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Oncocidin A1: A Novel Tubulin-binding Drug with Antitumor Activity against Human Breast and Ovarian Carcinoma Xenografts in Nude Mice

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ABSTRACT. We identified a structural analog of thyroid hormone, methyl-3,5-diiodo-4-(4′-methoxyphenoxy) benzoate (Oncocidin A1™), that inhibits human carcinoma cell proliferation and the growth of human breast (MDA MB-231) and ovarian (OVCAR-3) carcinoma xenografts in nude mice. This novel antitumor agent is orally bioavailable and well tolerated by animals. Exposure of MCF-7 and MDA MB-231 breast carcinoma cells to Oncocidin A1 *in vitro* caused a cell-cycle arrest in prometaphase (a G_2/M arrest) and apoptosis, suggesting a cytotoxic mechanism involving mitotic spindle function. The interaction of Oncocidin A1 with microtubules was demonstrated by: 1) immunofluorescence studies of microtubule assembly in the presence of the drug in cell-free and in cellular assays; and 2) *in vitro* binding inhibition studies involving radiolabeled Oncocidin A1 or colchicine and tubulin monomers. Taken together, these experiments indicate that Oncocidin A1 perturbs cellular microtubule assembly, possibly by binding to the colchicine site on tubulin. Three-dimensional structural modelling of Oncocidin A1 revealed that it can adopt a twisted conformation similar to that of combretastatin A-4, which binds to the colchicine site of tubulin. The novel structural features of Oncocidin A1 could guide the design of a new class of microtubule-binding antitumor agents having substantially reduced normal tissue toxicity upon oral administration. BIOCHEM PHARMACOL **56**;5:623–633, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. thyroid hormone; mitotic arrest; spindle assembly; tubulin; colchicine; combretastatin

Oncocidin A1™§ (formerly referred to as DIME; Fig. 1), was originally developed as a thyroxine analog [1], but failed to show any physiological activity associated with thyroid hormone metabolism [2, 3]. However, during an investigation of the antitumor effects of various iodinated aromatic compounds, we observed that Oncocidin A1 inhibits the proliferation of human carcinoma cells *in vitro* [4]. In the present study we report that Oncocidin A1 has potent growth delay activity in human MDA MB-231 breast and OVCAR-3 ovarian carcinoma xenografts in nude mice, and is orally bioavailable and well tolerated by animals treated with the drug for up to 6 months. We were prompted by these *in vivo* properties of Oncocidin A1 to investigate potential mechanisms of its cytotoxicity toward tumor cells, which presumably involve targets other than TRs [2, 4]. We report that exposure of human breast carcinoma cells to Oncocidin A1 causes a prometaphase arrest (i.e. possibly a spindle-assembly checkpoint arrest) and apoptosis. This mitotic arrest is associated with disruption of cellular microtubule assembly, and may involve specific binding of the drug to the colchicine site of tubulin. Taken together, these findings demonstrate that Oncocidin A1 represents a new class of microtubule binding agents having limited normal tissue toxicity upon oral administration and potential clinical value.

MATERIALS AND METHODS

Cell Lines and Materials

Human MDA MB-231 and MCF-7 breast, OVCAR-3 ovarian, and HeLa cervical carcinoma cells were obtained from the American Type Culture Collection. CV-1 African green monkey kidney cells were a gift from Dr. Xiao-kun Zhang (The Burnham Institute). The breast and cervical carcinoma cells were grown in DMEM (BioWhittaker) containing 1.5 g $NaHCO_3$/L, 10% FBS (Gemini Bioproducts), and 2 mM L-glutamine. OVCAR-3 cells were grown in RPMI (BioWhittaker) containing 1.5 g of $NaHCO_3$/L, 10% FBS, and 2 mM L-glutamine. Colchicine,

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§ *Abbreviations:* CAT, chloramphenicol acetyltransferase; CSA-4, combretastatin A-4; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IC_{50}, concentration of drug that inhibited cell proliferation by 50%; Oncocidin A1™, methyl-3,5-diiodo-4-(4′-methoxyphenoxy) benzoate; PIPES, 1,4-piperazinediethanesulfonic acid; T_2, L-3,5-diiodo thyronine; T_3, L-3,5,3′-thyronine; T_4, L-3,5,3′,5′-thyronine; TR, thyroid hormone receptor; and TRE, thyroid-hormone-response element.

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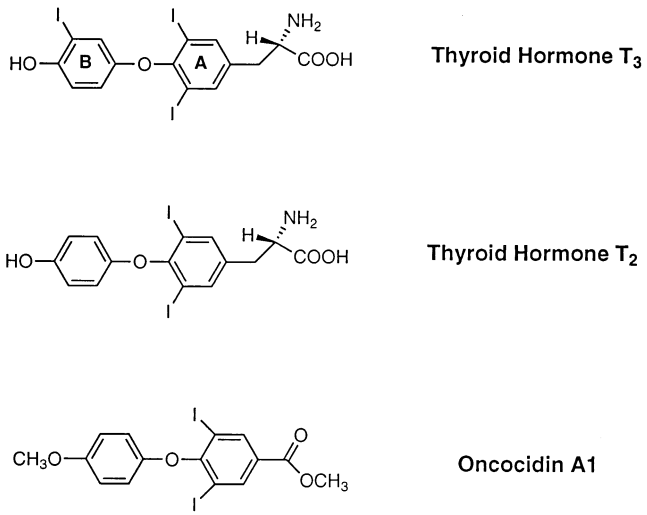


FIG. 1. Structures of thyroid hormones T_3 and T_2 and the hormone analog Oncocidin A1. The inner ring attached to the amino acid residue is termed ring A, and the outer ring is termed ring B.

vinblastine, Hoechst 33258, and propidium iodide were obtained from the Sigma Chemical Co. Oncocidin A1 was obtained from Biosource Technologies, Inc., and [^{14}C]Oncocidin A1 was synthesized by Dr. Lane Clizbe (Pharmaceutical Discovery Division, SRI International) according to literature procedures [1]. [^{3}H]Colchicine was obtained from NEN Life Science Products, Inc. (61.4 Ci/mmol). A TRE/CAT reporter vector (G17-2-CAT) and expression vectors for TRα and -β were gifts from Dr. Xiao-kun Zhang. Purified bovine brain tubulin (≥99% pure) was obtained from Cytoskeleton. Rhodamine-labeled bovine brain tubulin and anti-α-tubulin antibody were provided by Dr. Timothy Mitchison (Department of Molecular Pharmacology, University of California at San Francisco).

Bioavailability and Normal Tissue Toxicity Studies

To determine the bioavailability of Oncocidin A1, nude mice (NCR Swiss–Webster, 3–4 weeks of age) were injected subcutaneously with 5×10^6 MDA MB 231 human breast carcinoma cells. When palpable tumors developed (tumor size range: 70–200 mm^3), animals were treated with drug. The treatment regimen consisted of oral doses (gavage) of Oncocidin A1 in a corn oil carrier at 500 mg/kg twice daily at 12-hr intervals for 3 days (total of 6 doses). The first four doses were with nonradiolabeled drug; the last two doses included [^{14}C]Oncocidin A1. Mice were sacrificed 4 hr after the last dose, and their tissues were extracted, weighed, and counted, using standard pharmacological methods.

To determine normal tissue toxicity, 6-week-old female B6C3 F1 mice were treated orally and daily for 28 consecutive days with Oncocidin A1 at two dose levels (500 and 1000 mg/kg/day) using a corn oil vehicle. End points recorded during treatment included body weight, clinical observations, and hematology.

In Vivo *Tumor Xenograft Studies*

Swiss NCr nude (nu/nu) female mice, age 4–5 weeks, were purchased from Taconic. The animals were housed three per cage in sterile filter-topped cages in a barrier clean room (BioBubble, Inc.). Upon arrival, they were quarantined for 4 working days before use. The mice were fed sterile, certified Purina rodent chow *ad lib.* Drinking water was acidified and autoclaved, and the source water was recirculated, deionized, UV-treated, and filtered (5-μm filters). After the animals were released from quarantine, they were injected subcutaneously in the right flank with either 5×10^6 MDA MB-231 or 1×10^7 OVCAR-3 cells in PBS (0.1 mL injection volumes). Tumor sizes and animal weights were measured twice weekly. Vernier calipers were used to measure tumor dimensions in three orthogonal planes, and tumor volumes (V) were calculated by using the formula $V = \pi\ (x \times y \times z)/6$, where x, y, and z are the tumor measurements minus skin thickness. When the tumors reached a predetermined size (mean volumes of 50–60 mm^3), mice were divided into treatment groups of eight each.

Oncocidin A1 was suspended in corn oil (50 mg/mL) after being ground to a fine powder and were administered at a dosing volume of 10 mL/kg. Oncocidin A1 was given *per os* twice daily for at least 6 weeks; the total daily dosage was 1000 mg/kg (<25% of the LD_{50}). The tumors were measured twice weekly for at least 6 weeks after the first treatment, and the mean tumor volume for each control or treatment group was calculated for each time point. Comparisons between groups at specific times were made by using an unpaired, two-tailed *t*-test, and the results were analyzed by ANOVA. These statistical analyses were performed by using the JMP software package (SAS Institute Inc.). Systemic toxicity was assessed by determining whether treatments were associated with reductions in body weight. Mice were sacrificed at the end of the study or earlier if their tumor volumes reached 1600 mm^3 or became ulcerated.

In Vitro *Growth Inhibition and Cytotoxicity Assay*

Cytotoxicity was determined by using a trypan blue exclusion assay to measure the effect of various drug exposures on cell growth as a function of time after treatment. Cells were seeded into 24-well plates at 1×10^5 cells/well and incubated at 37° for 24 hr before treatment. At various times after treatment, cells were trypsinized, mixed with 0.2% trypan blue/PBS solution, and counted in a hemocytometer. All measurements were performed in triplicate. The IC_{50} value is the drug concentration at which the cell number was decreased by 50% relative to that of the vehicle-treated controls.

Nuclear Morphology

To visualize apoptotic cultures, MDA MB-231 cells were seeded into 12-well plates at 2×10^5 cells/well in phenol-red-free minimum essential Eagle's medium (MEM) containing 10% FBS, and treated with drugs at various doses. After 48 hr, the nuclear dye Hoechst 33258 (1 μg/mL) was added to each well, and a combination of light and fluorescence microscopy was used to obtain images of cells exhibiting nuclear morphology characteristic of apoptosis [5]. To visualize mitotically arrested cultures, MCF-7 cells were plated into 2-well chamber slides at 1×10^5 cells/well and incubated with drugs for 24 hr. Attached cells were washed with PBS twice, fixed in ice-cold methanol, and stained with Hoechst 33258 (2.5 μg/mL) for fluorescence microscopy.

Flow Cytometry

Treated and untreated cells (1×10^6 cells/sample) were trypsinized, washed with PBS, fixed in ice-cold 70% ethanol, and stored at 4°. For flow cytometry, cells were washed with PBS, treated with RNase A (1 mg/mL final concentration) at room temperature for 30 min, and stained with propidium iodide (10 μg/mL final concentration) for 20 min. Cell-cycle analysis was performed by measuring DNA content as propidium iodide fluorescence in a Coulter Epics Elite flow cytometer (10,000 cells/measurement). The MultiCycle program (Phoenix Flow Systems) was used for obtaining cell-cycle distributions.

Microtubule Assembly Inhibition Assays

A cell-free assay for measuring the inhibition of microtubule assembly was performed by first mixing tubulin and rhodamine-labeled tubulin at a ratio of 4:1 [6, 7]. This solution was then added on ice to a buffer [BRB 80; 80 mM potassium PIPES (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA] containing 1 mM GTP and 1 mM dithiothreitol, to a 15 μM final concentration. Drugs at various concentrations (0.5 μL) were added to 50-μL samples of the buffered tubulin, and 10 μL of each solution was transferred to microfuge tubes. Each tube received 0.4 μL of microtubule seeds [7]. The samples were incubated at 37° for 10 min before adding 100 μL of BRB 80 containing 1% glutaraldehyde. Each reaction mixture (2.5 μL) was transferred to a microscope slide for fluorescence microscopy.

Microtubules in intact cells were visualized by using a mouse anti-α-tubulin monoclonal antibody and a fluorescein-labeled donkey anti-mouse polyclonal antibody. Briefly, HeLa cells were plated in 2-well chamber slides at 1.5×10^4 cells/mL and incubated at 37° for 24 hr before exposure to drugs for 1 hr. After removing the medium, the cells were treated with BRB 80 containing 4 mM EGTA and 0.5% Triton X-100 [7]. The cells were fixed for 3 min in methanol chilled at −20°, and then washed with TBS [0.02 M Tris–HCl (pH 7.4), 0.15 M NaCl] and permeabilized with TBS/0.5% Triton X-100. After several washes with TBS/0.1% Triton X-100, the cells were treated with a blocking buffer (TBS, 0.1% Triton X-100, 2% BSA, 0.1% sodium azide) for 10 min. The cells were exposed in the dark to the primary antibody for 1.5 hr, and then the secondary antibody was added in the blocking buffer containing 1 μg/mL of Hoechst 33342. The cells were incubated in the dark for 45 min. The slides were mounted with *n*-propyl gallate [2% (w/v) in 30% 0.1 M Tris/glycerol (pH 9.0)] and sealed under glass coverslips for fluorescence microscopy.

Tubulin-Binding Assays

The binding of Oncocidin A1 to the colchicine binding site of tubulin was performed by using a spin column method [8, 9] and a filter-binding assay [10, 11]. In the spin column assay, [^{14}C]Oncocidin A1 (20 μM; 10 nCi) was added to tubulin (10 μM) and various concentrations of colchicine in a buffer containing 0.1 M MES (pH 6.8), 1 mM EGTA, 1 mM EDTA, and 1 mM $MgCl_2$, and incubated at room temperature for 1.5 hr. Each solution was loaded on a column containing 1 mL of Sephadex G50 equilibrated with a buffer containing 40 mM MES (pH 7.5), 40 mM Tris, and 1 mM $MgSO_4$. The columns were centrifuged at 900 *g* for 3 min, and each eluate was mixed with 3 mL of CytoScint fluid (ICN) for analysis by liquid scintillation counting. Only background radioactivity was detected in eluates from samples lacking tubulin, indicating that [^{14}C]Oncocidin A1 was quantitatively retained by the Sephadex. All experiments were performed in triplicate. In the filter-binding assay, [^{3}H]colchicine (5 μM; 50 nCi) was added to tubulin (10 μM) and various concentrations of Oncocidin A1 in a buffer containing 1.0 M glutamate (pH 6.6), 0.1 M glucose-1-phosphate, 1 mM GTP, and 0.5 mg/mL of BSA, and incubated at 37° for 1 hr in the dark. This solution (50 μL) was added to a stack of two DEAE filter papers in a 13-mm-diameter filter holder (Nucleopore; Corning Costar) attached to a vacuum manifold (J. T. Baker), and the tubulin was allowed to bind to the filters for 5 min at room temperature. The filters were washed three times with 150 μL of binding buffer by filtration at a partial pressure of 5 Torr, and then they were dried and counted in CytoScint fluid. All experiments were performed in triplicate.

Transient Transfection and CAT Assays

CV-1 cells were plated in DMEM/10% FBS at 1×10^6 cells/60-mm-diameter culture dish 16–24 hr before performing transfections. Three hours before the transfections, the medium was replaced with 5 mL of DMEM containing 10% charcoal-treated FBS. A modified calcium phosphate/DNA precipitation procedure was used for the transient transfections [12]. Briefly, 1 μg of reporter plasmid (G17-2-CAT), 0.5 μg of human TR-α or -β expression vector, 3 μg of luciferase control vector (pGL3-promoter; Promega),

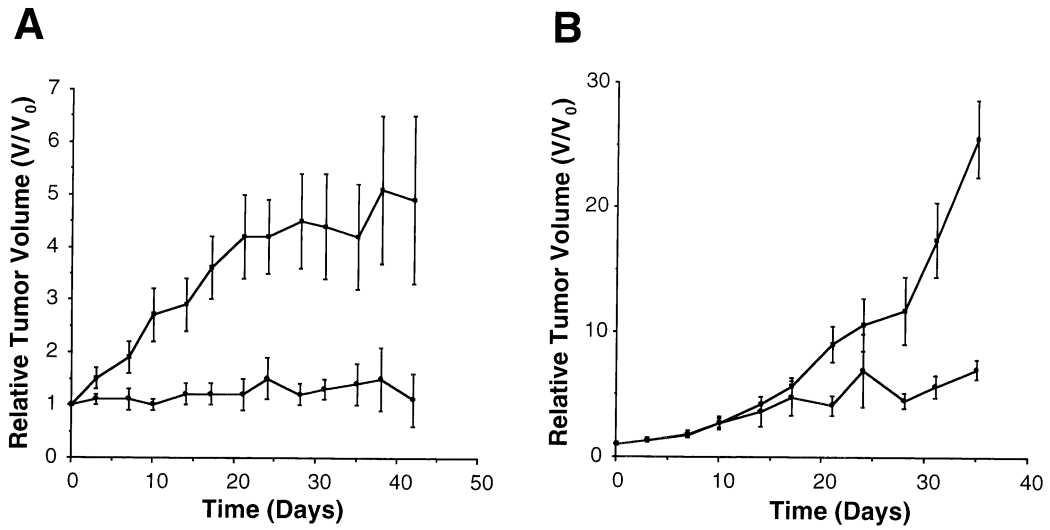


FIG. 2. Mean growth response as relative tumor volume of (A) MDA MB-231 and (B) OVCAR-3 tumors implanted in nude mice treated with Oncocidin A1. When tumors reached a size of 50 mm^3 (day 0), animals were treated twice daily with oral doses of vehicle (control) or Oncocidin A1. Key: upper line, control; lower line, treated; V_0, initial mean volume. Error bars represent the standard error of the mean for measurements of groups of animals consisting of eight members each.

and a variable amount of a Bluescript plasmid (Stratagene Cloning Systems) were mixed to obtain a total of 10 μg of DNA in 250 μL of 2 × HEBS buffer (137 mM NaCl, 5 mM KCl, 0.7 mM sodium phosphate, 6 mM glucose, and 42 mM HEPES acid; pH 7.00) and 250 μL of 0.25 M $CaCl_2$. The mixture was kept at room temperature for 20 min before adding 500 μL of precipitate to each plate and incubating the cells in 5% CO_2 at 37° for 16 hr. Then the medium was replaced with DMEM containing 10% charcoal-treated FBS with or without T_3. Incubation was continued in 5% CO_2 at 37° for 24 hr. Then the cells were harvested in 100 μL of 0.25 M Tris–HCl (pH 7.8), and lysed by three cycles of freeze/thawing. To normalize for transfection efficiency, luciferase activities of the cell extracts were measured by using a luminometer. CAT activities were measured by using acetyl CoA and [^{14}C]chloramphenicol (Amersham Life Science, Inc.) as a substrate.

Three-Dimensional Structural Modeling of Oncocidin A1 and Other Compounds

Three-dimensional structures of Oncocidin A1, CSA-4, and colchicine were modeled by using a molecular modelling software package (Insight II 95.0; Biosym/Molecular Simulations) operating on a Silicon Graphics workstation (Indigo^2, Silicon Graphics, Inc.). Two-dimensional structures of the molecules were first built by using Sketcher in the Builder module of Insight II. Distance-geometry algorithms were applied to generate three-dimensional coordinates based on assigned distance constraints. These models were refined and optimized to generate final structures.

RESULTS

Bioavailability of Oncocidin A1 and Normal Tissue Toxicity Studies

Orally administered [^{14}C]Oncocidin A1 was bioavailable to tumor tissue. Tumors contained an average of 9.53 μg equivalents of Oncocidin A1/g of tissue, or a tissue concentration equivalent to 18.7 μM. The tissue-to-plasma ratio was approximately 0.2. Since the *in vitro* efficacy of Oncocidin A1 against MDA MB 231 cells is in the submicromolar range, as shown below, the concentration of drug in the tumor appears sufficient to induce the observed toxic effects.

Gavage administration of Oncocidin A1 to nude mice at doses as high as 1000 mg/kg/day for 60–180 days produced only slight and transient increases in liver and kidney weights. No other gross pathology was observed (data not shown). No mortality was seen at a daily administration of 4000 mg/kg/day of Oncocidin A1 for 3 weeks. Because a common toxic effect of antiproliferative chemotherapeutic agents is myelosuppression caused by depletion of bone marrow stem cells, we tested the possibility that Oncocidin A1 may have bone marrow toxicity. Swiss Webster mice exposed to the drug at two dose levels (500 and 1000 mg/kg/day) by gavage for a period of 4 weeks showed none to only mild immunotoxic effects compared with vehicle-treated animals, and the bone marrow profiles of treated animals did not differ from those of control animals (data not shown).

Tumor Growth Delay Effects of Oncocidin A1 in Human Breast and Ovarian Carcinoma Xenografts

Mice implanted with MDA MB-231 human breast carcinoma xenografts showed a positive response to treatment

with Oncocidin A1 (Fig. 2A). Growth of tumors in the Oncocidin A1-treated group was suppressed during 6 weeks of therapy, while tumor growth in the control vehicle-treated animals increased approximately 5-fold on the average (relative mean tumor volume). Oncocidin A1 was also effective in inhibiting the growth of OVCAR-3 human ovarian carcinoma xenografts (Fig. 2B). Using the same schedules and doses as those described for the MDA MB-231 breast tumor study, but with shorter study duration, the relative mean tumor volume at 5 weeks of animals exposed to Oncocidin A1 was again approximately five-fold less than that of the vehicle-treated controls. Two important conclusions of this study are the following: 1) Oncocidin A1 is effective as a cytostatic/cytotoxic antitumor drug when administered orally; and 2) Oncocidin A1 is essentially nontoxic even at very high orally administered doses. Based on the *in vitro* cytotoxicity data described below, it seems possible that the effective dose of Oncocidin A1 is substantially lower than the oral doses given in this study, suggesting considerable scope for developing alternative formulations or routes of administration.

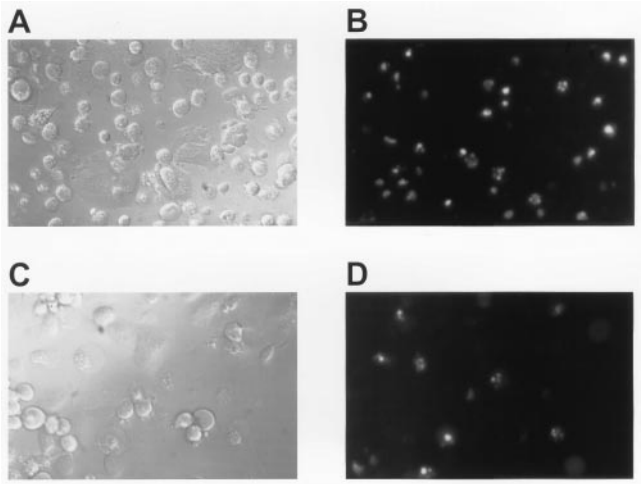


FIG. 3. Apoptosis of human breast cancer cells induced by Oncocidin A1. MCF-7 (A and B) and MDA MB-231 (C and D) cells were treated with Oncocidin A1 at 1 μM for 48 hr. Light microscopy images (A and C); fluorescence imaged from the same cells showing apoptotic morphology of nuclei stained with Hoechst 33258 (B and D). The untreated cells did not show fluorescence because they were not permeabilized prior to exposure to the dye (see Materials and Methods).

Apoptosis of Human Breast Carcinoma Cells Exposed to Oncocidin A1 In Vitro

Having demonstrated significant antitumor effects of Oncocidin A1 *in vivo*, we investigated its cytotoxicity toward human breast carcinoma cells *in vitro*. The IC_{50} of Oncocidin A1 in MDA MB-231 and MCF-7 cell cultures treated for 48 hr was 0.3 and 0.6 μM, respectively. To determine whether this growth inhibition correlated with a loss of viability and the induction of apoptosis, we investigated the effect of drug exposure on the nuclear morphology of both tumor cell types. These studies showed that cultures treated with 1 μM Oncocidin A1 for 48 hr contained a significant fraction of cells having condensed or fragmented nuclei characteristic of apoptotic cell death [13] (Fig. 3). In agreement with this result, analysis of the DNA content of Oncocidin A1-treated MDA MB-231 cells by flow cytometry showed a substantial accumulation of sub-G_1 genomic fragments (data not shown).

Mitotic Arrest Induced in MCF-7 Cells Exposed to Oncocidin A1

To test whether Oncocidin A1 exerts its cytotoxicity by perturbing the cell cycle, MCF-7 cultures were exposed to the drug at 1 μM for 24 hr. A large fraction of these cells showed chromosome condensation characteristic of a prometaphase arrest (Fig. 4B) [14, 15]. In agreement with this conclusion, treatment of MCF-7 cells with the antimitotic drug colchicine (1 μM) for 24 hr produced identical chromosome condensation patterns (Fig. 4C). After 48 hr of exposure to 1 μM Oncocidin A1, MCF-7 cells showed substantial cytotoxicity based on the criterion of loss of membrane integrity measured by a trypan blue exclusion assay. This result is in agreement with that of the cytotoxicity study described above, and demonstrates that Oncocidin 1A-induced cell death occurs subsequent to a prometaphase arrest in MCF-7 cultures.

To investigate the mitotic arrest caused by Oncocidin A1 in more detail, flow cytometry was used to determine the effect of the drug on the cell-cycle progression of MCF-7 and HeLa cells. These experiments showed that at least 60% of these cells were arrested in G_2/M within 24 hr of treatment with 1 μM Oncocidin A1 (Fig. 5). The same finding was obtained for MDA MB-231 cells exposed to the drug under the same conditions (data not shown). These results demonstrate that Oncocidin A1 causes human carcinoma cells to accumulate in the G_2/M cell-cycle phase, presumably by activating a mitotic spindle-assembly checkpoint [15, 16]. Arrested cells eventually undergo cell death, which can occur by an apoptotic mechanism.

Inhibition of Microtubule Assembly by Oncocidin A1

Microtubules are major cytoskeletal components composed of α- and β-tubulin heterodimers bound with GTP (reviewed in Ref. 17). Antimitotic drugs such as colchicine, *Vinca* alkaloids, and paclitaxel perturb the intrinsic dynamic instability of microtubules arising from GTP hydrolysis by binding directly to tubulin and causing inhibition of spindle formation with consequent mitotic arrest [18, 19]. To determine whether the antimitotic effect of Oncocidin A1 exposure could be explained by tubulin binding activity, two studies were performed. The effect of the drug on microtubule assembly was investigated directly by using a cell-free fluorescence assay involving the visualization of microtubule formation from rhodamine-labeled tubulin and

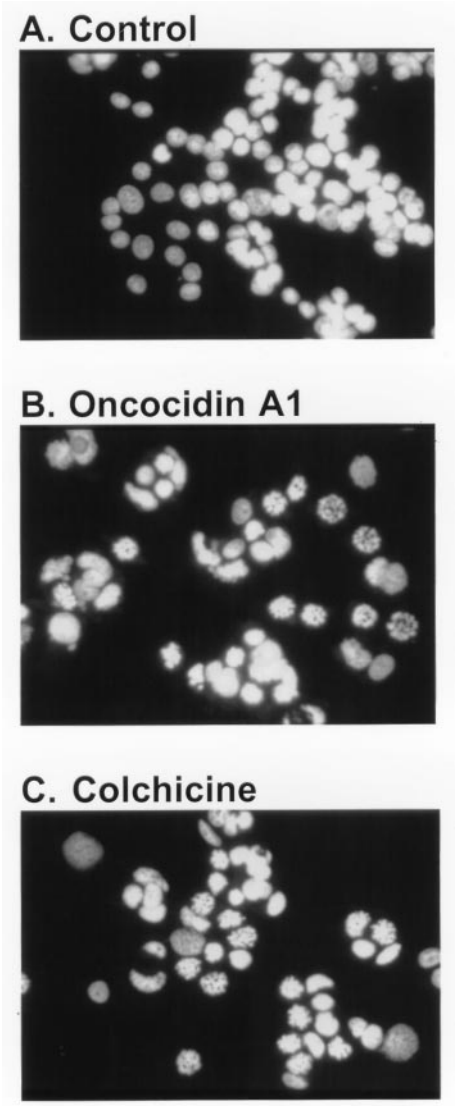


FIG. 4. Prometaphase arrest of MCF-7 cells caused by Oncocidin A1 treatment. MCF-7 cells were exposed to 1 μM Oncocidin A1 or 1 μM colchicine for 24 hr, and then fixed and stained with Hoechst 33342. (A) Untreated cells at 24 hr. (B) Oncocidin A1-treated MCF-7 cells at 24 hr of exposure. Condensed chromosomes are visible showing cells arrested in prometaphase. (C) Colchicine-treated cells at 24 hr of exposure.

microtubule nuclei in the presence of GTP [6, 7]. In this assay, 15 μM tubulin rapidly polymerized to form long and bright microtubule threads in the absence of a tubulin-binding agent (Fig. 6A), whereas in the presence of 1 μM colchicine microtubule formation was inhibited completely (Fig. 6B). The same effect was observed in the presence of 10 μM Oncocidin A1 (Fig. 6D). At a concentration of 1 μM, the drug was less potent, but nonetheless produced much shorter structures than those visible in the control experiment (Fig. 6C). In a second study, the ability of Oncocidin A1 to perturb cellular microtubule assembly was investigated by using HeLa cells, which also arrest at mitosis when exposed to the drug (data not shown). Figure 7 shows that HeLa cells exposed to 10 μM Oncocidin A1 for 1 hr had aberrant microtubule networks resembling those created by exposure to 1 μM colchicine (data not shown), and typical of those induced by other microtubule-binding agents [8, 20]. Taken together, these findings demonstrate that Oncocidin A1 can interact directly with tubulin *in vitro* and in cells to inhibit microtubule assembly, much like the antimitotic drug colchicine. However, it appears that Oncocidin A1 has lower affinity for tubulin than colchicine.

Cross Inhibition of Tubulin Binding between Oncocidin A1 and Colchicine

Tubulin-binding antimitotic drugs interact with the protein at diverse sites [21]. Colchicine binds to soluble tubulin heterodimers at a single high-affinity site (the colchicine binding site) to form a stable complex. Vinblastine binds to one or two identical high-affinity sites on tubulin (*Vinca* alkaloid binding sites) that are different from the colchicine site. To determine whether the effect of Oncocidin A1 on microtubule assembly is mediated by a specific tubulin binding site, the ability of ^{14}C-labeled drug to inhibit the binding of colchicine or vinblastine to purified tubulin was measured as a function of colchicine or vinblastine concentration. Figure 8A demonstrates that colchicine inhibited the binding of Oncocidin A1 to tubulin, whereas vinblastine had no effect. A complementary study was performed in which the ability of Oncocidin A1 to inhibit the binding of [^{3}H]colchicine to tubulin was measured. Figure 8B shows that Oncocidin A1 inhibited the binding of colchicine to tubulin. Based on these results, Oncocidin A1 appears to interact with the colchicine site but not the *Vinca* alkaloid high-affinity binding sites of tubulin *in vitro*.

Effect of Oncocidin A1 on Thyroid Hormone-dependent Transcriptional Activation

To confirm that Oncocidin A1 has no thyroid hormone activity at the molecular level, the effect of the drug on T_3-dependent transactivation was investigated in transient co-transfection experiments involving a CAT reporter vector containing a thyroid hormone responsive element and expression vectors for human TRs (TRα or TRβ) [22]. Figure 9 shows that T_3 stimulated CAT activity in transfected CV-1 cells at 10^{-7} and 10^{-8} M, but Oncocidin A1 had no significant agonist or antagonist activity. The same finding was obtained in experiments involving the TRβ receptor (data not shown).

DISCUSSION

The findings presented here demonstrated that a structural analogue of thyroid hormone, Oncocidin A1, has marked antitumor effects against human breast and ovarian carcinoma xenografts, and binds to tubulin to inhibit microtubule formation. It does not bind detectably to TRs. A

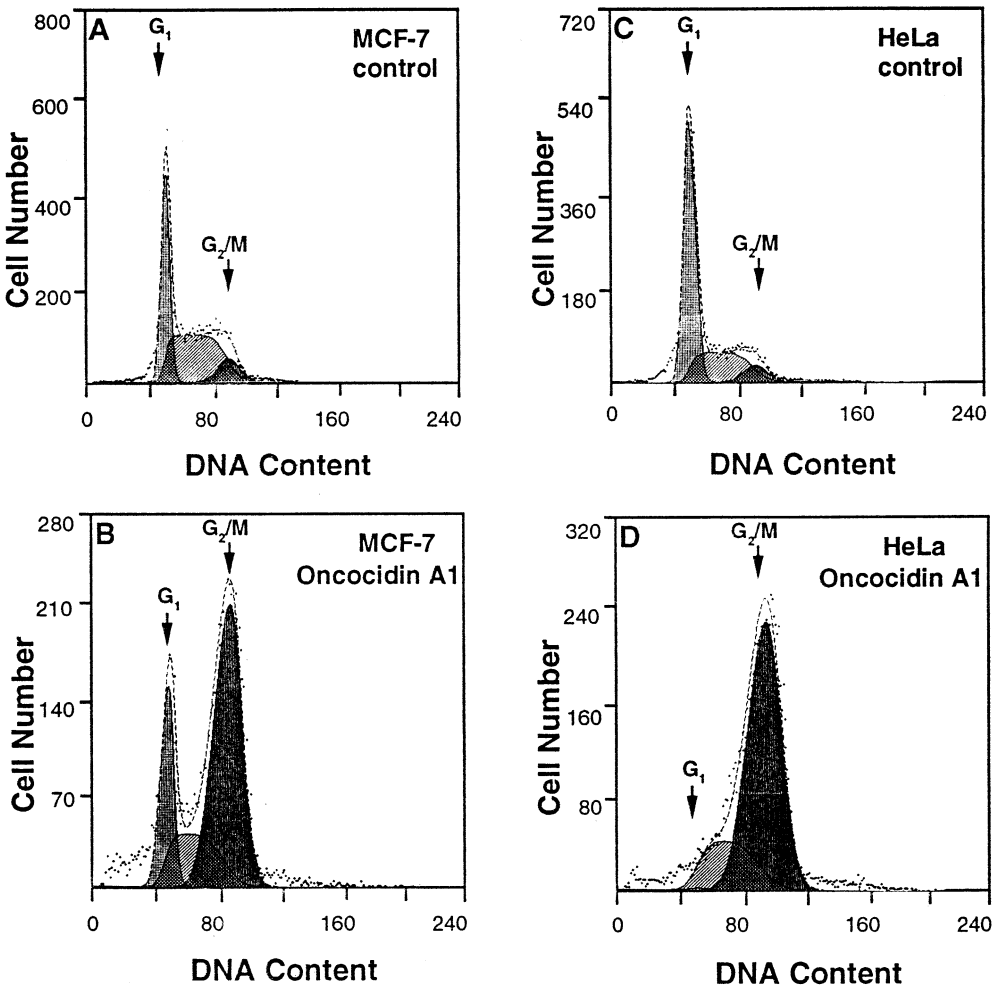


FIG. 5. Cell-cycle analyses of MCF-7 and HeLa cells exposed to Oncocidin A1. (A) Untreated MCF-7 cells at 24 hr. (B) Oncocidin A1-treated (1 μM) MCF-7 cells at 24 hr. Approximately 60% of the cells were arrested in mitosis. (C) Untreated HeLa cells at 24 hr. (D) Oncocidin A1-treated (1 μM) HeLa cells at 24 hr. Approximately 80% of the cells were arrested in mitosis.

comparison of the effect of Oncocidin A1 with that of colchicine on exponentially growing human tumor cells *in vitro* established that the compounds have similar antimitotic properties, consistent with similar mechanisms of cytotoxicity. Although Oncocidin A1 was less potent on a molar basis than colchicine in tubulin binding activity or as an *in vitro* cytotoxic agent, our *in vivo* toxicity and antitumor efficacy studies showed that, unlike colchicine, it is remarkably safe (LD_{50} ~4 g/kg) when administered orally to animals, causes very mild myelosuppression, and strongly inhibits or suppresses the growth of specific human breast and ovarian cancer xenografts in nude mice.

Neurotoxicity, a common effect of microtubule-binding agents, was not measured in this study. However, during the exposure period, animals were observed to behave normally and without overt symptoms of hyperaesthesia, analgesia, tremors, paralysis, or hyperactivity (unpublished results). Microtubule-binding agents currently used clinically, such as *Vinca* alkaloids and paclitaxel, also arrest proliferating cells in the mitotic phase of the cell cycle, but possess substantial dose-limiting toxicities and require administration by intravenous injection or infusion [23, 24]. Oncocidin A1 represents a new class of well-tolerated antimitotic agents having unique structural features that could guide the design of more effective versions of the parent molecule.

Oncocidin A1 causes mitotic arrest based on spindle disruption, as do drugs such as colchicine, vinblastine, and paclitaxel. The binding site of Oncocidin A1 on tubulin appears to be the same as that of colchicine, with concomitant inhibition of microtubule assembly. Oncocidin A1 inhibits microtubule formation *in vitro* at substoichiometric concentrations relative to tubulin, and it activates a G_2/M arrest in tumor cells at the same concentrations (Figs. 4 and 5). In addition, human carcinoma cells treated with Oncocidin A1 under these conditions remained arrested at G_2/M for at least 24 hr after removal of the drug before showing direct signs of cytotoxicity (unpublished observations).

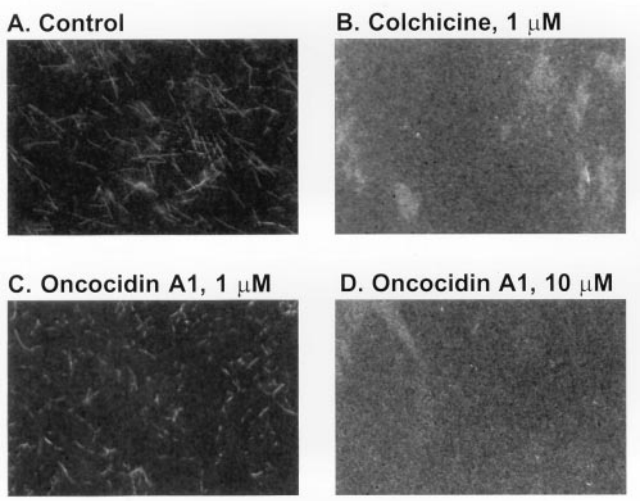


FIG. 6. Inhibition of microtubule assembly by Oncocidin A1. Rhodamine-labeled tubulin mixed with tubulin, GTP, and microtubule seeds was allowed to polymerize at 37° in the presence or absence of Oncocidin A1 as described in Materials and Methods. (A) Buffer control. (B) Colchicine at 1 μM completely blocked the polymerization of tubulin, leaving a blank background. (C) Oncocidin A1 at 1 μM markedly inhibited microtubule formation. (D) Oncocidin A1 at 10 μM. The images were obtained by using a fluorescence microscope.

These results indicate that Oncocidin A1 perturbs microtubule assembly and eventually causes irreversible changes in mitotic spindle function with associated cell death. Interestingly, Oncocidin A1 has been found to induce serine/threonine phosphatase PP2A activity and apoptosis in H-*ras* transformed bovine endothelial cells [4]. PP2A activity is important for cell-cycle progression and seems to be involved in some forms of apoptosis [25, 26], suggesting a link between PP2A activation by a microtubule-binding agent and apoptotic signaling pathways. Determining the molecular mechanisms involved in the cytotoxic effects of microtubule-binding agents such as Oncocidin A1 is an important area for further research.

Our finding indicating that Oncocidin A1 binds to the colchicine site on tubulin provides important information for the rational design of new antimitotic chemotherapeutic drugs based on structure–activity relationships established for thyroxine analogs. x-Ray crystallographic structures of T_2, T_3, and T_4 reveal that the C4–O–C1′ bond angle between the two phenyl rings is 120°, and the planes of the phenyl rings are essentially perpendicular [27, 28]. The bulky 3,5-iodines provide steric constraints that stabilize this twisted conformation. Molecular modelling using distance-geometry and energy minimization calculations show that Oncocidin A1 can adopt a twisted conformation similar to that of T_2 (Fig. 10). It has been established that a major determinant of tubulin binding by the colchicine-class of antimitotic drugs is a bicyclic ring system in which the two planar aromatic rings are tilted with respect to each other [11, 29]. Thus, the twisted conformation of Oncocidin A1 may be responsible for its affinity for the colchicine binding site of tubulin. Presumably, Oncocidin A1 lacks critical functional groups, such as a carboxylate on ring A and a 4′-hydroxyl on ring B, that favor productive binding to a TR [30]. We postulate that the substitution of a methoxy group in Oncocidin A1 for the ring B hydroxyl of T_3 promotes an interaction with tubulin similar to that of the twisted phenyl-ring model described for colchicine [29].

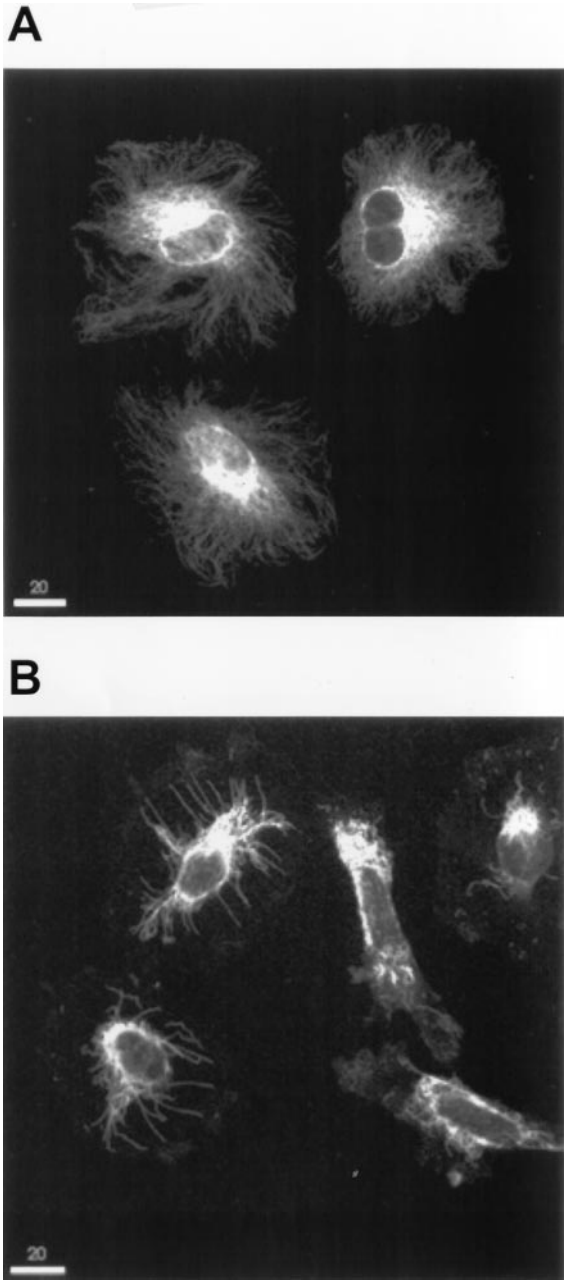


FIG. 7. Rapid depletion of cellular microtubules by Oncocidin A1. HeLa cells were exposed to 10 μM Oncocidin A1 for 1 hr, and then were fixed, permeabilized, and stained with an anti-α-tubulin antibody. (A) Untreated cells. (B) Oncocidin A1 inhibited polymerization of cellular microtubules, producing aberrant structures. The images were obtained by using a confocal microscope. The variation of shades is due to different intensities of fluorescein immunofluorescence in the image.

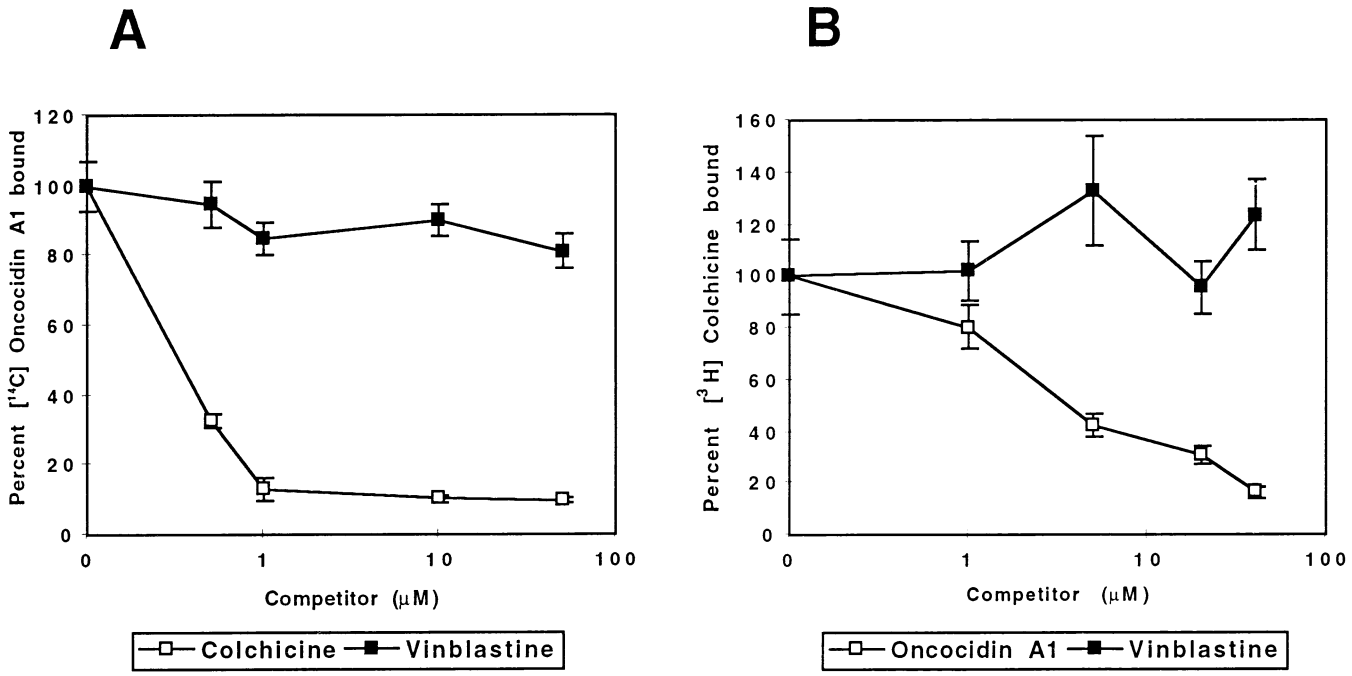


FIG. 8. Competition of Oncocidin A1 with colchicine for the colchicine binding site of tubulin. (A) Bovine brain tubulin was mixed with [^{14}C]Oncocidin A1 (20 μM, 10 nCi) and competitors (colchicine or vinblastine) at the concentrations indicated. The binding of [^{14}C]Oncocidin A1 to tubulin was determined using a Sephadex G50 mini-column assay as described in Materials and Methods. (B) Bovine brain tubulin was mixed with [^{3}H]colchicine (5 μM; 50 nCi) and Oncocidin A1 at the concentrations indicated. The binding of [^{3}H]colchicine to tubulin was determined by using a filter-binding assay as described in Materials and Methods. Values are means ± SD of triplicate determinations.

Another antimitotic drug having potential as an anticancer agent is the natural product CSA-4 (*cis*-1-(3,4,5-trimethoxyphenyl)-2-(3′-hydroxy-4′-methoxyphenol)ethene). The *cis*- rather than the *trans*-isomer of CSA-4 is required for binding to tubulin and inhibition of microtubule assembly [8]. In addition, like colchicine, the methoxy group on the A ring of CSA-4 is necessary for tubulin binding [8]. Comparison of the structure of Oncocidin A1 with that of CSA-4 or colchicine shows a similar structural relationship between the 4′-methoxy group of Oncocidin A1 and an equivalent methoxy group in each of the other molecules (Fig. 10). Removal of the 4′-methoxy group of Oncocidin A1 or substitution with a 4′-trifluoromethoxy group eliminates the antimitotic activity of the drug, suggesting that this group is critical for the binding of Oncocidin A1 to tubulin (Chen *et al.*, unpublished observations).

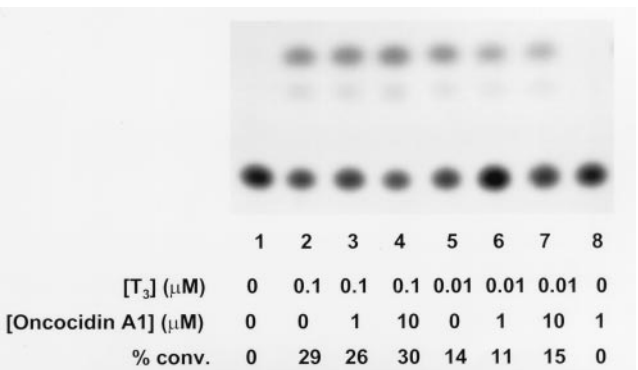
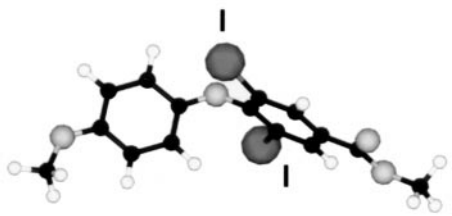
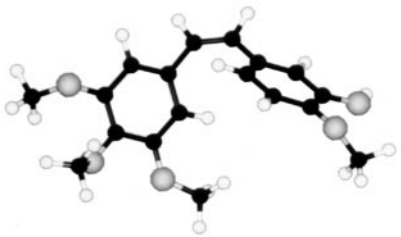


FIG. 9. Effect of Oncocidin A1 on T_3-dependent transcriptional activation. A TRE/CAT reporter gene vector and an expression vector for TRα were transfected by the calcium phosphate method into CV-1 cells together with a pGL3-promoter luciferase reporter vector. After transfection, the cells were incubated in DMEM plus 10% charcoal-treated FBS for 24 hr in the presence or absence of ligands at the concentrations indicated. Transfection efficiency was monitored by luciferase activity, and the amount of cell extract used for the CAT assays was normalized to those activities. % conv. = [cpm for acetylated [^{14}C]chloramphenicol]/[cpm for [^{14}C]chloramphenicol].

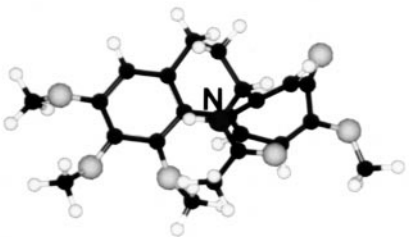
Recently an endogenous estrogen metabolite, 2-methoxyestradiol (2-ME), a weak competitor of colchicine binding to tubulin [31], was reported to inhibit proliferating tumor cells *in vitro* and the growth of human and murine tumors *in vivo* with minimal toxicity [32]. These findings were explained by antimitotic and antivascular effects of 2-ME involving both tumor and endothelial compartments of the experimental tumors [32]. Consistent with the effect of this antimitotic agent, CSA-4 was reported to cause vascular damage and dysfunction in experimental tumors, leading to extensive tumor cell death [33]. The structural and functional similarities of Oncocidin A1 with CSA-4 suggest that it may also exert its antitumor effects partially through interactions with tumor microvascular endothelial cells. These possible antiangiogenic effects of Oncocidin A1 are currently under investigation in our laboratory.



Oncocidin A1



Combretastatin A-4



Colchicine

FIG. 10. Three-dimensional structural models of Oncocidin A1, combretastatin A-4, and colchicine. The molecular structures were modeled by using a molecular modeling program provided by BioSym/Molecular Simulation (Insight II 95.0). Shown are the ball-and-stick representations of the molecules. The drawing shows the orientation of the 4′-methoxy group of Oncocidin A1 with respect to the backbone of CSA-4. For clarity, iodine and nitrogen are labeled. Carbon is shown in black; oxygen, gray; and hydrogen, white.

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