LU103793 (NSC D-669356): A Synthetic Peptide That Interacts with Microtubules and Inhibits Mitosis

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ABSTRACT

LU103793 (NSC D-669356) is a new synthetic derivative of Dolastatin 15, an antiproliferative compound which was isolated from the mollusk Dolabella auricularia. Like Dolastatin 15, LU103793 is highly cytotoxic in vitro (IC_{50} = 0.1 nM). To investigate the mechanism of action of LU103793, we used a combination of biochemical and cellular methods. Turridity assays with bovine brain microtubules demonstrated that LU103793 inhibits microtubule polymerization in a concentration-dependent manner (IC_{50} = 7 µM). Treatment with this compound also induced depolymerization of preassembled microtubules. Cell cycle analysis of tumor cell lines treated with LU103793 indicated a block in the G2-M phase. At the cellular level, it induced depolymerization of microtubules in interphase cells and development of abnormal spindles and chromosome distribution in mitotic cells. Although these effects are very similar to the cellular alterations caused by vinblastine, LU103793 does not inhibit vinblastine binding to unpolymerized tubulin in vitro. Our results suggest that LU103793 exerts its cytotoxic activity primarily through disruption of microtubule organization.

INTRODUCTION

Since the establishment of the screening and discovery program for cancer chemotherapeutics at the National Cancer Institute in 1960, several antimitotic drugs have been identified through the screening of natural products from plants, fungi, cyanobacteria, and marine organisms. Among them are the Dolastatin peptides, isolated from the marine shell-less mollusk Dolabella auricularia. Pettit and collaborators initially demonstrated that crude extracts of this sea hare strongly inhibited proliferation of the murine P388 lymphocytic leukemia cells. After more than a decade of continuous effort, they isolated and characterized the structures of 15 novel cytotoxic peptides designated Dolastatin 1–15 (1, 2). The most studied members of this family are the linear peptides Dolastatin 10 and Dolastatin 15 (3, 4). These compounds are potent inhibitors of cell proliferation at concentrations ranging from 10^{-11} to 10^{-9} M, in a wide variety of leukemia cell lines (5). When tested against different lymphoma cell lines, both drugs induced a block in mitosis, with more than 70% of the cells arrested in the G2-M phase (6).

As in the case of many other antimitotic drugs, the target for Dolastatin 10 and Dolastatin 15 appears to be microtubules. Treatment of cultured cells with either compound induced depolymerization of the microtubule network (7). Dolastatin 10 and Dolastatin 15 inhibit microtubule assembly in vitro, as well as tubulin-dependent GTP hydrolysis. However, only Dolastatin 10 inhibits nucleotide exchange and binding of vinblastine or vincristine to tubulin heterodimers (8).

Although the synthetic Dolastatin 10 and Dolastatin 15 display the same cytotoxic activity in vitro as the naturally occurring products (7), their chemical synthesis will be described elsewhere;2 vinblastine sulfate was purchased from Perkin Elmer Cetus (Emeryville, CA), and taxol was a gift from Bristol-Myers Squibb (Willington, CT). [γ-32]P-GTP was purchased from DuPont New England Nuclear (Bedford, MA), and G-32P-labeled Vinblastine sulfate was from Amersham (Little Chalfont, England). Drug stock solutions were made in deionized water (LU 103793, vinblastine) or DMSO (taxol) and kept in aliquots at -20°C. For each experiment, fresh solutions were made in buffer or medium to an adequate concentration.

Antibodies. Mouse anti-α-tubulin antibody (DM1A) was purchased from Sigma Chemical Co. (St. Louis, MO); human autoimmune antinuclear carcinoma (SNP) was a gift from Dr. R. Balczon (University of South Alabama), FITC-conjugated horse anti-mouse antibody was from Vector Laboratories (Burlingame, CA), and tetramethylrhodamine B isothiocyanate-conjugated goat anti-human antibody was from Pierce Chemical Co. (Rockford, IL).

ASSAY FOR CYTOTOXICITY. The effect of LU103793 on cell proliferation was determined with the use of a standard MTT-based colorimetric assay (9). Briefly, diluted drug (50 µl; 10^{-4} to 10^{-6} M final concentrations) or medium alone was added to exponentially growing cells (3.0 x 10^5/well) in a 96-well plate. After incubation for 72 h at 37°C, 5% CO_{2} in 95% air plate. After incubation for 72 h at 37°C, 5% CO_{2}, MTT (50 µl, 3 mg/ml; Sigma) was added to each well and incubated for 5 h at 37°C. SDS (2%, 50 µl, pH 2) was then added to the cultures and incubated for 16–18 h at 37°C to allow formazan solubilization. The absorbance at 550 nm of each well was measured with the use of a microplate reader (Molecular Devices) interfaced with a computer. Data were analyzed, and the IC_{50} was determined by an in-house computer program.

Flow Cytometry. Flow cytometric analysis of cellular DNA content by propidium iodide (50 µg/ml, Sigma) staining was performed as described elsewhere.

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1 To whom requests for reprints should be addressed, at BASF Bioresearch Corporation, 100 Research Drive, Worcester, MA 01605.

2 B. Janssen and A. Haupt, unpublished observations.

3 The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo- lium bromide; MAP, microtubule-associated proteins; MTP, microtubule protein; PENG, 100 mM PIPES-NaOH-1 mM EGTA-1 mM MgSO_{4}-1 mM GTP (pH 6.9); FACS, fluorescence-activated cell sorting; PI, phosphate.

MATERIALS AND METHODS

Cell Culture. HeLa S3 (epithelial-like cells from carcinoma of human cervix) and HT-29 (human colon adenocarcinoma) were purchased from American Type Culture Collection (Rockville, MD); MDA-MB 345 (human mammary carcinoma) was obtained from M. D. Anderson Cancer Center (Houston, Texas). Cells were grown in monolayers at 37°C, 5% CO_{2} in DMEM (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM glutamine, and 10 mM HEPES (pH 7; GIBCO-BRL).

Drugs and Other Chemicals. LU103793 was made at BASF and its synthesis will be described elsewhere;2 vinblastine sulfate was purchased from Perkin Elmer Cetus (Emeryville, CA), and taxol was a gift from Bristol-Myers Squibb (Wallingford, CT). [γ-32P]-GTP was purchased from DuPont New England Nuclear (Bedford, MA), and G-32P-labeled Vinblastine sulfate was from Amersham (Little Chalfont, England). Drug stock solutions were made in deionized water (LU 103793, vinblastine) or DMSO (taxol) and kept in aliquots at -20°C. For each experiment, fresh solutions were made in buffer or medium to an adequate concentration.

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Flow Cytometry. Flow cytometric analysis of cellular DNA content by propidium iodide (50 µg/ml, Sigma) staining was performed as described elsewhere.
Previously (10). Cells were grown at 5 x 10⁴ cells/ml in 6-well plates for 24 h and treated as described in "Results." After treatment, cells were trypsinized, rinsed with PBS, and fixed with ice-cold 70% ethanol. After 1 h at 4°C, cells were rinsed with PBS and treated with RNase (25 µg/ml, DNase free) for 30 min at room temperature to decrease RNA fluorescence. After propidium iodide staining, cells were analyzed in a Becton Dickinson FACS II. The percentage of cells in G₁, S, and G₂-M were calculated with the use of the CellFIT program (Becton Dickinson).

**Indirect Immunofluorescence.** Cells were plated on glass coverslips and grown for 24–30 h before drug exposure. After treatment, cells were fixed and stained as described previously (11). Briefly, cells were fixed for 10 min in 10% formalin-2 mM EGTA in PBS (EM Sciences, Fort Washington, PA). The formalin solution was removed, replaced by cold methanol, and the plate was incubated at −20°C for 10 min. After fixation, the cells were rinsed with PBS in 37°C for 1 h, and was followed by 3 rinses in PBS/BSA. The secondary antibodies, FITC conjugated and/or tetramethylrhodamine B isothiocyanate conjugated, (diluted 1:1500) were added at 37°C for 1 h. After they were stained with 4',6-diamidino-2-phenylindole (0.1 µg/ml) for 2 min, the cover-slips were mounted on glass slides with the use of Vectashield (Vector Laboratories). The slides were analyzed in a Zeiss Axioplan microscope equipped with an epillumination, ×63 Plan-Apochromat and Plan-Neofluor ×100 objectives and the appropriate filters.

**Purification of Microtubule Protein and Tubulin.** Bovine MTP consisting of approximately 70–80% tubulin and 20–30% MAP was isolated by two cycles of depolymerization and polymerization following the Tiwari and Suprenant protocol (12) with minor changes. The second polymerization cycle was carried out in the absence of DMSO, and polymerized microtubules were stored frozen as pellets at −80°C. Tubulin was purified from MTP suspension by ion exchange chromatography with the use of P11-phosphocellulose as described previously (13).

Before use, MTP pellets were submitted to one cycle of depolymerization in PEMG. After 20–30 min at 4°C, suspensions were centrifuged at 39,000 x g for 30 min at 4°C, and the protein concentration in the supernatant was estimated with the use of the Coomassie Plus kit (Pierce) and BSA as a standard.

**Turbidity Assays.** The in vitro assembly of microtubules was followed by turbidimetry at 350 nm for 20 min at 37°C in an AVIV spectrophotometer according to Gaskin et al. (14). Reaction mixtures contained 2 mg/ml of unpolymerized MTP or 3–4 mg/ml of tubulin in PEMG and various concentrations of drug. The mixtures were preincubated at 4°C for 5–10 min, transferred to the cuvettes, and assayed. IC₅₀ was determined by comparison of the final 350 nm reading of the reactions in the presence and absence of drug.

**Microtubule Disassembly Assays.** Microtubule disassembly was measured by collecting assembled microtubules by centrifugation through a sucrose cushion (15). Briefly, the MTP (2–2.5 mg/ml) suspension was polymerized for 20 min at 37°C in the presence or absence of either 10 µM taxol or 5% DMSO. After 20-min incubation with or without LU103793, samples (50 µl) were immediately layered on top of a 100-µl cushion of 50% sucrose in PEMG and centrifuged (Rotor TLA-100; 150,000 x g for 1 h at 37°C; Beckman TL100 Optima ultracentrifuge). The supernatants were discarded, and the pellet and walls of the tubes were carefully rinsed with warm PEMG (200 µl). The MTP pellets were resuspended in 0.1 N NaOH, and the protein concentration was determined with the use of the Coomassie Plus kit (Pierce).

**Centrifugal Gel Filtration.** The assays were performed with the use of BioSpin6 columns (Bio-Rad, Hercules, CA) as described previously (16). Columns were equilibrated in reaction buffer [100 mM 2-(N-morpholino)ethanesulfonic acid-0.5 mM MgCl₂ (pH 6.9)] and centrifuged at 1100 x g for 2 min. Meanwhile, reactions (220 µl) containing 2 µg purified tubulin in reaction buffer, 1 µM β3-labeled vinblastine sulfate (5 Ci/mmol), and the drug of interest were incubated at 22°C. After 20-min incubation, triplicate 50-µl aliquots of each reaction were applied onto precentrifuged columns and spun at 1110 x g for 4 min. The radioactivity and protein concentration in the column filtrates was determined and averaged.

**GTP Hydrolysis Measurements.** Microtubule-assembly-dependent GTPase activity was determined by measuring the Pi release from [γ-32P]GTP after drug incubation, with the use of the method of Penningroth and Peterson (17). Reactions (120 µl) contained 240 µg purified tubulin; 100 µM [γ-32P]GTP (100 Ci/mmol); and 0, 10, or 20 µM LU103793 in PEM. A mock reaction containing only buffer and 100 µM [γ-32P]GTP was included in each experiment. Samples were incubated at 37°C, and at timed intervals, 10-µl aliquots were removed and quenched in 400 µl of 6% (v/v) acetic acid, 2.5 mM KH₂PO₄, and 10% (w/v) activated charcoal. The slurries were mixed, spun for 15 min, and γ-32P released during the reaction was measured in the supernatant.

**Electron Microscopy.** Samples from microtubule disassembly assays were either diluted 1:5 in PEMG or directly pipetted onto Formvar carbon-coated grids. The microtubules were negatively stained with 1% uranyl acetate and examined in a Phillips EM301 electron microscope.

**RESULTS**

**Effects of LU103793 on Cell Proliferation and Cell Cycle Progression.** We evaluated the antiproliferative effect of LU103793 on three human carcinoma cell lines: colon (HT-29); cervical (HeLa S3); and breast (MDA-MB435) using a MTT assay. Cells were grown in the continuous presence of LU103793 (10⁻¹² to 10⁻⁴ M) for 72 h. Proliferation was inhibited by 50% at concentrations of 0.62 nM (HT-29), 0.7 nM (HeLa S3) and 0.12 nM (MDA-MB435). This activity is similar to that reported for Dolastatin 10 and Dolastatin 15 on leukemia cells, where IC₅₀ ranged between 0.02 and 0.8 nM for Dolastatin 10 and between 0.08 and 1 nM for Dolastatin 15 (5).

The effects of LU103793 on the cell cycle was investigated by flow cytometric analysis of cellular DNA content with the use of propidium iodide staining. In all three cell lines studied, 24 h incubation with LU103793 induced a dose-dependent arrest in G₂-M (Fig. 2). Control cells and cells incubated with 1 nM LU103793 had 10–20% of the population in G₂-M (Fig. 2, a and b), whereas cells treated with 10 or 100 nM for 24 h had 70–90% of the cells in mitosis (Fig. 2, c and d). This mitotic arrest was also time dependent; cell lines treated with 10 mM LU103793 for 4, 7, and 24 h had approximately 30, 50, and 80% of the cells arrested in G₂-M, respectively (data not shown).

**Inhibition of Microtubule Assembly and GTP Hydrolysis in Vitro.** Several drugs that induce accumulation of cells in G₂-M are known to interact with microtubules (18, 19). We used turbidity measurements to investigate the effect of LU103793 on microtubule polymerization in vitro. Fig. 3A shows the concentration-dependent inhibition of microtubule assembly by LU103793 with the use of MTP at 2 mg/ml. The concentration of LU103793 necessary to inhibit the microtubule polymerization by 50% was approximately 7 µM. The IC₅₀ determined for LU103793 is between the reported IC₅₀ for Dolastatin 10 and Dolastatin 15, 1.2 and 23 µM, respectively (8). An IC₅₀ of approximately 5 µM was observed with purified tubulin (3 mg/ml), suggesting that the inhibitory effect is due to a direct interaction with tubulin and not with MAPs (data not shown).
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Fig. 2. Dose-dependent effect of LU103793 treatment on cell cycle. HT-29, HeLa S3, and MDA-MB435 cells were incubated for 24 h in the absence (column a) or presence of 1, 10, and 100 nM LU103793 (columns b–d, respectively).

We measured the Pi release during assembly of microtubules from MAP-depleted tubulin (2 mg/ml). As a consequence of the inhibitory effect on microtubule assembly, there was a decrease in the tubulin-dependent GTP hydrolysis rates in the presence of LU103793. As shown on Fig. 3B, this decrease was dose dependent. A 37 and 70% inhibition of Pi release was observed when 10 and 20 μM LU103793, respectively, were added to the reaction containing tubulin and [γ-32P]GTP.

Induction of Microtubule Depolymerization. We further investigated the in vitro interaction of LU103793 with microtubules by centrifugation assay (see “Materials and Methods”). As shown in Fig. 4A, LU103793 caused a concentration-dependent disassembly of microtubules that had been polymerized previously to steady state. Stabilization of microtubules by either 5% DMSO or 10 μM taxol during the assembly period significantly reduced the disassembly effect observed with LU103793. Electron microscopic examination of samples before centrifugation showed that most of the microtubules incubated in the presence of 50–100 μM LU103793 were disassembled, and protofilaments were transformed into ring structures. The remaining microtubules often displayed curled protofilaments at the ends (Fig. 4B).

Binding of Vinblastine to Tubulin in the Presence of LU103793. Several antimitotic drugs of unrelated chemical structure are known to inhibit the binding of vinblastine to tubulin (8, 20). As shown in Table 1, LU103793 did not inhibit binding of 3H-labeled vinblastine to tubulin, even at concentrations as high as 200 μM, although addition of 100 μM vinblastine to the reaction mixture almost abolished the binding of labeled vinblastine to tubulin. In additional experiments, we did not detect significant inhibition of colchicine binding to tubulin by LU103793 (data not shown).

Effect of LU103793 on Cellular Microtubules and Chromosome Distribution. Microtubules play a vital role in several cellular processes, such as determination of cell shape, motility, intracellular transport, and accurate chromosomal segregation during mitosis. Using indirect immunofluorescence and 4',6-diamidino-2-phenylindole staining, we analyzed the effect of LU103793 on the organization of the cellular microtubule network and localization of the chromosomes on the mitotic spindles. The three cell lines studied (MDA-MB435, HeLa S3, and HT-29) were very sensitive to LU103793 treatment at concentrations as low as 0.5 nM. MDA-MB435 cells were selected to analyze the effect on interphasic microtubules because of their high

Fig. 3. Inhibition of microtubule assembly (A) and tubulin-dependent GTP hydrolysis (B) by LU103793. A, microtubule proteins (2 mg/ml) were incubated at 37°C in the absence or presence of 5–10 μM LU103793, and the turbidity was monitored at 350 nm, 37°C. Abs, absorbance units. B, P11-phosphocellulose-tubulin was incubated in the absence (○) or the presence of 10 (●) or 20 (■) μM LU103793 at 37°C. Tubulin-dependent GTPase activity was determined by measuring the Pi release from [γ-32P]GTP after drug incubation at 37°C as described in “Materials and Methods.” Data are from one representative experiment repeated three times with similar results.
Fig. 4. Effect of LU103793 on preassembled microtubules. A, microtubule protein (2.5 mg/ml) was assembled to steady state for 20 min, incubated with different concentrations of LU103793 for 20 min, and centrifuged as described in “Materials and Methods.” Microtubules preassembled in the absence of stabilizing agents (•) are sensitive to LU103793 and tend to depolymerize. Microtubules assembled in the presence of 5% DMSO (○) or 10 μM taxol (□) were protected against the destabilization caused by LU103793. B, electron micrographs of negative stained nonstabilized microtubules after incubation for 20 min in the presence of 50 μM LU103793. Coiled protofilaments at the ends (short arrow) and ring structures in the background (long arrow) are indicated. Data are from one representative experiment repeated three times with similar results. Bar, 0.25 μm.

Table 1 Effect of LU103793 on the binding of ^3H-labeled vinblastine to tubulin

<table>
<thead>
<tr>
<th>Addition</th>
<th>^3H-labeled vinblastine (nmol bound/mol tubulin)</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.6 ± 0.9</td>
<td>100 ± 3.4</td>
</tr>
<tr>
<td>Vinblastine, 100 μM</td>
<td>1.45 ± 0.11</td>
<td>5.5 ± 1.4</td>
</tr>
<tr>
<td>LU103793, 5 μM</td>
<td>28.3 ± 1.3</td>
<td>108.8 ± 4.6</td>
</tr>
<tr>
<td>LU103793, 50 μM</td>
<td>26.7 ± 1.7</td>
<td>102.7 ± 6.5</td>
</tr>
<tr>
<td>LU103793, 100 μM</td>
<td>27.1 ± 0.9</td>
<td>104.2 ± 3.3</td>
</tr>
<tr>
<td>LU103793, 200 μM</td>
<td>22.7 ± 1.7</td>
<td>87.3 ± 6.6</td>
</tr>
</tbody>
</table>

* Binding of ^3H-labeled vinblastine to tubulin was determined by centrifugal gel filtration. Reactions containing 2 mg/ml tubulin, 1 μM ^3H-labeled vinblastine (5 Ci/mmole). After 20-min incubation at 22°C, the bound vinblastine was determined as described in “Materials and Methods.” Values are expressed as mean ± SD of two separate experiments carried out in triplicates.

expression of tubulin and extensive network of microtubules, whereas HT-29 and HeLa S3 cells were used for studies on the mitotic spindles.

The network of MDA-MB435 cells was disrupted by incubation with 2–50 nM LU103793 for 20 h, with microtubules much shorter than the untreated controls. As shown on Fig. 5, the degree of microtubule depolymerization was concentration dependent. Treatment with 2 nM LU103793 induced slight depolymerization of microtubules in interphasic MDA-MB435 cells (Fig. 5, A and B). After 18–20 h incubation with 5 or 10 nM LU103793, significant effect on the microtubules integrity was observed. In general, the microtubule network was considerably less dense and the individual microtubules...
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Fig. 5. Microtubules and centrosome distribution in MDA-MB435 cells after 18 h incubation in the presence of the following amounts of LU103793: A, 0 nM (untreated cells); B, 2 nM; C and D, 5 nM; E and F, 10 nM. A, B, C and E, antitubulin immunofluorescence; D and F, ant centrosome immunofluorescence.

were much shorter than the untreated controls. It is interesting that cells treated with 5 or 10 nM drug often displayed more than one microtubule-organizing center (Fig. 5, C and E). Indirect immunofluorescence of centrosomes using a human autoimmune anticentrosome serum showed staining for centrosomal material in the multiple microtubule-organizing centers (Fig. 5, D and F). Incubation with 25 or 50 nM LU103793 for 20 h caused complete depolymerization of microtubules in most cells (not shown). Addition of LU103793 (2–50 nM) caused a large proportion of the cells to detach. In agreement with the block in G2-M observed by FACS analysis, fixation and staining of the cells in suspension revealed that they were in mitosis and displayed abnormal spindle morphology (data not shown).

Time course experiments revealed that 2-h treatments with either 10, 25, and 50 nM LU103793 were enough to cause severe microtubule depolymerization (Fig. 6, A–C). After 6-h treatment, cells treated with 10 and 25 nM displayed no microtubule (Fig. 6, D and E). Cells treated with 5 nM LU103793 for 6 h had a dense but less organized microtubule network (Fig. 6F).

HeLa S3 cells have been used as a cell model to analyze the effect of Vinca alkaloids and other antimitotic drugs on microtubules of mitotic cells (11, 19). We have observed that the effects of LU103793 on HeLa S3 cells were very similar to those induced by vinblastine, in that both drugs caused formation of abnormal mitotic spindles and chromosomal distribution (Fig. 7). The frequency and severity of abnormalities in mitotic spindle morphology and the chromosomal arrangement after 20-h treatment was dose dependent. At a low concentration (2 nM LU103793), there was a high percentage of mitotic cells with chromosomes located outside the equatorial metaphase plate, near the poles (Fig. 7, C and D). With 10 nM LU103793, many spindles were bipolar, but astral microtubules were longer than in the controls and central spindles were shorter. Some spindles were no longer bipolar and had chromosomes arranged in a circular fashion (Fig. 7, E and F). As the drug concentration was increased (≥25 nM), the spindle microtubules were depolymerized, and in some cases spindles were reduced only to spots (Fig. 7, G and H). Curiously, many of the cells that were not in mitosis after 20-h treatments with 2, 5, or 10 nM drug had a normal density of microtubules and were often multinucleated (Fig. 7, I and J).

HT-29 cells responded to LU103793 treatments in a manner similar to MDA-MB435 and HeLa S3 cells. They were also blocked in mitosis, displaying abnormal mitotic spindles and abnormal chromosomal distribution (data not shown).

Discussion

LU103793, a synthetic analogue of the natural peptides Dolastatin 10 and 15, retains the ability to inhibit cell proliferation in vitro and has excellent antitumor activity in a number of in vivo tumor models when administered systemically.4 Here, we suggest that the cytotoxic activity of this compound is likely to be associated with disruption of the microtubule assembly/disassembly process.

Interactions with Microtubules in Vitro. Turbidity assays showed potent inhibition of microtubule polymerization that was

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Fig. 6. Microlubule arrangement on MDA-MB435 cells treated with LU103793 at the indicated dose and period: A, 50 nM for 2 h; B, 25 nM for 2 h; C, 10 nM for 2 h; D, 25 nM for 6 h; E, 10 nM for 6 h; F, 5 nM for 6 h.

Independent of the presence of MAPs. Similar to vinblastine and Dolastats (8), concentrations of drug that are substoichiometric to tubulin-inhibited microtubule assembly. LU103793 induced a long lag phase (10–15 min) and a slow rate of polymerization, whereas the untreated microtubules reached steady state in about 2–3 min. Thus, we suggest that LU103793 may interfere with both the nucleation and elongation phases of microtubule assembly.

LU103793 also induced microtubule depolymerization in vitro, as do several other tubulin-binding agents (18, 21–24). Ultrastructural analysis of microtubules treated with LU103793 revealed protofilaments and ring structures similar to the ones reported for microtubules in the process of shortening (25, 26). Mandelkow et al. (25) have shown that during stochastic transition from growth to shortening ("catastrophe"), microtubules disassemble primarily into oligomers. The ends of the microtubules in the process of shortening appear to have protofilaments coiled inside out. Thus, binding of LU103793 to microtubules may destabilize the interactions between protofilaments, either by a conformational change in the tubulin heterodimers or by the formation of a break point. As with vinblastine, the depolymerization of microtubules into helically coiled protofilaments and ring structures upon drug incubation is proposed to be a result of the weakening of the lateral interactions between protofilaments after drug binding (27, 28).

Photoaffinity labeling and competition studies have shown that multiple drug-binding sites exist on tubulin (27–32). LU103793 did not inhibit binding of radiolabeled vinblastine to tubulin heterodimers, nor did it interfere with colchicine and taxol binding (data not shown). This implies that either LU103793 binds to different sites on tubulin, or that binding of LU103793 to unpolymerized tubulin is too weak to enable the displacement of bound vinblastine. Very little is currently known about the binding of Dolastatin 15 to tubulin. Bai et al. (7) showed that Dolastatin 15 did not have biochemical effects on tubulin in contrast to Dolastatin 10. The authors speculated that the difference must be due to a weaker binding of Dolastatin 15. Availability of radiolabeled LU103793 and Dolastatin 15 should help in the future to elucidate the binding sites of these compounds on tubulin.

Effect of LU103793 on Cytoplasmic Microtubules and Correlation with Mitotic Arrest. In the case of MDA-MB435 and HT-29 lines, immunofluorescence staining at moderate drug concentrations (5–25 nM) indicated a severe disintegration of the cellular microtubule network. In agreement with the FACS analysis, a considerable increase in the number of mitotic cells on LU103793-treated cultures was evident. In most HeLa S3 and HT-29-mitotic cells, the spindles and the distribution of metaphase chromosomes were irregular and strongly resembled the effects caused by vinblastine, podophyllotoxin, and nocodazole (19), suggesting that the G2–M arrest is caused by the generation of nonfunctional mitotic spindles.

Several other antimitotic drugs such as the Vinca alkaloids, Dolastats, cryptophycin, or estramustine are known to induce microtubule depolymerization in vivo (7, 19, 20, 22, 23). In the case of the Dolastatin 10 and Dolastatin 15, it has been reported that treated Chinese hamster ovary cells showed a depolymerized microtubule network and are frequently multinucleated, but there are no observations about the effect on spindles and chromosomal organization (7).
It is of interest that aberrant spindles and mitotic arrest were observed at concentrations 10-fold lower than necessary to trigger visible microtubule depolymerization. The results of Jordan et al. (11, 33–35) indicate that the generation of aberrant spindles occurs due to suppression of microtubules dynamics and stabilization of the attenuated stage. Considering that microtubules from spindles are highly dynamic and display a turnover rate 20-fold faster than interphasic microtubules (36), interference with their dynamic behavior could result in morphologically abnormal spindles. Consequently, the anti-proliferative effect of these drugs could be caused primarily by
interference with the dynamics of spindle microtubules rather than depolymerization of the microtubules. Studies of the effects of LU103793 on microtubules dynamics in vitro are currently ongoing. A lack of quantitative correlation between inhibition of cell proliferation and inhibition of microtubule assembly in vitro is common among other drugs. In the case of LU103793, the concentration necessary to affect microtubule polymerization/depolymerization in vitro is about 1000-fold higher than the concentration necessary to induce cytotoxicity in cultured cells. There are different hypotheses to explain this discrepancy: (a) LU103793 could be accumulated intracellularly to significantly higher concentrations, as occurs with taxol and vinblastine in HeLa S3 cells (11, 35). Using the same methodology described by these authors, we found that LU103793 indeed accumulated in HeLa S3, but only to levels that did not exceed 10–20-fold. Therefore, it is unlikely that intracellular accumulation alone can account for the difference; (b) LU103793 binds to other targets which would then contribute to the inhibition of proliferation and to the destabilization of microtubules. Such a target could be a microtubule severing and/or disassembly factor, functionally similar to those purified from Xenopus extracts (37, 38); (c) LU103793 binds with much higher affinity to tubulin from human tumor cell lines than to tubulin from bovine brain. Differences in isotype representation between these tissues as well as species-specific sequence differences have been reported (39) and could be responsible for differential binding affinity of a drug; and (d) intracellular metabolism to a more active compound could account for the limited correlation between observed in vitro and in vivo effects. In fact, a major metabolite of LU103793 has a lower IC50 in the turbidity assays than does LU103793. Further studies with this derivative are in progress.

Our studies indicate that LU103793 is a potent new antimitotic compound. The therapeutic value of LU103793 for cancer treatment is now being examined in clinical trials.

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REFERENCES

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