, inhibit microtubule polymerization (2). However, only a small number of distinct chemotypes have been discovered that, like the taxanes, stimulate microtubule polymerization. Significantly, most microtubule-stabilizing agents are structurally very complex, creating considerable challenges to their preclinical and clinical development. Using a phenotypic cell-based assay for agents that block cells in mitosis, we have screened extracts from marine invertebrates and recently isolated the novel marine sponge heterocyclic alkaloids ceratamines A and B (3). This paper describes their identification as microtubule-stabilizing agents with unusually simple chemical structures and with effects on interphase and mitotic microtubules that are distinct from those of taxanes and other antimitotics described to date.

## Materials and Methods

***In vitro* stimulation of tubulin polymerization.** On ice, 35  $\mu$ L buffer A [80 mmol/L PIPES (pH 6.8), 1 mmol/L  $MgCl_2$ , 1 mmol/L EGTA] were added to wells in a 96-well plate, followed by 5  $\mu$ L of 10 mg/mL bovine brain tubulin (>99% pure, TL238, Cytoskeleton, Inc., Denver, CO) in buffer A containing 10% glycerol, 5  $\mu$ L of a 10  $\times$  solution of ceratamine A or B in buffer A, and 5  $\mu$ L of 10 mmol/L GTP in buffer A. The absorbance at 340 nm was followed at 32°C over time.

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***In vitro* inhibition of tubulin polymerization.** This assay was done as above with the following differences. On ice, 25  $\mu$ L buffer A plus 10% glycerol were added to wells of a 96-well plate, followed by 15  $\mu$ L of 10 mg/mL tubulin in buffer A containing 10% glycerol, 5  $\mu$ L of 10  $\times$  ceratamine A or B solution in buffer A containing 10% glycerol, and 5  $\mu$ L of 10 mmol/L GTP in buffer A containing 10% glycerol. The absorbance at 340 nm was followed at 37°C over time.

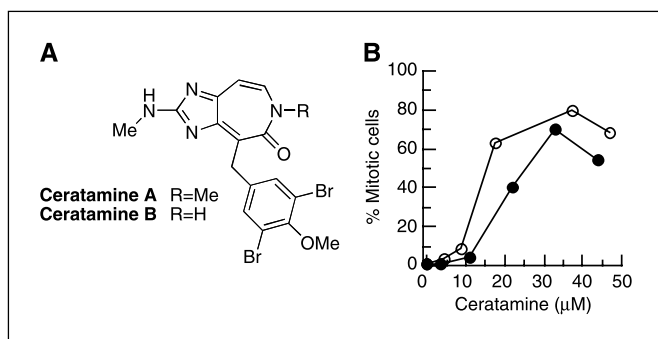
**Negative staining electron microscopy.** A 10  $\mu$ L sample of microtubules assembled in the presence of ceratamine A was placed on a Formvar-coated copper grid and left for 1 minute at room temperature. The fluid was wicked off by Whatman filter paper and 10  $\mu$ L of 2% uranyl acetate solution were added and immediately removed by Whatman filter paper wicking. The sample was examined by electron microscopy using a Hitachi H7600 transmission electron microscope.

**Three-dimensional confocal microscopy.** Cells cultured on glass coverslips were treated with or without ceratamine and their microtubules were immunostained using the E7 monoclonal  $\beta$ -tubulin antibody. Stacks of 0.35- $\mu$ m-thick optical sections were collected using a Bio-Rad radiance plus confocal microscope (Bio-Rad, Hercules, CA) and analyzed using ImageJ software (NIH, Bethesda, MD).

## Results and Discussion

The chemical structures of ceratamine A and B are shown in Fig. 1A. Exposure of MCF-7 breast carcinoma cells (4) to ceratamine A or B for 16 hours caused a concentration-dependent accumulation of cells with condensed mitotic chromosomes as determined by microscopy (Fig. 1B). The effect of ceratamines on cell cycle progression was investigated in more detail using flow cytometry (5). Cells treated with 0.1% DMSO (drug solvent) showed a profile typical of asynchronously proliferating cells, with large populations of cells in  $G_1$ , S, and  $G_2$  and a small M cell population (Fig. 2). Exposure to ceratamine A or B for 16 hours caused an almost complete disappearance of the  $G_1$  and S peaks with a correspondingly large increase in the  $G_2$ -M peak (Fig. 2B, left). Staining with the GF-7 antibody to distinguish between  $G_2$  and M (5) showed the arrest to be at M and not  $G_2$  (Fig. 2, right) as for the known microtubule-targeting agent nocodazole (Fig. 2). Arrest of cell cycle progression exclusively at M shows that the ceratamines are bona fide antimitotic agents.

Most antimitotic agents target microtubules directly and either inhibit or stimulate microtubule polymerization *in vitro* (2). To investigate the mechanism of action of ceratamines, we first examined their effect on the polymerization of pure tubulin into microtubules *in vitro*. Purified brain tubulin (3 mg/mL) was preincubated with DMSO, nocodazole, or ceratamine A or B on ice and the temperature was raised to 37°C. Microtubules polymerized spontaneously in the presence of DMSO, whereas 10  $\mu$ mol/L nocodazole prevented the assembly of microtubules (Fig. 3A). However, the ceratamines did not inhibit tubulin polymerization at any concentration tested (up to 110  $\mu$ mol/L). We next determined whether the ceratamines can stimulate microtubule polymerization. Tubulin (1 mg/mL) was preincubated with DMSO, paclitaxel,



**Figure 1.** Antimitotic activity of ceratamines. *A*, structural formulas of ceratamine A and B. *B*, cells were exposed to different concentrations of ceratamine A (○) or B (●) for 16 hours and the percentage of mitotic cells was counted in the microscope after fixation and staining of DNA with Hoechst 33258.

or ceratamine A or B on ice and the temperature was raised to 37°C. At this low tubulin concentration, microtubules did not polymerize spontaneously but polymerization was stimulated by 10 μmol/L paclitaxel (Fig. 3B). Ceratamine A and B at a concentration of 100 μmol/L also promoted microtubule polymerization (Fig. 3B), but to a lesser extent than paclitaxel.

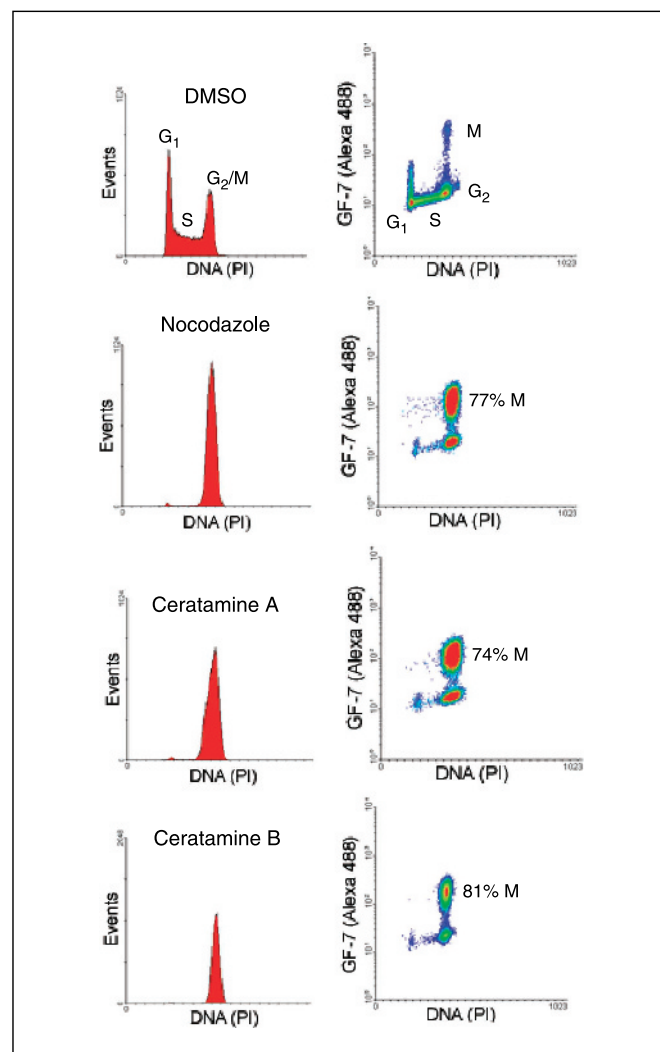
To determine whether the increase in light scattering measured in this assay was indeed due to the formation of microtubules, samples obtained at the end of the experiment described in Fig. 3B were examined by transmission electron microscopy. As shown in Fig. 3C for ceratamine A, the ceratamines indeed caused the formation of microtubules. No microtubules were observed in samples treated with DMSO only (not shown). These results show that the ceratamines belong to the microtubule-stabilizing class of antimitotic agents and that they target microtubules directly.

The taxanes, epothilones, eleutherisides, and discodermolide are microtubule-stabilizing agents that bind to the same site on β-tubulin, whereas the macrolides laulimalide and peloruside A do not compete with paclitaxel for binding to microtubules (2, 6, 7). To investigate whether the ceratamines and paclitaxel bind to microtubules competitively, we used an assay whereby microtubules assembled in the presence of [<sup>3</sup>H]paclitaxel (NSC-125973, 16.2 Ci/mmol, National Cancer Institute, Bethesda, MD) are exposed to other microtubule-stabilizing agents (8). Agents that compete for binding to the same site cause a release of radioactive paclitaxel. Microtubules were assembled using 2 μmol/L tubulin and 2 μmol/L [<sup>3</sup>H]paclitaxel. Addition of 4 μmol/L eleutherobin, a microtubule-stabilizing agent previously shown to bind to the paclitaxel site (8), caused the release of 29 ± 1% of the radiolabeled paclitaxel from the microtubules (Fig. 3D). By contrast, ceratamine A added at 20 or 50 μmol/L, the highest concentration that could be tested in this assay without compound precipitation, caused no statistically significant release of radiolabeled paclitaxel (Fig. 3D). These results indicate that ceratamine A either does not bind to the paclitaxel site or that it binds with an affinity too low to be detected with this assay.

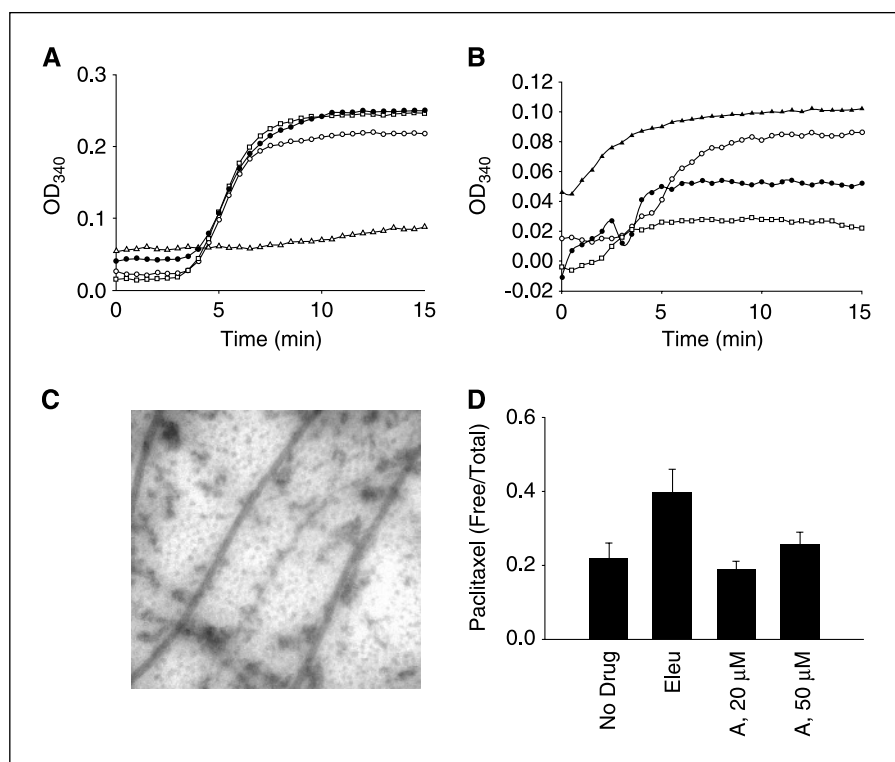
We next examined the effect of ceratamines on cellular microtubule morphology. Cells treated or not with 20 μmol/L ceratamine A for 5 hours were prepared for immunofluorescence microscopy using a β-tubulin antibody to visualize microtubules and Hoechst 33258 to stain DNA. Untreated interphase cells showed a well-defined microtubule network (Fig. 4A). The ceratamines caused noticeable changes in the interphase microtubule network. In most cells, a pronounced accumulation of

microtubules was observed that partially or completely encircled the nucleus (Fig. 4B). In the confocal microscope, the interphase microtubules of ceratamine-treated cells seemed shorter than controls and were disorganized (Fig. 4C and D). This microtubule network morphology was unlike the bundling of interphase microtubules typically caused by microtubule-stabilizing agents.

In untreated populations, cells at all stages of mitosis were observed (Fig. 4E and F shows a metaphase cell and an anaphase cell), with clearly defined mitotic spindles (Fig. 4E) and chromosomes at different stages of segregation (Fig. 4F). In cells treated with ceratamine A, all mitotic cells had condensed chromosomes that were not aligned at the equator (Fig. 4H) and no spindles or microtubules were visible (Fig. 4G). Rather, multiple foci of β-tubulin staining were dispersed throughout the cells (Fig. 4G and L). The effect of ceratamine A on mitotic microtubules was different from that typically observed with



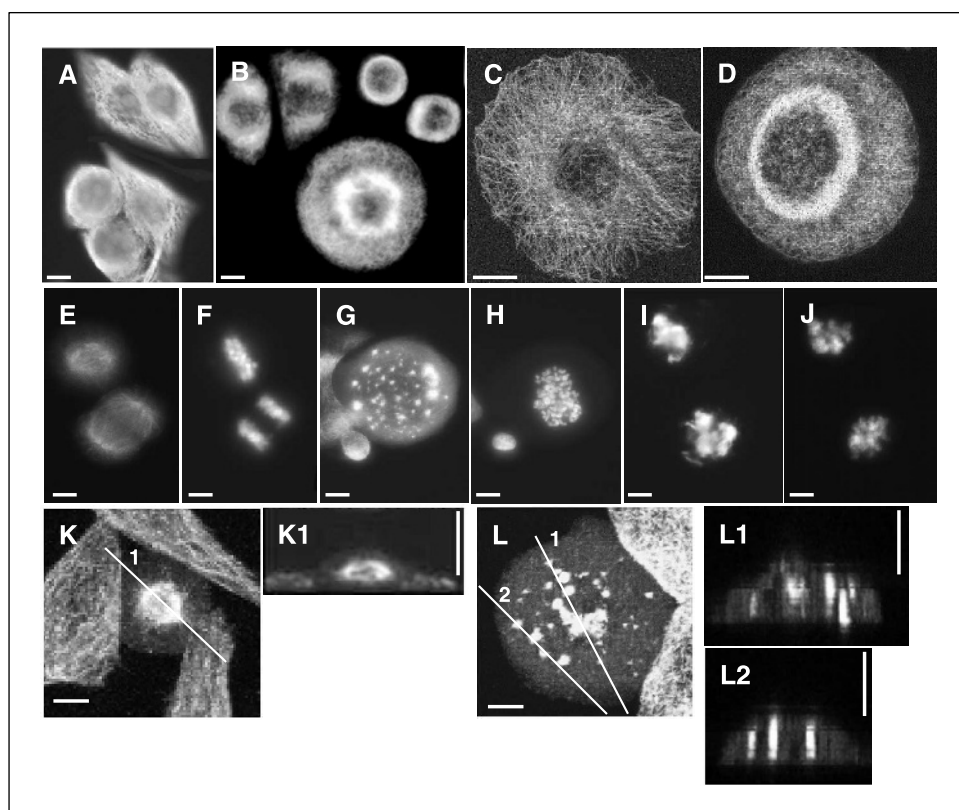
**Figure 2.** Effect of ceratamines on cell cycle progression measured by flow cytometry. Cells were treated for 16 hours with 0.1% DMSO (negative control), 1 μmol/L nocodazole (positive control), 20 μmol/L ceratamine A, or 23 μmol/L ceratamine B. *Left*, histograms of DNA (PI) profiles with the G<sub>1</sub>, S, and G<sub>2</sub>-M populations indicated in the top panel. *Right*, DNA was stained with propidium iodide (PI, X-axis), and mitotic cells were detected by Alexa 488 GF-7 immunofluorescence labeling (Y-axis). The number of events associated with a coordinate on the plot is indicated by the color scale: blue, low density; red, high density.



**Figure 3.** Stimulation of microtubule polymerization by ceratamines *in vitro*. Microtubule polymerization was carried out with 3 mg/mL tubulin (A) or 1 mg/mL tubulin (B) with DMSO ( $\square$ ) or with 10  $\mu$ M nocodazole ( $\triangle$ ), 10  $\mu$ M paclitaxel ( $\blacktriangle$ ), 100  $\mu$ M ceratamine A ( $\circ$ ), or 100  $\mu$ M ceratamine B ( $\bullet$ ). C, negative staining electron micrographs of microtubules induced by ceratamine A (magnification,  $\times 120,000$ ). D, paclitaxel binding competition assay. [ $^3$ H]paclitaxel-labeled microtubules were incubated without drug, with 4  $\mu$ M eleutherobin (*Eleu*), 20  $\mu$ M ceratamine A (A, 20  $\mu$ M/L), or 50  $\mu$ M ceratamine A (A, 50  $\mu$ M/L). The percent inhibition of paclitaxel binding was determined as described in Materials and Methods. Bars, SE with  $n \geq 4$ .

microtubule-stabilizing agents, such as paclitaxel, which cause the appearance of dense, "bushy" mitotic spindles and often extra-numerary spindles (Fig. 4I) with chromosomes that fail to align (Fig. 4J). The number of foci in cells arrested in M phase by

ceratamine varied from 3 to  $\sim 30$  per cell. Cells did not all contain foci of equal staining intensity, but when foci were few, they were large and intensely stained; on the other hand, when foci were numerous, they were small and less intensely stained (not shown).



**Figure 4.** Effect of ceratamine A on cellular microtubules. Cells were incubated without drug (A, E, F, K), with 20  $\mu$ M ceratamine A (B-D, G, H, L), or with 1  $\mu$ M paclitaxel (I, J) for 5 hours. Microtubules were visualized by immunofluorescence microscopy using the E7 monoclonal  $\beta$ -tubulin antibody. DNA was stained with Hoechst 33258 as described (9). Images were obtained using a standard (A, B, E-J) or confocal microscope (C, D, K, L). A-D, interphase cells; E-L, mitotic cells. K1, L1 and L2, stacks of 0.32- $\mu$ m-thick optical sections "sliced" along the lines shown in (K) and (L) and spanning the entire cell. The glass surface upon which the cells are attached is at the bottom of the stacks. Bars, 5  $\mu$ m.

Examination of the foci by three-dimensional confocal microscopy showed that they were pillar-like structures that extended vertically from the basal surface of the cells (Fig. 4L1 and L2), clearly different from the spindles seen in untreated mitotic cells (Fig. 4K1). The intensely staining foci spanned the entire thickness of the cell, whereas smaller and less intensely staining foci did not. Incubation with 20  $\mu\text{mol/L}$  ceratamine A for 30 minutes was sufficient to induce multiple foci of tubulin staining. Interestingly, some mitotic cells with apparently normal microtubules were observed but these were in anaphase or telophase, suggesting that ceratamines might preferentially affect microtubules before metaphase (data not shown). When cells were treated for longer than 10 hours, only cells with a small number of foci of intense tubulin staining were observed. These data show that the ceratamines affect the microtubule network of interphase and mitotic cells in ways that are different from those described for other microtubule-stabilizing agents.

In conclusion, the ceratamines are microtubule-stabilizing agents with novel and simple structures and with unusual effects on cellular microtubules. Although additional work will be required to understand why the ceratamines affect cellular microtubules differently from paclitaxel and to ascertain whether they bind a

distinct site in tubulin, these unusual characteristics make these compounds attractive as research tools and experimental drug candidates. Unlike other microtubule-stabilizing agents identified thus far, the ceratamines are structurally simple and contain no chiral centers. The synthesis of ceratamines A, B, and analogues in amounts sufficient for *in vivo* testing is forthcoming. In addition, their unusual effects on cellular microtubules raise the possibility that they might act differently on normal and cancer cells than other microtubule-targeting antimitotic agents and show a different spectrum of toxicity and antitumor activity.

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