Diazonamide A and a Synthetic Structural Analog: Disruptive Effects on Mitosis and Cellular Microtubules and Analysis of Their Interactions with Tubulin

ZOBEIDA CRUZ-MONSERRATE, HÉLÈNE C. VERVOORT, RUOLI BAI, DAVID J. NEWMAN, STEPHEN B. HOWELL, GERRIT LOS, JEFFREY T. MULLANEY, MICHAEL D. WILLIAMS, GEORGE R. PETTIT, WILLIAM FENICAL, and ERNEST HAMEL

Screening Technologies Branch (Z.C.-M., R.B., E.H.) and Natural Products Branch (D.J.N.), Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute at Frederick, National Institutes of Health, Frederick, Maryland; Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography (H.C.V., W.F.) and Department of Medicine and the Cancer Center (S.B.H., G.L.), University of California at San Diego, La Jolla, California; and Department of Chemistry and Biochemistry and Cancer Research Institute, Arizona State University, Tempe, Arizona (J.T.M., M.D.W., G.R.P.)

Received November 4, 2002; accepted February 24, 2003 This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The marine ascidian *Diazona angulata* was the source organism for the complex cytotoxic peptide diazonamide A. The molecular structure of this peptide was recently revised after synthesis of a biologically active analog of diazonamide A in which a single nitrogen atom was replaced by an oxygen atom. Diazonamide A causes cells to arrest in mitosis, and, after exposure to the drug, treated cells lose both interphase and spindle microtubules. Both diazonamide A and the oxygen analog are potent inhibitors of microtubule assembly, equivalent in activity to dolastatin 10 and therefore far more potent than dolastatin 15. This inhibition of microtubule assembly is accompanied by potent inhibition of tubulin-dependent GTP hydrolysis, also comparable with the effects observed with dolastatin 10. However, the remaining biochemical properties of diazonamide A and its analog differ markedly from those of dolastatin 10 and closely resemble the properties of dolastatin 15. Neither diazonamide A nor the analog inhibited the binding of $[^3H]$vinblastine, $[^3H]$dolastatin 10, or $[^8]$GTP to tubulin. Nor were they able to stabilize the colchicine binding activity of tubulin. These observations indicate either that diazonamide A and the analog have a unique binding site on tubulin differing from the vinca alkaloid and dolastatin 10 binding sites, or that diazonamide A and the analog bind weakly to unpolymerized tubulin but strongly to microtubule ends. If the latter is correct, diazonamide A and its oxygen analog should have uniquely potent inhibitory effects on the dynamic properties of microtubules.

The complex natural product peptide diazonamide A (NSC 700089) was originally isolated from the marine ascidian *Diazona angulata* and was found to have potent cytotoxic activity (Lindquist et al., 1991) [originally, the voucher specimen was misidentified as *Diazona chinensis* (Vervoort, 1999)]. A reisolation of diazonamide A was sponsored by the Natural Products Branch, DTP, National Cancer Institute. The compound was evaluated in the DTP drug screen, and analysis by the COMPARE algorithm of the resulting differential cytotoxicity pattern (Paull et al., 1992) indicated that diazonamide A was probably a tubulin-active agent. Cells treated with diazonamide A were examined by flow cytometry and immunofluorescence microscopy. Such cells accumulated at the G/M phase of the cell cycle and had major distortions of both their mitotic spindles and their interphase microtubule network (Vervoort, 1999).

The unusual structure originally proposed for diazonamide A (Fig. 1), as well as its cytotoxic properties, generated intense interest in synthesis of the compound (for review, see Ritter and Carreira, 2002). Harran and coworkers succeeded in developing a synthesis for the proposed structure (Lindquist et al., 1991), but the resulting compound had little cytotoxic activity and was highly unstable (Li et al., 2001b). The reported spectral data for diazonamide A was therefore reevaluated, and it was concluded that the compound probably had the structure shown in Fig. 1. These workers went on to synthesize compound 1 (Fig. 1), an analog of diazonamide A in which a single nitrogen atom was replaced with an oxygen atom. Compound 1 was essentially identical to natural diazonamide A in inhibiting the growth of NIH:OVCAR-3 human ovarian carcinoma cells. Like diazonamide A-treated cells, those treated with compound 1 showed major disruption of both spindle and interphase micro-

ABBREVIATIONS: DTP, Developmental Therapeutics Program; NSC 700089, diazonamide A; NSC 376128, dolastatin 10; NSC 617668, dolastatin 15; MAP, microtubule-associated protein; DAPI, 4,6-diamidino-2-phenylindole; MES, 4-morpholineethanesulfonate; FITC, fluorescein isothiocyanate; TLC, thin-layer chromatography; GI50, in the terminology of the DTP drug screen, the drug concentration that inhibits cell growth by 50% after 48 h.
We have now examined the interactions of diazonamide A and compound 1 with purified tubulin, comparing the effects of these peptides with those of dolastatins 10 (NSC 376128) (Bai et al., 1990) and 15 (NSC 617668) (Bai et al., 1992). Dolastatins 10 and 15 are highly cytotoxic, antimitic peptides originally isolated from the sea hare Dolabella auriculata (Pettit et al., 1987, 1989a) that have apparently distinct interactions with tubulin. Both dolastatins inhibit tubulin assembly and tubulin-dependent GTP hydrolysis, although dolastatin 10 is 10-20-fold more potent in its inhibitory effects than dolastatin 15. Dolastatin 10 also inhibits, in a noncompetitive manner, the binding of vinblastine to tubulin and nucleotide exchange on β-tubulin. Dolastatin 15 lacks these activities. Dolastatin 10 readily promotes the aberrant assembly of tubulin into ring and spiral structures, whereas dolastatin 15 does not (Bai et al., 1995). Dolastatin 10, but not dolastatin 15, stabilizes the colchicine binding activity of tubulin, which is probably related to induction of these tubulin oligomers (the binding sites for colchicine and vinblastine are entirely distinct regions of the tubulin α-β-dimer). Finally, [³H]dolastatin 10 binds avidly to tubulin with negligible dissociation even under nonequilibrium conditions (Bai et al., 1995); thus far, however, we have only been able to document binding of [³H]dolastatin 15 to tubulin by equilibrium (Hummel-Dreyer) gel filtration chromatography (Z.

Materials and Methods

Materials. Tubulin without MAPs and heat-treated MAPs were isolated from bovine brain (Hamel and Lin, 1984b), and synthetic dolastatin 10 (Pettit et al., 1989b) and dolastatin 15 (Pettit et al., 1991) were prepared as described previously. Compound 1 (Li et al., 2001a) was a generous gift of Dr. P. G. Harran (Southwestern Medical Center at Dallas, Dallas, TX). Maytansine and [³H]dolastatin 10 were supplied by the Drug Synthesis and Chemistry Branch, National Cancer Institute. In some studies, as indicated, the tubulin used had been freed of unbound nucleotide (primarily residual GDP from the final assembly cycle) by gel filtration chromatography on Sephadex G-50 (superfine), as described elsewhere (Grover and Hamel, 1994). Phomopsin A was from Calbiochem (San Diego, CA); [³H]colchicine and [³H]vinblastine were from Perkin Elmer Life Sciences (Boston, MA); GTP and [³H]GTP were from Sigma (St. Louis, MO) and Moravek Radiochemicals (Brea, CA), respectively, and both nucleotides were repurified from about 90 to >98% purity by triethylammonium bicarbonate gradient chromatography on DEAE-cellulose. All antibodies and DAPI were from Sigma. Polyethenylamine-cellulose TLC sheets were from Brinkmann Instruments (Westbury, NY). Burkitt lymphoma CA46 and PtK2 cells were obtained from the American Type Culture Collection (Manassas, VA) and MCF7 breast carcinoma, PC-3 prostate carcinoma, and A549 lung adenocarcinoma cells were obtained from the drug screening program of the Screening Technologies Branch, National Cancer Institute. Cells were maintained as recommended by the supplier.

Methods. The procedure outlined by Lindquist et al. (1991) for isolation of diazonamide A from specimens of D. angulata was modified as described in detail elsewhere (Vervoort, 1999). Frozen specimens of the ascidian were mixed with crushed dry ice and ground. The crushed mass was held at –20°C until the dry ice had sublimed (~2.5 days). The residue was stirred with 1 liter of water at 4°C for 30 min. A basket centrifuge was used to separate solids from supernatant. The solids were successively extracted at room temperature with a 1:1 mixture of methanol and methylene chloride and with methanol. The organic extracts were pooled, and the solvents removed under vacuum. Most of the crude extract (3.18 g) was fractionated between methanol and isooctane. The methanol fraction (2.36 g after solvent removal) was partitioned between water and butanol. The butanol fraction (1.22 g after solvent removal) was chromatographed on Sephadex LH-20 using a 3:1 mixture of isooctane, methanol, and toluene. Fractions containing diazonamide A, identified by ¹H nuclear magnetic resonance spectroscopy, were combined and rechromatographed on Sephadex LH-20 using 100% methanol. Fractions containing diazonamide A were combined, and the final purification of 20 mg of the peptide was accomplished by reverse phase high-performance liquid chromatography on a C18 column, which was developed isocratically with 74% methanol.
Flow cytometry was performed on human ovarian carcinoma 2008 cells (DiSaia et al., 1972). The cells were seeded at 2 × 10^3 cells per culture in 10 ml of RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 200 units/ml of penicillin G, and 200 μg/ml of streptomycin. Diazonamide A or an equivalent amount of dimethyl sulfoxide [the drug solvent, final concentration 0.01% (v/v)] was added; after another 24 h of growth, cells were harvested by trypsinization. The cells were washed three times with cold phosphate-buffered saline and fixed in ethanol at 0°C. Subsequently the cells were treated with RNase at 1.0 mg/ml for 30 min at 37°C and stained with propidium iodide at 50 μg/ml at 0°C for at least 30 min. DNA content of the cells was measured using a FACScan instrument (BD Biosciences, San Jose, CA) and MultiCycleAV software from Phoenix Flow Systems (San Diego, CA).

The MCF7, PC-3, and A549 cells were grown in microtiter plates, and drug IC_{50} values were obtained as described by Skehan et al. (1990), except that cells were treated with drug for 72 h. Cells were seeded into the microtiter plates 24 h before drug addition, and cell protein was the parameter measured, with sulforhodamine B. The IC_{50} was defined as the drug concentration that reduced increase in cell protein by 50% at 72 h after drug addition. The final experiments to determine IC_{50} values were done with successive 2-fold dilutions of drug into growth medium, with 10 concentrations used per drug. At the highest concentration of each drug, the dimethyl sulfoxide concentration was 1% (v/v).

The Burkitt lymphoma CA46 cells were grown in 5-ml flasks, and IC_{50} values were determined by counting the cells in a Coulter counter (Beckman Coulter, Fullerton, CA), with the IC_{50} value defined as the drug concentration that reduced increase in cell number by 50% at 16 h after drug addition. The cells were grown in RPMI 1640 medium supplemented with 17% fetal bovine serum and 2 mM L-glutamine at 37°C in a 5% CO_2 atmosphere. The dimethyl sulfoxide concentration was 0.1% (v/v) in all culture flasks.

The mitotic index in the Burkitt cell cultures was also determined at 16 h, the time that produces a near-maximal value after treatment with antitubulin drugs. About 4.5 ml of cell culture medium was centrifuged at 1000 rpm for 1 min. The pelletted cells were resuspended in 5 ml of phosphate-buffered saline at room temperature, and the cells were harvested by centrifuging the suspension as before. The cell pellet was suspended in 0.5 ml of half-strength phosphate-buffered saline, and the cells were allowed to swell for 10 min. The cells were then fixed by adding 6 ml of 0.5% acetic acid-1.5% ethanol. After 30 min, the cells were harvested by centrifuging as before. The cells were resuspended in 25% acetic acid/75% ethanol, and a droplet of the cell suspension was spread on the slide. The slide was air-dried and stained with Giemsa. The slide was examined under a light microscope, with mitotic cells defined as those with condensed chromosomes and no nuclear membrane. At least 200 cells were counted for each condition examined.

Visualization of the microtubule and actin filament networks of Potorous tridactylis kidney epithelial PtK2 cells was performed as described previously (Bai et al., 2001). The dimethyl sulfoxide concentration in all slide chambers was 1% (v/v). In brief, appropriately fixed cells were stained with DAPI and appropriate antibodies or a fluorescent derivative of phalloidin and examined under a 100× oil objective (numerical aperture, 1.30) with an Eclipse E800 microscope (Nikon, Tokyo, Japan) equipped with epifluorescence and appropriate filters. Images were captured with a Spot digital camera, model 2.3.0, using version 3.0.2 software. The tubulin antibody used was a Cy3 conjugate of anti-β-tubulin clone TUB2.1 monoclonal antibody (Sigma). The actin antibody used was a FITC conjugate of anti-β-actin clone Ac-15 monoclonal antibody (Sigma). The actin antibody and the fluorescent phalloidin derivative resulted in identical staining patterns of the microfilaments, and the images presented here were obtained with the antibody.

Tubulin assembly was followed turbidimetrically in Beckman DU7400/7500 spectrophotometers (Beckman Coulter) equipped with electronic temperature controllers. The latter instruments are driven by software provided by MDB Analytical Associates (South Plainfield, NJ). The spectrophotometers contain a software patch to permit them to maintain a temperature of 0°C and have been modified so that the electronic controllers are water-cooled for more precise temperature maintenance.

The binding of [3H]vinblastine, [3H]dolastatin 10, and [8-14C]GTP to tubulin was measured by centrifugal gel filtration on microcolumns of Sephadex G-50 (superfine) prepared in tuberculin syringes, as described previously (Hamel and Lin, 1984a). The binding of [3H]colchicine to tubulin was measured by filtration through a stack of two DEAE-cellulose filters under reduced vacuum, as described previously (Bai et al., 1990).

Hydrolysis of [8-14C]GTP was followed by measuring formation of [8-14C]GDP by TLC on polyethyleneamine-cellulose plates, as described previously (Bai et al., 1992). Aliquots of reaction mixtures were added to 25% acetic acid to stop the reaction, and, after TLC, the GDP and GTP spots were located by autoradiography and cut from the plates to quantitate extent of hydrolysis.

### Results

**Cellular Effects of Diazonamide A in Comparison with Those of Compound 1, Dolastatin 10, and Dolastatin 15.** The cytotoxicity of diazonamide A had previously been assessed against only a few selected cell lines (Li et al., 2001a; Lindquist et al., 1991). This evaluation was extended to the 60 cell lines in the DTP drug screen. In the cell lines successfully evaluated, a mean GI50 value of about 5 nM was obtained. [The complete data set can be found in Vervoort (1999) and on the DTP web site (http://www.dtp.nci.nih.gov/dtpstandard/cancerscreeningdata/index.jsp).] The data obtained were analyzed with the COMPARE algorithm; this analysis indicated that diazonamide A had a high probability of being an antitubulin agent, because all correlation coefficients >0.6 (Paull et al., 1992) were with well known antitu-
The effects of the peptide were first evaluated on cell cycle progression of the human ovarian carcinoma line 2008. In a clonogenic assay, the IC$_{50}$ value obtained for diazonamide A was 10 nM. When cells treated with 4- and 10-fold higher concentrations for 24 h were examined by flow cytometry, there was a concentration-dependent increase in the fraction of cells arrested at the G2/M phase of the cell cycle (compare those shown in Table 1. The data are presented as mean ± S.D. of three independent experiments. In each experiment, the mitotic index of the control culture was determined. The average value was 2.4 ± 0.2. In the Burkitt cells, dolastatin 15 was nearly as least active. In the A549 cell line, except for the potent values obtained for diazonamide A averaged about 65% of the values obtained for dolastatin 10, and compound 15 (see below), it seemed desirable to perform direct cellular comparisons as well. We examined the effects of the four compounds on the growth of four human cancer cell lines. These were Burkitt lymphoma CA46 cells (selected because they generally yield a very high mitotic index when treated with antitubulin agents), MCF7 breast carcinoma cells, PC-3 prostate carcinoma cells, and A549 lung adenocarcinoma cells. The data obtained are shown in Table 1; for further details, see Vervoort, 1999).

When we decided to compare the antitubulin properties of diazonamide A with those of compound 1 and dolastatins 10 and 15 (see below), it seemed desirable to perform direct cellular comparisons as well. We examined the effects of the four compounds on the mitotic index, using equitoxic drug concentrations (the IC$_{50}$ value and a concentration 10-fold greater). Similar results were obtained with all four drugs: when cells were treated with the IC$_{50}$ concentrations, the mitotic index ranged from 38 to 46% and, when the drug concentrations were 10-fold higher, from 74 to 79% (Table 2).

The effects of diazonamide A and compound 1 on cytoskeletal integrity were studied in more detail using PtK2 cells, in which the microtubule network and actin filament stress fibers are exceptionally well visualized. The IC$_{50}$ values of diazonamide A and compound 1 with the PtK2 cells were 0.3 and 1.0 nM, respectively. For immunofluorescence microscopy studies, DNA was stained with DAPI, tubulin with a Cy3 conjugate of an anti-β-tubulin monoclonal antibody, and F-actin with a FITC-conjugate of an anti-β-actin monoclonal antibody with increased affinity for F-actin. Cells were examined after 16 h of treatment at the IC$_{50}$ concentrations (Fig. 3) or at 10-fold higher concentrations (Fig. 4). Significant and similar effects on cellular microtubules were observed with both compounds at both concentrations, but there were no significant effects on F-actin filaments (shown only at the higher concentration). Cells treated at the IC$_{50}$ concentrations showed a thinning of microtubules and what seemed to be disorganization of the overall microtubule network (Fig. 3). At the higher concentrations (Fig. 4), few if any microtubules remained after treatment with either peptide.

**Effects of Diazonamide A and Compound 1 on Tubulin Assembly: Comparison with Dolastatin 10 and Dolastatin 15.** The effects of diazonamide A and compound 1 on tubulin assembly were examined in two systems: assembly dependent on MAPs, both with and without exogenous Mg$^{2+}$ added to the reaction mixture, and assembly of purified tubulin dependent on a high concentration of glutamate (Hamel and Lin, 1984b) to establish that tubulin itself was interacting with diazonamide A and compound 1. The glutamate-dependent reaction has been routinely used in our laboratory for extensive structure-activity comparisons with many different classes of antimitotic drugs. Table 3 summarizes the IC$_{50}$ values (50% inhibition of extent of assembly after a 20-min incubation at 30°C) obtained with the four peptides in the two systems, as well as values obtained in the glutamate system with a drug-tubulin preincubation before addition of the GTP required for assembly.

As before, the IC$_{50}$ values obtained with dolastatin 10 were substantially lower than those obtained with dolastatin 15. The values obtained for diazonamide A under all conditions were lower than those obtained for dolastatin 10 (the IC$_{50}$ values obtained for diazonamide A averaged about 65% of the values obtained for dolastatin 10), and compound 1 yielded

### Table 1: Inhibition of human cancer cell growth by diazonamide A, compound 1, dolastatin 10, and dolastatin 15

<table>
<thead>
<tr>
<th>Drug</th>
<th>CA46 IC$_{50}$</th>
<th>MCF7 IC$_{50}$</th>
<th>PC-3 IC$_{50}$</th>
<th>A549 IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazonamide A</td>
<td>3.8 ± 0.3</td>
<td>1.9 ± 1</td>
<td>2.3 ± 0.6</td>
<td>4.5 ± 2</td>
</tr>
<tr>
<td>Compound 1</td>
<td>24 ± 0.5</td>
<td>15 ± 10</td>
<td>9.6 ± 5</td>
<td>5.5 ± 4</td>
</tr>
<tr>
<td>Dolastatin 10</td>
<td>0.21 ± 0.2</td>
<td>0.06 ± 0.03</td>
<td>0.11 ± 0.08</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Dolastatin 15</td>
<td>0.46 ± 0.4</td>
<td>0.90 ± 0.1</td>
<td>6.0 ± 2</td>
<td>5.8 ± 2</td>
</tr>
</tbody>
</table>

### Table 2: Effect on mitotic index of treatment of CA46 cells with diazonamide A, compound 1, dolastatin 10, or dolastatin 15

Cells were treated with the IC$_{50}$ concentrations equal to and 10-fold greater than those shown in Table 1. The data are presented as mean ± S.D. of three independent experiments. In each experiment, the mitotic index of the control culture was determined. The average value was 2.4 ± 0.56%.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC$_{50}$ % mitotic cells</th>
<th>10 × IC$_{50}$ % mitotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazonamide A</td>
<td>43 ± 5</td>
<td>74 ± 4</td>
</tr>
<tr>
<td>Compound 1</td>
<td>46 ± 3</td>
<td>78 ± 5</td>
</tr>
<tr>
<td>Dolastatin 10</td>
<td>40 ± 4</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>Dolastatin 15</td>
<td>38 ± 2</td>
<td>77 ± 4</td>
</tr>
</tbody>
</table>
IC\textsubscript{50} values higher than those for dolastatin 10 (the IC\textsubscript{50}
values for compound 1 on average were about 55% higher than those obtained for dolastatin 10). Note that in the glutamate system, a preincubation did not substantially alter the IC\textsubscript{50} values obtained for any of the peptides, unlike the significant reduction that occurs with colchicine (Grover et al., 1992). This indicates that binding of the peptides to tubulin occurs relatively rapidly, in contrast to the slow, temperature-dependent binding of colchicine to the protein (Hastie, 1991). Although it must be noted that the relative potency of diazonamide A and dolastatin 10 is not reflected in their relative potency as inhibitors of cell growth (dolastatin 10 is significantly more potent than diazonamide A), the two activities have shown similar divergence in many other studies (see, for example, Pettit et al., 1998).

**Effects of Diazonamide A and Compound 1 on Tubulin-Dependent GTP Hydrolysis: Comparison with Dolastatin 10 and Dolastatin 15.** GTP hydrolysis was examined in a glutamate-dependent reaction, under reaction conditions somewhat different from those used in the above assembly studies. All the peptides inhibited GTP hydrolysis in a concentration-dependent manner (Fig. 5). The IC\textsubscript{50} values derived from these data were higher than was observed for the assembly reaction, but the relationship between the four antimitotic peptides was the same as in the assembly reactions (values presented as mean ± S.D.): diazonamide A (2.4 ± 0.7 μM) < dolastatin 10 (3.4 ± 0.9 μM) < compound 1 (7.3 ± 1.4 μM) << dolastatin 15 (33 ± 7 μM). Note that most colchicine site compounds that have been examined stimulate rather than inhibit tubulin-dependent GTP hydrolysis under the reaction condition used here. Because the differences between the assembly assay and the GTPase assay (higher temperature and higher glutamate concentration) result in higher drug IC\textsubscript{50} values for assembly with other drugs (E. Hamel, unpublished data), it is likely that the inhibition of GTP hydrolysis with the peptides is reasonably concordant with inhibition of the associated assembly reaction.

**Fig. 3.** Treatment of PtK2 cells with the IC\textsubscript{50} concentrations of diazonamide A and compound 1 causes the partial disappearance of cellular microtubules. A, no drug. B, cells were treated with 0.3 nM diazonamide A for 16 h. C, cells were treated with 1.0 nM compound 1 for 16 h. DNA and tubulin fluorescence shown.

**Fig. 4.** Treatment of PtK2 cells with 10 times the IC\textsubscript{50} concentrations of diazonamide A and compound 1 causes disappearance of cellular microtubules with little effect on the actin filament network. A and B, no drug. C and D, cells were treated with 3 nM diazonamide A for 16 h. E and F, cells were treated with 10 nM compound 1 for 16 h. A, C, and E, DNA and tubulin fluorescence shown. B, D, and F, DNA and F-actin fluorescence shown.
of activity of the tubulin in binding colchicine. As shown previously (Bai et al., 1990), was able to prevent loss of colchicine binding to tubulin in the presence of all drugs except for 5. Without a preincubation, there was some enhancement reaction mixture. These experiments are summarized in Table 3.

**TABLE 3**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MAP-Induced Assembly</th>
<th>Glutamate-Induced Assembly</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ 0.5 mm MgCl₂</td>
<td>µM</td>
</tr>
<tr>
<td></td>
<td>No MgCl₂</td>
<td>µM</td>
</tr>
<tr>
<td>Diazonamide A</td>
<td>0.30 ± 0.01</td>
<td>0.75 ± 0.2</td>
</tr>
<tr>
<td>Compound 1</td>
<td>0.69 ± 0.01</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Dolastatin 10</td>
<td>0.49 ± 0.05</td>
<td>1.3 ± 0.05</td>
</tr>
<tr>
<td>Dolastatin 15</td>
<td>11 ± 0.2</td>
<td>6.2 ± 1</td>
</tr>
</tbody>
</table>

**MAP-Induced Assembly**

**Glutamate-Induced Assembly**

**Preincubation**

**No Preincubation**

**Discussion**

Diazonamide A and compound 1 are potent inhibitors of tubulin assembly, with diazonamide A yielding even lower IC₅₀ values than dolastatin 10. Diazonamide A and compound 1 were also effective inhibitors of tubulin-dependent GTP hydrolysis, but neither compound had further biochemical similarity to dolastatin 10 (Bai et al., 1990, 1995). They had no significant inhibitory effects on the binding of [³H]vinblastine or [³H]dolastatin 10 to tubulin. Despite strongly inhibiting GTP hydrolysis, diazonamide A and compound 1 had little effect on nucleotide exchange on β-tubulin. Nor were these complex peptides able to stabilize the [³H]colchicine binding activity of tubulin. Finally, the ability of diazonamide A to induce formation of stable tubulin aggregates that could be demonstrated by gel filtration high-performance liquid chromatography was evaluated, but the peptide was inactive in this assay, too.

In short, except for their potency as inhibitors of tubulin assembly and the associated hydrolysis of GTP, diazonamide A and compound 1 are more comparable, in terms of their biochemical properties, with dolastatin 15, which has no detectable effect in the interactions of the radiolabeled ligands with tubulin and is unable to induce formation of tubulin aggregates (Bai et al., 1995).

It is likely that the differences between dolastatin 10 and dolastatin 15 in their interactions with tubulin derive from a much weaker binding of the latter drug to tubulin. Dolastatin 10 binds avidly to tubulin, with little bound drug lost during gel filtration chromatography, and the apparent Kᵦ for the peptide is about 25 nM (Bai et al., 1995). In contrast, it is difficult to demonstrate the binding of [³H]dolastatin 15 to tubulin; such binding is only demonstrable by Hummel-Dreyer chromatography. Analogously, Jordan et al. (1998) examined the binding to tubulin of an analog of dolastatin 15,
The recently described vitilevuamide (Edler et al., 2002) differs in its behavior with tubulin. Although vitilevuamide noncompetitively inhibited the binding of \(^{3}H\)vinblastine to tubulin, stabilized colchicine binding activity, and inhibited the binding of \(^{3}H\)dolastatin 10 (type of inhibition not determined), vitilevuamide was unable to inhibit nucleotide exchange on β-tubulin.

The last two peptide families, the tubulysins (Sasse et al., 2000) and the colgelotins (Morita et al., 2000; Kobayashi et al., 2001) have not yet been adequately characterized, although tubulysin A inhibited the binding of vinblastine to tubulin (Khalil, 1999).

From this summary of the known interactions of antimitotic peptides with tubulin, it seems that diazonamide A and compound 1 have distinctive properties. Their behavior is superficially similar to that of dolastatin 15. However, their powerful inhibitory effects on tubulin assembly, with diazonamide A the most potent peptide we have ever evaluated, argue against a weak interaction at the dolastatin 10 binding site. There are two distinct possibilities for the interaction of diazonamide A and its analog with tubulin. First, these peptides could have a unique binding site on the α-β-tubulin dimer. Their failure to inhibit vinblastine, colchicine, or dolastatin 10 binding and their potent inhibitory effect on assembly support this hypothesis. A second possibility is that diazonamide A and compound 1 do bind in the postulated “peptide site” adjacent to the vinca site (Bai et al., 1990), but only when this site is at the growing ends of microtubules. This would imply that these peptides should have a particularly potent effect on microtubule dynamics. Panda and colleagues have shown that cryptophycins 1 and 52 are exceptionally effective agents for suppressing microtubule dynamics (Panda et al., 1997, 1998). With \(^{3}H\)cryptophycin 52, they obtained an apparent \(K_d\) for microtubule ends of 47 nM, 2- to 9-fold lower than the \(K_d\) for the binding of the drug to unassembled tubulin. In view of the differences between diazonamides and cryptophycins (the latter are qualitatively similar in behavior to dolastatin 10) in their interactions with tubulin, this hypothesis would predict a much greater difference in \(K_d\) values between microtubule ends and unassembled tubulin for the diazonamides than was observed with cryptophycin 52.

TABLE 4

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Vinblastine Binding</th>
<th>Dolastatin 10 Binding</th>
<th>GTP Binding % inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazonamide A</td>
<td>4 ± 10</td>
<td>10 ± 4</td>
<td></td>
</tr>
<tr>
<td>Compound 1</td>
<td>0</td>
<td>0</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Dolastatin 15</td>
<td>0</td>
<td>0</td>
<td>98 ± 0.5</td>
</tr>
<tr>
<td>Dolastatin 10</td>
<td>99 ± 0.2</td>
<td>96 ± 2</td>
<td></td>
</tr>
<tr>
<td>Phomopisin A</td>
<td>99 ± 0.2</td>
<td>98 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

The tubulin concentration was 10 μM, and the [\(^{3}H\)]vinblastine concentration was 6 nM. The indicated potential stabilizing drugs were present at 50 μM. Incubation after colchicine addition was for 2 h at 37°C. If a preincubation without colchicine was performed, it was for 3 h at 37°C. Except as indicated, three independent experiments were performed. In the unpreincubated control, 0.35 μl of colchicine was bound per μl of tubulin.

References


Edler MC, Fernandez AM, Lassota P, Ireland CM, and Barrows LR (2002) Inhibition of tubulin polymerization by vitilevuamide, a bicyclic marine peptide, at a site

TABLE 5

The reaction conditions were essentially those described by Ludueña et al. (1989). The tubulin concentration was 4 μM, and the [\(^{3}H\)]colchicine concentration was 6 μM. The indicated potential stabilizing drugs were present at 50 μM. Incubation after colchicine addition was for 2 h at 37°C. If a preincubation without colchicine was performed, it was for 3 h at 37°C. Except as indicated, three independent experiments were performed. In the unpreincubated control, 0.35 μl of colchicine was bound per μl of tubulin.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Not Preincubated</th>
<th>Preincubated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of nonpreincubated control</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>47 ± 5</td>
</tr>
<tr>
<td>Diazonamide A</td>
<td>138 ± 6</td>
<td>64 ± 3</td>
</tr>
<tr>
<td>Compound 1</td>
<td>125</td>
<td>49</td>
</tr>
<tr>
<td>Dolastatin 10</td>
<td>134 ± 9</td>
<td>121 ± 6</td>
</tr>
<tr>
<td>Dolastatin 15</td>
<td>111 ± 8</td>
<td>56 ± 3</td>
</tr>
<tr>
<td>Maytansine a</td>
<td>126 ± 7</td>
<td>62 ± 2</td>
</tr>
</tbody>
</table>

* A single experiment was performed.

* Two experiments were performed.

\(^a\) a single experiment was performed.

\(^b\) Two experiments were performed.
Address correspondence to: Dr. E. Hamel, Building 469, Room 104, National Cancer Institute at Frederick, Frederick, MD 21702. E-mail: hamele@mail.nih.gov