Mechanism of Action of the Microtubule-Targeted Antimitotic Depsipeptide Tasidotin (Formerly ILX651) and Its Major Metabolite Tasidotin C-Carboxylate


Abstract

Tasidotin (ILX-651), an orally active synthetic microtubule-targeted derivative of the marine depsipeptide dolastatin-15, is currently undergoing clinical evaluation for cancer treatment. Tasidotin inhibited proliferation of MCF7/GFP breast cancer cells with an IC_{50} of 63 nmol/L and inhibited mitosis with an IC_{50} of 72 nmol/L in the absence of detectable effects on spindle microtubule polymer mass. Tasidotin inhibited the polymerization of purified tubulin into microtubules weakly (IC_{50} ~ 30 \mu mol/L). However, it strongly suppressed the dynamic instability behavior of the microtubules at their plus ends at concentrations ~ 5 to 10 times below those required to inhibit polymerization. Its major actions were to reduce the shortening rate, the switching frequency from growth to shortening (catastrophe frequency), and the fraction of time the microtubules grew. In contrast with all other microtubule-targeted drugs thus far examined that can inhibit polymerization, tasidotin did not inhibit the growth rate. In contrast to stabilizing plus ends, tasidotin enhanced microtubule dynamics, tasidotin did not inhibit the growth rate. In contrast to stabilizing plus ends, tasidotin enhanced microtubule dynamic instability at minus ends, increasing the shortening length, the fraction of time the microtubules shortened, and the catastrophe frequency and reducing the rescue frequency. Tasidotin C-carboxylate, the major intracellular metabolite of tasidotin, altered dynamic instability of purified microtubules in a qualitatively similar manner to tasidotin but was 10 to 30 times more potent. The results suggest that the principal mechanism by which tasidotin inhibits cell proliferation is by suppressing spindle microtubule dynamics. Tasidotin may be a relatively weak prodrug for the functionally active tasidotin C-carboxylate. [Cancer Res 2007;67(8):3767–76]

Introduction

Antimitotic peptides and depsipeptides are microtubule-targeted compounds that are receiving considerable attention for the possible treatment of cancer. Originally isolated as natural products from marine sources, they are linear structures composed of five to seven amino acid residues. The depsipeptide dolastatin-15 (Fig. 1), initially isolated from the Indian Ocean sea hare, inhibits polymerization of human malignant cell lines in vitro and is active in a broad range of animal tumor models (1–3). Dolastatin-15 inhibits cell cycle progression at mitosis and seems to target microtubules and their tubulin subunits (2, 4). A synthetic analogue of dolastatin-15, cemadotin (LU103793), has been evaluated in phase I and II clinical trials for the possible treatment of cancer (5, 6). Whereas several patients achieved stable disease, there were no objective responses, and the drug caused significant toxicities, most notably, hypertension (5).

Because of their attractive therapeutic potential, a new generation of dolastatins represented by tasidotin (N,N-dimethyl-L-valyl-l-valyl-N-methyl-l-valyl-l-prolyl-l-proline-tert-butylamide hydrochloride, formerly ILX651; Fig. 1) was developed that possesses several advantages over earlier dolastatins (7, 8). Preclinical studies in which the cell growth–inhibitory activity of tasidotin was analyzed in human tumor cell lines using a standard tetrazolium dye/growth inhibition assay, including K562 erythroid-leukemia (IC_{50}, 20 nmol/L), OVCAR-3 ovarian carcinoma (IC_{50}, 60 nmol/L), HT-29 colon carcinoma (IC_{50}, 60 nmol/L), H460 lung carcinoma (IC_{50}, 0.5 \mu mol/L), and LOX melanoma (IC_{50}, 1 \mu mol/L), indicated that tasidotin possesses good cytotoxic activity against a wide range of solid tumor cells. In mouse xenograft models, tasidotin induced complete responses in early- and late-stage breast carcinoma, melanoma, and prostate cancer. In the P388 murine leukemia model, tasidotin significantly increased survival time (9). Importantly, toxicity studies in rats and dogs showed that tasidotin exerts <10% of the cardiovascular toxicity of cemadotin (7). Because of its promising antineoplastic activity and reduced toxicity, tasidotin was recently evaluated in phase I clinical trials and is currently undergoing phase II trials for the treatment of cancer (7, 8, 10).

Both dolastatin-15 and cemadotin bind to tubulin with moderate affinity and inhibit microtubule polymerization both in vitro and in cultured tumor cells (2, 11, 12). Cemadotin also suppresses microtubule dynamic instability in vitro (11). Microtubules display two unusual dynamic behaviors that are critical for proper spindle function during mitosis, dynamic instability, and treadmilling (13–17). Cells with impaired spindle microtubule dynamics induced by very low concentrations of antimitotic drugs, such as the vinca alkaloids or taxol, display misaligned chromosomes with abnormal spindles and are either completely arrested at mitosis or exhibit slow progression through mitosis (14, 15). Suppression of spindle microtubule dynamics with minimal effect on the microtubule mass is thought to be a principal mechanism by which many microtubule-targeted drugs, acting through different molecular interactions with tubulin and microtubules, inhibit cell cycle progression at mitosis and kill tumor cells (14–16).

Only limited data have been published on the ability of tasidotin to inhibit microtubule polymerization (18, 19), and the effects of

Requests for reprints: Leslie Wilson, Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, CA 93106-9610. Phone: 805-893-2819; Fax: 805-893-8949; E-mail: Wilson@ilfisc.ucsb.edu.

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4 Genzyme, unpublished data.

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tasidotin on microtubule dynamics have not yet been studied. Here, we examined the ability of tasidotin to inhibit proliferation and mitotic spindle organization in MCF7/GFP breast tumor cells in relation to its ability to alter microtubule polymerization and dynamic instability. The actions of the major metabolite of tasidotin, tasidotin C-carboxylate (also called N-desbenzylaminocemudotin; refs. 20, 21), was also examined. We found that at its IC50 for inhibition of proliferation, tasidotin inhibited mitosis without appreciably depolymerizing or disorganizing the spindle microtubules. In addition, tasidotin only weakly inhibited tubulin polymerization into microtubules in vitro, but it strongly suppressed dynamic instability at microtubule plus ends and less strongly enhanced it at minus ends. Interestingly, tasidotin C-carboxylate altered microtubule dynamic instability in a manner qualitatively similar to that of tasidotin but was >10 times more potent. Thus, tasidotin may be a relatively weak prodruk, and its metabolite, tasidotin C-carboxylate, may be the more active intracellular form of the compound.

Materials and Methods

Purification of microtubule protein and tubulin. Bovine brain microtubule protein consisting of ~70% tubulin and 30% microtubule-associated proteins (MAPs) was purified and stored as described previously (22). MAP-free tubulin (>99% tubulin) was purified from the microtubule protein by phosphocellulose column chromatography (23). Microtubule protein was polymerized at 37°C in PMME buffer [86 mmol/L PIPES, 36 mmol/L Mes, 1.4 mmol/L MgCl2, 1 mmol/L EGTA, and 1 mmol/L GTP (pH 6.8; ref. 24)] in the absence or presence of tasidotin or tasidotin C-carboxylate. MAP-depleted tubulin was polymerized in PMME buffer by nucleation with ~2- to 3-μm-long sea urchin axonemal microtubule seeds prepared in 10% DMSO and 10% glycerol (24). The final volume of seed to tubulin solution was 1:50, which reduced the glycerol and DMSO concentrations in the final suspension to 0.2% each. Polymerization was monitored turbidimetrically at 330 nm at 37°C.

Analysis of dynamic instability. The dynamic instability behavior of individual microtubules was carried out by video microscopy as described previously (11, 22, 25). Briefly, tubulin (20 μmol/L) was mixed with sea urchin axonemal seeds and polymerized to steady state (40 min, 37°C) in PMME buffer in the presence or absence of tasidotin or its metabolite. Dynamics were then measured for a maximum of 60 min. Plus ends were distinguished from minus ends as described previously (25). The catastrophe frequency (the switching frequency from the growing or attenuated states to shortening) and the rescue frequency (switching frequency from shortening to the growing or attenuated state) were determined as described previously (25). Between 30 and 40 microtubules were analyzed for each condition.

Cell culture. An MCF7 cell line stably transfected with green fluorescent protein (GFP)/α-tubulin (MCF7/GFP cells), which exhibit good sensitivity to microtubule targeted antimitotic drugs (e.g., see refs. 24, 26), were grown in DMEM supplemented with 5% fetal bovine serum (Atlanta Biological, Atlanta, GA), MEM nonessential amino acid solution (Sigma, St. Louis, MO; M7145), and Pen/Strep (Sigma; P0781) in a humidified incubator (37°C, 5% CO2) for 24 to 72 h. Doubling time was 29 h.

Immunofluorescence and electron microscopy. MCF7/GFP cells were prepared for immunofluorescence microscopy by a formalin/methanol fixation as previously described (24, 26). Fixed cells were treated with a mouse monoclonal antibody cocktail of anti-α-tubulin and anti-β-tubulin (DM1A/DM1B; 1:1,000; Sigma) followed by addition of FITC-conjugated secondary antibody (Sigma). Cells were visualized with a Nikon Eclipse E800 fluorescence microscope, and images were acquired using a Photometrics CoolSNAP HQ digital camera (Tucson, AZ). Microtubule mean lengths and number concentrations were determined by negative-stain electron microscopy (27).

Cell proliferation and mitotic arrest. MCF7/GFP cells were seeded at 3 × 104 per mL (2 mL per well) in six-well Costar culture dishes (Fisher Scientific, Pittsburgh, PA). After 24 h, control wells were harvested for counting, and fresh medium plus/minus tasidotin (0, 10, 25, 50, 75, 100, 200, and 300 mmol/L) was added to duplicate wells and incubated for an additional 20 to 24 h. Tasidotin was diluted from a 100 μmol/L stock solution in water. Media with detached cells were collected. Attached cells were collected by rinsing twice with 1 mL Versene (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L KH2PO4, 8.1 mmol/L Na2PO4, and 0.5 mmol/L EDTA), 1 mL trypsin, 1 mL trypsin-neutralizing solution, and 1 mL PBS. All solutions containing adherent and floating cells were pooled, centrifuged, resuspended in 100 μL PBS, and counted using a hemacytometer. Inhibition of proliferation was calculated by dividing the increase in cell number in the presence of tasidotin over 24 h by the increase in cell number in controls. The stated IC50 is the average of the IC50s determined by linear extrapolation for each of three experiments.

To determine mitotic indices (26), MCF7/GFP cells were plated, incubated with tasidotin for 20 to 24 h, and collected as described above. Cells were fixed with 10% formalin in PBS (20 min) followed by 10% methanol (10 min) and stained with 4,6-diamidino-phenylindole (Sigma) to visualize the DNA. Cells were scored as being either in interphase or in mitosis. Results are the mean ± SD of three independent experiments in which 300 cells were counted for each drug concentration.
Results

Effects of tasidotin and tasidotin C-carboxylate on microtubule nucleation and the final extent of polymerization. A major goal of this work was to determine how tasidotin and its major metabolite modulate the dynamic instability behavior of individual microtubules at their opposite ends. We wanted to use conditions in which the soluble tubulin and polymer mass were held constant, and we required optimal numbers and lengths of microtubules to measure their growing and shortening dynamics (25). Based upon the initial report indicating that 25 to 40 μmol/L tasidotin suppressed nucleation but did not appreciably affect the final extent of polymerization (19), we first determined the effects of tasidotin and its metabolite on the polymerization of self-nucleating MAP-rich microtubules in vitro. As previously reported (19), concentrations of tasidotin of 5 and 10 μmol/L delayed formation of microtubules for 10 to 20 min but did not reduce the eventual polymer mass (Fig. 2). If the polymerization delay were due to suppression of nucleation, the mean lengths of the microtubules would be expected to be longer than controls, and the number of microtubules would be reduced. As expected, 5 μmol/L tasidotin increased the mean length from 15.8 ± 0.3 μm (controls) to 37.5 ± 1.1 μm, and 10 μmol/L tasidotin increased it to 49.2 ± 1.7 μm (Fig. 2B). Tasidotin also decreased the microtubule number concentration from 5.2 × 10−10 per mL (controls) to 2.1 × 10−10 and 1.2 × 10−10 per mL at 5 and 10 μmol/L tasidotin, respectively (data not shown). Thus, tasidotin suppresses nucleation of MAP-rich microtubules. There were no appreciable differences in the MAP content of the microtubules polymerized in the absence and presence of tasidotin as determined by SDS-PAGE (data not shown). Thus, suppression of nucleation by tasidotin does not seem to be due to depletion of the MAPs.

As a result of suppression of nucleation, the effects of tasidotin on the final extent of polymerization were difficult to assess in the foregoing experiments. To determine the ability of tasidotin to reduce the polymer mass independent of its ability to suppress nucleation, we bypassed the nucleation reaction by initiating polymerization of pure tubulin with nucleating seeds (Materials and Methods). As shown in Fig. 2C, tasidotin did inhibit polymerization, but it did so weakly. Specifically, 10 μmol/L tasidotin reduced the final extent of polymerization by only ~10%. Substantial (45%) inhibition of polymerization required tasidotin concentrations of 25 μmol/L and higher (Fig. 2C).

Tasidotin C-carboxylate also inhibited polymerization of MAP-rich microtubules (Fig. 3A), but in contrast to tasidotin, it did not inhibit nucleation of the microtubules as determined by analysis of the lengths of the microtubules formed in the presence of the compound. Rather than increasing the mean lengths as would be expected if the metabolite suppressed nucleation, the mean lengths of the microtubules decreased. For example, 0.35 μmol/L metabolite, which inhibited polymerization by ~10%, reduced the mean length of the microtubules ~20% from 12.9 to 10.5 μm (data not shown).

In addition, tasidotin C-carboxylate inhibited polymerization much more strongly than tasidotin. Specifically, 5 μmol/L tasidotin did not detectably inhibit the final extent of polymerization of MAP-rich tubulin (Fig. 2A), whereas the same concentration of tasidotin C-carboxylate inhibited polymerization by ~60% (Fig. 3A). Furthermore, only 2 μmol/L metabolite was required to inhibit the assembly of MAP-free seeded microtubules by 60% to 70% (Fig. 3B). Half-maximal inhibition of seeded polymerization by tasidotin required >25 μmol/L compound but required only ~1 to 2 μmol/L metabolite (compare Fig. 2C and Fig. 3B). The potencies of tasidotin and tasidotin C-carboxylate on the polymerization of MAP-free microtubules are shown together in Fig. 3C. It is clear that tasidotin C-carboxylate inhibits polymerization ~20 to 40 times more strongly than the parent compound.

Tasidotin and its major metabolite strongly suppress dynamic instability at microtubule plus ends and moderately enhance dynamic instability at minus ends. We analyzed the effects of tasidotin and tasidotin C-carboxylate on the growing and shortening dynamics of individual microtubules at polymer mass steady state at plus and minus ends (Materials and Methods) by using compound concentrations and conditions in which the polymer mass was unaffected or only minimally reduced. The quantitative effects of tasidotin and its major metabolite on the plus-end dynamic instability variables are shown in Table 1A and B, respectively, and in Fig. 4A and B. Quite surprisingly, 5 μmol/L tasidotin (Table 1A) and tasidotin concentrations as high as 10 μmol/L (data not shown) did not inhibit the growth rate and only weakly decreased the length the microtubules grew during growth events. The lack of effect on the growth rate is unusual because all other microtubule-targeted drugs that can reduce the polymer mass, including cemadotin (10, 14), also reduce the growth rate. In contrast with its lack of effect on the growth rate, tasidotin strongly reduced the rate and extent of shortening (Table 1A; Fig. 4A). At a concentration of 10 μmol/L, tasidotin suppressed the shortening rate by 53% (from 28.4 to 13.3 μm/min; Fig. 4A). Tasidotin also significantly reduced the catastrophe frequency (Table 1; Fig. 4B). Tasidotin also strongly decreased the fraction of time the microtubules grew (Fig. 4C, C) and increased the fraction of time the microtubules spent in an attenuated (paused) state (Fig. 4C, D). At 2 μmol/L, tasidotin reduced the overall dynamicity, the total detectable extent of growth and shortening per unit time by 63% (Table 1A).

Interestingly, tasidotin C-carboxylate suppressed plus-end dynamics in a manner that was qualitatively similar to, but much stronger than, the parent compound (Table 1B; Fig. 4A–C). For example, a concentration of only 0.5 μmol/L tasidotin C-carboxylate suppressed the shortening rate by 82%, whereas a similar degree of suppression of this variable required a 10- to 20-fold higher concentration of tasidotin. The concentration of tubulin used in the assay was 20 μmol/L, far higher than the concentration of tasidotin C-carboxylate (0.05–0.5 μmol/L) required to suppress dynamics, indicating that the metabolite suppresses dynamics substoichiometrically to tubulin and thus must be acting directly on the microtubules rather than on the soluble tubulin pool.

We also determined the effects of tasidotin and tasidotin C-carboxylate on dynamic instability at minus ends. As shown in Table 1C, tasidotin enhanced dynamic instability at minus ends, predominantly by increasing the catastrophe frequency and the percentage of time the microtubules shortened. Tasidotin also increased the average length of the microtubules shortened during shortening events and decreased the rescue frequency. The metabolite enhanced dynamic instability at minus ends in a fashion qualitatively similar to tasidotin but was ~10 times more potent than the parent compound (Table 1C). In addition, the enhancement of dynamic instability at minus ends required considerably higher concentrations both of tasidotin and tasidotin C-carboxylate than those required to suppress dynamics at plus ends.

Inhibition of cell proliferation and mitosis in MCF7/GFP cells by tasidotin and its effects on microtubule organization in mitotic and interphase cells. We determined the relationship
between the ability of tasidotin to inhibit proliferation of MCF7/GFP cells and its ability to block cells at mitosis after 1 day of incubation with tasidotin. As shown in Fig. 5A, tasidotin inhibited proliferation with an IC\textsubscript{50} of 63 nmol/L and induced half-maximal accumulation of cells at mitosis at a similar concentration of 72 nmol/L.

In the absence of tasidotin, control mitotic metaphase cells displayed normal spindles with tightly congressed chromosomes and...
full arrays of kinetochore and interpolar microtubules with few astral microtubules (Fig. 5B). At approximately one half the IC_{50} (Fig. 1B; 37 nmol/L) and near the IC_{50} (Fig. 1B; 76 nmol/L) for tasidotin, most cells had normal or nearly normal bipolar spindles. A few of the spindles were abnormal, and some spindles were somewhat smaller than spindles in control cells. The fraction of cells with abnormal spindles increased as the tasidotin concentration was increased. In those spindles that remained bipolar, the chromosomes were loosely organized at the metaphase plate (Fig. 5B; 300 nmol/L). At tasidotin concentrations higher than the IC_{50} (Fig. 1B; 300 and 600 nmol/L tasidotin, respectively), mitotic cells contained mostly ball-shaped spheres of chromosomes with a few arrays of microtubules that were mostly disorganized; however, in a few spindles, most or all of the chromosomes had congressed to a loosely organized bipolar metaphase plate, with some chromosomes remaining at the poles and associated with astral microtubules that sometimes emanated from only one pole. At ~10 times the IC_{50} (Fig. 1B; 760 nmol/L tasidotin), very few microtubules remained in the spindles; those spindles that were bipolar had a few erratically curved thick microtubule fibers and ball-shaped spheres of chromosomes containing several foci of short, stubby microtubules. The presence of robust, normal, or nearly normal bipolar spindles at the IC_{50} for tasidotin indicates that the drug does not act primarily by depolymerizing the spindle microtubules but rather acts by modulating the dynamics of the spindle microtubules.

The organization of microtubules in tasidotin-treated interphase cells was also examined. Control interphase cells were well spread

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**Figure 3.** Inhibition of microtubule polymerization by tasidotin C-carboxylate. A, MAP-rich microtubule protein (3 mg/mL) was polymerized in the absence (○) or presence of 0.5 μmol/L (△), 2.0 μmol/L (△), 5 μmol/L (▼), or 10 μmol/L (○) tasidotin C-carboxylate. Polymerization was measured turbidimetrically at 350 nm. B, MAP-free tubulin (3 mg/mL) was polymerized by addition of microtubule seeds in the absence (○) or presence of 0.2 μmol/L (□), 0.5 μmol/L (△), 1 μmol/L (▼), or 2 μmol/L (○) tasidotin C-carboxylate. C, concentration dependence for the ability of tasidotin C-carboxylate (□) and tasidotin (○) to inhibit polymerization of purified tubulin into microtubules.
and contained normal dense networks of very fine microtubules (Fig. 5C). At the IC_{25} (Fig. 1C; 37 nmol/L) and close to the IC_{50} for tasidotin (Fig. 1C; 76 nmol/L), cells remained well spread, and whereas there was no depolymerization of the microtubules, they were more distinct and somewhat thicker or coarser than those in untreated interphase cells. At higher tasidotin concentrations (Fig. 1C, bottom right), the cells were often significantly smaller in area (they seemed to have “pulled back” or rounded), but the

### Table 1. Modulation of dynamic instability at plus and minus microtubule ends at steady state in vitro by tasidotin and tasidotin C-carboxylate

#### A. Effects of tasidotin at plus ends

<table>
<thead>
<tr>
<th>Concentration (µmol/L)</th>
<th>0 (Control)</th>
<th>0.5</th>
<th>2</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate (µm/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td>1.7 ± 0.8</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Shortening</td>
<td>28.4 ± 2.0</td>
<td>20.7 ± 2.0</td>
<td>23.5 ± 2.0</td>
<td>9.0 ± 0.7*</td>
</tr>
<tr>
<td>Length change (µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td>2.3 ± 0.7</td>
<td>2.2 ± 0.9</td>
<td>2.1 ± 0.8</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>Shortening</td>
<td>5.0 ± 0.5</td>
<td>4.6 ± 0.4</td>
<td>4.4 ± 0.4</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>Catastrophe frequency (events per min)</td>
<td>0.5 ± 0.01</td>
<td>0.4 ± 0.01</td>
<td>0.2 ± 0.01</td>
<td>0.2 ± 0.01*</td>
</tr>
<tr>
<td>Rescue frequency (events per min)</td>
<td>1.8 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Dynamicity (µm/min)</td>
<td>2.3</td>
<td>1.1</td>
<td>0.8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

#### B. Effects of tasidotin C-carboxylate at plus ends (control values are the same as in A)

<table>
<thead>
<tr>
<th>Concentration (µmol/L)</th>
<th>0.025</th>
<th>0.05</th>
<th>0.1</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate (µm/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td>1.7 ± 0.7</td>
<td>1.7 ± 0.7</td>
<td>1.5 ± 0.5</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>Shortening</td>
<td>25.4 ± 1</td>
<td>9.1 ± 0.5*</td>
<td>10.5 ± 0.7*</td>
<td>5.0 ± 0.5*</td>
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<tr>
<td>Length change (µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td>2.2 ± 0.6</td>
<td>1.7 ± 0.8</td>
<td>1.6 ± 0.4</td>
<td>1.5 ± 0.8</td>
</tr>
<tr>
<td>Shortening</td>
<td>4.6 ± 0.5</td>
<td>4.0 ± 0.4</td>
<td>4.7 ± 0.4</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Catastrophe frequency (events per min)</td>
<td>0.4 ± 0.01</td>
<td>0.2 ± 0.01*</td>
<td>0.2 ± 0.01*</td>
<td>0.2 ± 0.01*</td>
</tr>
<tr>
<td>Rescue frequency (events per min)</td>
<td>1.6 ± 0.1</td>
<td>1.3 ± 0.08†</td>
<td>1.2 ± 0.06†</td>
<td>0.9 ± 0.2*</td>
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<tr>
<td>Dynamicity (µm/min)</td>
<td>2.1</td>
<td>0.5</td>
<td>0.6</td>
<td>0.4</td>
</tr>
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</table>

#### C. Effects of tasidotin and tasidotin C-carboxylate at minus ends

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Tasidotin</th>
<th>Tasidotin C-carboxylate</th>
</tr>
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<tbody>
<tr>
<td>Rate (µm/min)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td>0.6 ± 0.05</td>
<td>1.5 ± 0.3</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Shortening</td>
<td>13.8 ± 1.1</td>
<td>17.4 ± 2.4†</td>
<td>15.5 ± 3.3</td>
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<tr>
<td>Length change (µm)</td>
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<td></td>
</tr>
<tr>
<td>Growth</td>
<td>0.9 ± 0.07</td>
<td>1.5 ± 0.1</td>
<td>0.4 ± 0.02</td>
</tr>
<tr>
<td>Shortening</td>
<td>2.8 ± 0.3</td>
<td>3.0 ± 0.1</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>Catastrophe frequency (events per min)</td>
<td>0.03 ± 0.003</td>
<td>0.1 ± 0.01†</td>
<td>0.1 ± 0.01†</td>
</tr>
<tr>
<td>Rescue frequency (events per min)</td>
<td>2.8 ± 0.5</td>
<td>1.8 ± 0.4*</td>
<td>0.3 ± 0.3*</td>
</tr>
<tr>
<td>Percentage time</td>
<td></td>
<td></td>
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<tr>
<td>Growing</td>
<td>11</td>
<td>15</td>
<td>0.4</td>
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<tr>
<td>Shortening</td>
<td>0.6</td>
<td>2.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Attenuated</td>
<td>88</td>
<td>82.5</td>
<td>97</td>
</tr>
<tr>
<td>Dynamicity (µm/min)</td>
<td></td>
<td>0.2</td>
<td>0.4</td>
</tr>
</tbody>
</table>

NOTE: Data are mean ± SE. Tests of significance were not done on dynamicity, an overall variable.  
*Significantly different than controls at the 99.99% (Student’s t test).  
†Significantly different than controls at the 97% confidence levels (Student’s t test).
interphase microtubules were still prominent and densely arrayed. At first glance, microtubules seemed not to have depolymerized significantly, although such an appearance is probably misleading because the cells were rounded, smaller in area, and thus thicker. However, in the regions of the nucleus (particularly visible over the nucleus), it was clear that many of the microtubules were short and stubby and often had completely depolymerized into aggregates. Many cells were multinucleate after incubation with tasidotin (Fig. 5C; 37, 300, and 600 nmol/L).

**Discussion**

We have analyzed the mechanism of action of tasidotin (formerly ILX651), a synthetic microtubule-targeted derivative of dolastatin-15, and its major metabolite, tasidotin C-carboxylate, on the polymerization and dynamics of purified microtubules and on spindle microtubule organization in mitotically blocked MCF/GFP tumor cells. We find that both tasidotin and tasidotin C-carboxylate suppress dynamic instability at plus ends of purified microtubules in vitro at concentrations that are 10 to 20 times lower than the concentrations that inhibit microtubule polymerization, with the metabolite being considerably more potent than the parent compound. Tasidotin inhibited proliferation of MCF/GFP cells by 50% at 63 nmol/L and induced half-maximal accumulation of cells at mitosis at a similar concentration of 72 nmol/L. These results indicate that the principal antiproliferative mechanism is inhibition of mitotic progression. At the antimitotic IC50 for tasidotin, blocked spindles seemed normal or nearly normal (Fig. 5B), indicating that tasidotin was not inhibiting mitosis by depolymerizing the spindle microtubules but most likely was acting by modulating their dynamics.

Effects of tasidotin and tasidotin C-carboxylate on dynamic instability at opposite microtubule ends at steady state in vitro. Tasidotin strongly stabilized the microtubules at their plus ends. Its most significant actions were reduction of the rate and length of shortening, reduction of the fraction of time the microtubules grew, and reduction of the catastrophe frequency (Table 1A; Fig. 4). The strong suppressive effects of tasidotin on plus end dynamics (e.g., those induced by 5 μmol/L tasidotin) occurred at tasidotin concentrations that minimally affected the polymer mass either with MAP-rich microtubules (Fig. 2A) or MAP-free seeded microtubules (Fig. 2C). The inability of tasidotin to inhibit the plus end

**Figure 4.** Major dynamic instability parameters affected by tasidotin and tasidotin C-carboxylate. Effects of tasidotin and its metabolite on (A) the rate of shortening and (B) the catastrophe frequency. C, effects of tasidotin and tasidotin C-carboxylate on the percentage of time microtubules grew (○), shortened (△), and remained in an attenuated state, neither growing nor shortening detectably (□; Materials and Methods).
growth rate is surprising because the related compound cemadotin as well as compounds thus far studied that are able to depolymerize microtubules all inhibit the plus-end growth rate (11, 22).

Although the major cellular metabolite of tasidotin, tasidotin C-carboxylate, suppressed plus end dynamics in a manner that was qualitatively similar to that of tasidotin, it was much more potent than tasidotin (Table 1B). For example, 75% suppression of overall dynamics required 5 μmol/L tasidotin, but only 0.5 μmol/L tasidotin C-carboxylate. Like tasidotin, tasidotin C-carboxylate stabilized the plus ends at concentrations that were much lower than those required to depolymerize the microtubules.

The minus ends of microtubules in many animal cells are considered to be nondynamic during interphase (28). However, during mitosis, whereas the microtubule minus ends remain...
tethered at the spindle poles, they shorten rapidly (29). Tasidotin and its metabolite enhanced minus-end microtubule dynamics, an action that could be important in the ability of tasidotin to inhibit mitosis. The action of tasidotin and its metabolite on opposite microtubule ends resembles that of vinblastine, which also stabilizes plus-end dynamics but enhances minus-end dynamics (23). However, tasidotin and its metabolite act differently on minus ends from vinblastine. Like vinblastine, tasidotin and tasidotin C-carboxylate increased the catastrophe frequency and decreased the rescue frequency. However, tasidotin and its metabolite also increased the minus-end growth rate and increased the length of minus end loss during shortening events, which are different from vinblastine. The abilities of tasidotin and tasidotin C-carboxylate to enhance minus-end dynamics were ~10- to 20-fold weaker than their abilities to stabilize the plus ends.

**How might tasidotin and its metabolite mechanistically stabilize plus ends and destabilize minus ends?** The suppression of plus end dynamics by tasidotin and tasidotin C-carboxylate occurred at concentrations well below the concentration of total tubulin. For example, at a concentration of 0.5 μmol/L tasidotin and 20 μmol/L tubulin (1:40, tasidotin/tubulin), tasidotin induced >50% reduction in the dynamicity (Table 1A). Tasidotin C-carboxylate suppressed plus end dynamics at drug: tubulin ratios that were even lower than those for tasidotin. Specifically, ~0.03 to 0.04 μmol/L tasidotin C-carboxylate reduced the dynamicity ~50%, and at these concentrations, the tubulin concentration exceeded the tasidotin C-carboxylate concentration by 500-fold. These data indicate that tasidotin and its metabolite do not suppress plus end dynamics by acting on soluble tubulin, but rather, they act directly on the microtubules.

Interestingly, unlike other known antimitotic drugs that inhibit microtubule polymerization, including cemadotin and vinblastine (e.g., refs. 11, 16, 23), neither tasidotin nor its metabolite inhibited the plus-end growth rate. The results suggest that tasidotin and its metabolite may not act by binding at the plus ends but rather by binding along the length of the microtubules. In this way, the actions of tasidotin and its metabolite on dynamics resemble the action of taxol, which at low concentrations stabilizes microtubule plus ends but increases the catastrophe frequency at minus ends by binding at very low stoichiometry to tubulin along the microtubule surface (30, 31).

Recent studies have indicated that dolastatin-15 binds in the vicinity of the Vincra binding domain of tubulin (12). Based upon the available X-ray crystal structure (32), the Vincra binding domain seems to be in β-tubulin at the inter-dimer interface between α-tubulin and β-tubulin subunits along the lengths of microtubules (also see ref. 33). The major contacts of vinblastine are with residues 177, 179, 210, and 214 in β-tubulin. It is reasonable to think that tasidotin, which is an analogue of dolastatin-15, could also bind in the vicinity of this domain and thus might be capable of binding to this site along the entire microtubule surface. If so, stabilization at the plus ends of microtubules could be due to an ability of tasidotin to strengthen the intra-protofilament interactions within the microtubule lattice as it seems to occur with vinblastine.

With purified microtubules, destabilization at the minus ends by tasidotin, and its metabolite required 10- to 20-fold higher concentrations than those required to stabilize plus ends, which indicates that the consequences of tasidotin binding to microtubule surfaces are different at opposite ends. This is reasonable because tasidotin most likely binds to the β-subunits of tubulin, which are oriented outward at the plus ends and inward at the minus ends. Tubulin subunits within the core of the microtubule lattice are believed to be in a strained conformation (34, 35). It is conceivable that tasidotin binding to the β-tubulin subunits at the minus ends, in contrast to the plus ends, may exaggerate such strain and thus weaken the lateral interactions between the protofilaments.

**Effects of tasidotin on MCF7/GFP cell proliferation and mitosis.** Tasidotin inhibited proliferation of MCF7/GFP cells with an IC50 of 63 nmol/L (Fig. 5A). It arrested the cells in mitosis at a similar concentration (IC50, 72 nmol/L). This indicates that mitotic inhibition is the primary mechanism by which tasidotin inhibits cell proliferation. Tasidotin induced aberrant organization of the spindle microtubules but did not depolymerize them. At the mitotic IC50 for tasidotin, the major fraction of microtubules in interphase cells seemed normal. These results are consistent with our *in vitro* data showing that tasidotin and its metabolite suppress microtubule dynamics at concentrations well below those required to depolymerize the microtubules and indicate that tasidotin blocks mitosis by modulating spindle microtubule dynamics.

**Functional significance of tasidotin C-carboxylate.** Despite the high potency of tasidotin C-carboxylate in modulating microtubule polymerization and dynamics *in vitro*, its IC50 for inhibition of cell proliferation is very weak, ~4 mmol/L (data not shown; i.e., 50 times weaker than the parent compound). Thus, it is reasonable to think that the metabolite must penetrate into cells very poorly. Recent studies have indicated that in various tumor cells, tasidotin is transformed into tasidotin C-carboxylate by the cytosolic endopeptidase prolyloligopeptidase (36, 37). Thus, tasidotin may be considered to be a relatively weak prodrug that when administered to cancer cells containing high expression levels of prolyloligopeptidase (38) is readily transformed into tasidotin C-carboxylate. Interestingly, tasidotin C-carboxylate is also formed by metabolism of cemadotin (20, 21). Cemadotin was found too toxic for use in cancer treatment because of its cardiovascular effects (5). It is significant that such toxicities do not occur with tasidotin (7), but the reasons for the improved toxicity profile are not yet understood. Thus, tasidotin seems to be a considerably more acceptable drug than cemadotin.

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**References**


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Anasuya Ray, Tatiana Okouneva, Tapas Manna, et al.


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