Kinetic Stabilization of Microtubule Dynamics by Estramustine Is Associated with Tubulin Acetylation, Spindle Abnormalities, and Mitotic Arrest

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Abstract

Estramustine (EM) alone or in combination with other anticancer agents is clinically used for the treatment of hormone refractory prostate cancer. Furthermore, EM has been shown to potently inhibit the proliferation of different types of cancer cells in culture apparently by targeting microtubules; however, the antiproliferative mechanism of action of EM is not clear. In this work, we have shown that EM strongly suppressed the dynamic instability of individual microtubules in MCF-7 cells by reducing the rates of growing and shortening excursions and increasing the time microtubule spent in the pause state. At its half maximal proliferation inhibitory concentration (IC50), EM exerted strong suppressive effects on the dynamics of microtubules in MCF-7 cells without detectably affecting either the organization or the polymerized mass of microtubules. At relatively high concentrations (5 × IC50), EM significantly depolymerized microtubules in the cells. Furthermore, the microtubules were found highly acetylated, supporting the conclusion that they were stabilized by the drug. EM treatment induced spindle abnormalities in MCF-7 cells, and a major population of the arrested mitotic cells was multipolar. EM also perturbed the microtubule-kinetochore interaction, thereby activating the spindle assembly checkpoint and leading to apoptotic cell death. [Cancer Res 2008; 68(15):6181–9]

Introduction

Estramustine (EM), a conjugate of nor-nitrogen mustard and estradiol phosphate, has become one of the most valuable drugs for the treatment of hormone refractory prostate cancer (HRPC). When used alone to treat HRPC, it has shown response rates ranging from 19% to 69% (1). EM has also shown promising activity against HRPC in combination with other drugs and is currently undergoing clinical trials in combination with docetaxel, etoposide, carboplatin, and vinblastine (2, 3). Fizazi and colleagues (2007) have reported that the overall survival rate for metastatic HRPC is significantly increased when EM is combined with docetaxel, paclitaxel, vinblastine, or ixabepilone rather than used by itself (4). The promising response of the combined application of EM with docetaxel led to the Southwest Oncology Group trial, which was a phase 3–randomized study for evaluating the combination of EM and docetaxel in 770 HRPC patients. Patients treated with this combination showed a significant increase in the overall survival and also a significant reduction in the risk of death (5). In addition, EM when used singly was found effective in advanced breast cancer (6) and in combination with docetaxel was found to increase the overall survival and improve the quality of life of patients having refractory metastatic breast carcinoma (7). EM is given via the oral route in the form of EM phosphate (EMP) with 70% to 75% of the oral dose absorbed. EMP is more soluble than the parent compound but is not active in cells because it does not penetrate the plasma membrane. However, it is rapidly dephosphorylated in the gastrointestinal tract and the dephosphorylated form predominates ~4 h after ingestion (8). The most important adverse effects of EM are cardiovascular and gastrointestinal toxicities, which can be avoided by careful treatment measures (9).

Chemically, EM consists of an estradiol moiety linked to nor-nitrogen mustard by a carbamate bridge. Originally, it was designed to treat breast cancer based on the notion that the estradiol moiety may specifically direct the nor-nitrogen mustard to the breast cancer cells, wherein the alkylating activity of the nor-nitrogen mustard can kill the breast cancer cells (10). However, contrary to this idea, EM was found to be highly effective in treating prostate cancer patients. It inhibits cell proliferation and induces mitotic arrest in many types of cancer cells but is especially active in prostate cancer cells (11). The high efficacy of EM against prostate cancer cells is thought to be due to the presence of an EM binding protein in these cells (12).

The antitumor activity of EM is thought to be due to its action on microtubules. EM has been found to bind weakly to microtubule-associated proteins (MAP) and to inhibit microtubule assembly in vitro (13, 14). EM has been shown to bind to tubulin dimers (15, 16) and weakly inhibits the polymerization of MAPs-free tubulin into microtubules (16). Furthermore, EM has been shown to suppress the dynamic instability of individual MAP-free microtubules in vitro (16). The EM binding site on tubulin has been suggested to be distinct from the colchicine and vinblastine sites (16) and may partially overlap with the Taxol-binding site in tubulin (15). Interestingly, EMP has been shown to bind to brain MAPs and to depolymerize MAP-rich microtubules in vitro (17).

Whereas the antimitotic activity of EM seems to be due to its actions on microtubules, the mechanism by which it inhibits cell cycle progression and mitosis is poorly understood. In this study, we have shown that EM suppresses the dynamic instability of individual microtubules in living MCF-7 cells. The kinetic stabilization of microtubule dynamics occurred in the absence of a significant depolymerization of the microtubules. EM also increased the acetylation levels of the interphase microtubules in the MCF-7 cells, further supporting the idea that EM kinetically stabilizes the microtubules. EM also interfered with the microtubule-kinetochore interaction, thereby activating the spindle checkpoint leading to apoptosis.
Materials and Methods

Reagents. EM was a kind gift from Dr. Leslie Wilson (University of California-Santa Barbara). Paclitaxel, sulforhodamine B (SBRB), mouse monoclonal anti-α-tubulin antibody, rabbit anti-γ-tubulin antibody, alkaline phosphatase (ALP)–conjugated anti-mouse IgG, ALP conjugated anti-rabbit IgG, FITC-conjugated anti-rabbit IgG, fetal bovine serum, and bovine serum albumin were purchased from Sigma. Anti-BubR1 antibody and Annexin V were purchased from BD PharMingen. Anti-mouse IgG-Alexa 568 conjugate and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes. All other reagents were of analytic grade.

Cell culture. Human breast cancer (MCF-7) cells were cultured in MEM (Hi Media) supplemented with 10% FCS, 1.5 g/L sodium bicarbonate, 10 μg/mL of human insulin, and 1% antibiotic-antimycotic solution containing streptomycin, amphotericin B, and penicillin. EM stock solution was prepared in 100% DMSO and was added to the culture medium (0.1% v/v) 24 h after seeding. DMSO (0.1%) was used as a vehicle control.

Cell proliferation assay. MCF-7 cells were seeded on glass coverslips at a density of 10^5 cells/mL for one cell cycle. The cells were incubated with different concentrations of EM at 37°C for one cell cycle (48 h). The inhibition of cell proliferation was measured by a widely used sulforhodamine assay (18, 19).

Immunofluorescence microscopy. Microtubules, centrosomes, kinetochores, BubR1, and DNA were visualized, as described previously (19, 20). Briefly, MCF-7 cells (0.6 × 10^5 cells/mL) seeded on glass coverslips were exposed to different concentrations of EM for one cell cycle at 37°C. Cells were stained with the following antibodies: mouse monoclonal anti-α-tubulin antibody (1:300), anti-BubR1 antibody (1:1,000), anticientromere antibody (1:1,500) kindly provided by Dr. K.F. Sullivan (Scripps Research Institute), mouse monoclonal anti-acetylated tubulin (1:600), and rabbit anti-γ-tubulin antibody (1:2,000). The secondary antibodies used were FITC-conjugated anti-mouse IgG, FITC-conjugated anti-rabbit IgG, goat anti-human–FITC conjugate, and Alexa 568 conjugate sheep antimouse IgG. To visualize nuclei and DNA, cells were stained with 1 μg/mL DAPI or Hoechst 33258 (0.8 μg/mL). Immunostained cells were examined with a Nikon Eclipse 2000-U fluorescence microscope and the images were analyzed with Image-Pro Plus software. Mitotic index was estimated by determining percentage of mitotic cells in a population (19).

The colocalization of acetylated tubulin and tubulin upon EM treatment was analyzed using GFP-tubulin transfected MCF-7 cells. MCF-7 were transfected with a plasmid (pEGFP-Tub) encoding a fusion protein consisting of human α-tubulin and a green fluorescent protein (gifted by Prof. Leslie Wilson, University of California-Santa Barbara). Cells stably expressing the EGFP-tubulin were selected and maintained in media containing G418. The stable cell lines were treated with different concentrations of EM, and 48 h after the drug addition, the cells were fixed and stained with antibody against acetylated tubulin. Level of tubulin or acetyl tubulin was obtained by measuring the total fluorescence intensity (mean intensity × area of the cell) of each of ~50 cells observed for each drug concentration.

Measurement of microtubule dynamics. GFP-tubulin transfected MCF-7 cells were seeded on to glass coverslips and treated with EM for 24 h. Before recording the dynamics, coverslips were transferred to culture media lacking phenol red in a glass-bottomed culture dish. Time lapse imaging of microtubules was carried out using an FV-500 laser scanning confocal microscope (Olympus) with a 60 × water immersion objective. Fifty images of each cell were acquired at 4-s interval using fluoroview software. The position of the plus ends of microtubules was tracked by Image J software, and the dynamic variables were calculated using Microsoft Excel. Life history traces were obtained by plotting the lengths of individual microtubules against time. Length changes of ≥0.5 μm were considered as growth or shortening events and changes of <0.5 μm for a minimum of two scans were considered to be in the pause state (neither growing nor shortening detectably). A transition from a growth (G) or a paused (P) state to a shortening (S) is called a "catastrophe," and a transition from a shortening state to a growth or a pause state is called a "rescue." The catastrophe frequency per unit time was calculated by dividing the number of catastrophes (transition from G to S and from P to S) by the sum total time spent in G plus P. The rescue frequency was calculated by dividing the numbers of rescues (transition from S to G and from S to P) by the total time spent in S. The catastrophe and rescue frequencies per unit length were determined by dividing the number of transitions by the length the microtubules have grown or shortened. Thirty microtubules, which were visible for ≥190 s, were analyzed for each condition, and various dynamic variables were calculated as previously described (16, 21, 22). Statistical significance was calculated by the Student's t test.

Western analysis. The effects of EM on the polymerized mass of microtubules in MCF-7 cells were determined using established protocol (23). Western blot analysis was done as described previously (20). The blots were incubated with mouse monoclonal anti-α-tubulin antibody (1:1,000) or mouse monoclonal anti-acetyl tubulin antibody (1:1,000). Intensity of the blot was determined using the Image J software, and the intensity was plotted against the EM concentration.

Annexin V/propidium iodide staining. MCF-7 cells were grown in the absence and presence of different concentrations of EM for 48 h. Annexin V/propidium iodide staining of the treated cells was carried out using the Annexin V apoptosis kit, as described recently (24), and the cells were examined under a fluorescence microscope. A flow cytometric analysis (FACS Caliber, Becton Dickinson) of live MCF-7 cells stained with Annexin V–FITC, and propidium iodide was performed to examine EM-induced cell death (25). The data were analyzed using the ModFit LT program.

Cell cycle analysis. MCF-7 cells without or with different concentrations of EM were grown for one cell cycle, fixed in 70% (v/v) ethanol, and incubated with 10 μg/mL RNase and 400 μg/mL propidium iodide at 37°C for 30 min. DNA content of the cells was quantified in a flow cytometer (FACS Caliber, Becton Dickinson; ref. 26), and the cell cycle distribution was washed using the ModFit LT program.

Results

EM inhibited proliferation of MCF-7 cells at mitosis. EM inhibited the proliferation of MCF-7 cells in a concentration-dependent manner with a half-maximal inhibitory concentration (IC50) of 5 ± 1 μmol/L (Fig. 1). EM arrested the cell cycle progression at mitosis (Fig. 1). For example, 49 ± 3% and 63 ± 3% of the MCF-7 cells were arrested at mitosis in the presence of 5 and 10 μmol/L EM, respectively, indicating that the inhibition of cell proliferation by EM occurred in association with the inhibition of cell cycle progression at mitosis. A flow cytometry analysis using propidium iodide staining showed that EM inhibited MCF-7 cells at
the G2-M phase (Supplementary Fig. S1). For example, 1.6%, 54.8%, and 57% of the cells were found to be in the G2-M phase in the absence and presence of 5 and 10 \( \mu \text{mol/L} \) EM, respectively.

**Effects of EM on MCF-7 cell microtubules.** Untreated MCF-7 cells displayed a regular network of interphase microtubules and a normal mitotic spindle with proper chromosome alignment at the metaphase plate (Fig. 2A and B). In the presence of 5 \( \mu \text{mol/L} \) EM, the interphase microtubules were similar to those in control cells (Fig. 2A). EM (10 \( \mu \text{mol/L} \), 2 \( \times \text{IC}_{50} \)) had a modest depolymerizing effect on the interphase microtubules. EM (25 \( \mu \text{mol/L}, 5 \times \text{IC}_{50} \)) caused a significant depolymerization of the microtubules (Fig. 2A), and the cells exhibited a spherical morphology. Disruption of the mitotic spindles in the cells occurred at much lower EM concentrations than those required to cause a significant depolymerization of the interphase microtubule network (Fig. 2B). Mitotic cells treated with EM (5 \( \mu \text{mol/L} \)) showed abnormal bipolar spindles, as well as unipolar and multipolar spindles with misaligned chromosomes around the spindle. Cells treated with 10 \( \mu \text{mol/L} \) EM also showed spindle microtubule abnormalities. Spindles formed had either unipolar or multipolar organization. At 25 \( \mu \text{mol/L} \) EM, a major proportion (>80%) of the mitotic cells had multipolar spindles and the chromosomes were not congressed to the metaphase plate.

EM reduced the polymerized fraction of cellular tubulin. At a concentration of 5 \( \mu \text{mol/L} \) EM, the mass of polymerized microtubules was similar to that of control cells (Fig. 2C and D). Relative to the control values, 10 \( \mu \text{mol/L} \) EM decreased the microtubule polymer mass by 15% and 25 \( \mu \text{mol/L} \) EM reduced it by 29% (Fig. 2D). These results were in agreement with the depolymerizing effects of EM on interphase microtubules as visualized by immunofluorescence microscopy.

**EM induces apoptotic cell death.** Control MCF-7 cells remained viable after 48 h, as seen by the absence of Annexin V and propidium iodide staining (Supplementary Fig. S2A). After 48 h of EM treatment, cells were found at various stages of apoptosis. After 48 h, 5 \( \mu \text{mol/L} \) EM–treated cells were stained positive for Annexin V alone, indicating that the cells were in early apoptosis, whereas cells treated with 10 \( \mu \text{mol/L} \) EM–treated cells were stained positive for both Annexin V and propidium iodide, indicating them to be in the later apoptotic stages. Cells treated with 25 \( \mu \text{mol/L} \) EM–stained positive for propidium iodide alone, suggesting that these cells were dead. Furthermore, a flow cytometric analysis of live MCF-7 cells stained with Annexin V/propidium iodide confirmed that EM treatment killed MCF-7 cells through apoptosis (Supplementary Fig. S2B). In the absence of EM, 83% of the cells were live and 4% and 10% of the cells were in early apoptosis.

![Figure 2. Effects of EM on cellular microtubules. MCF-7 cells were incubated in the absence and presence of different concentrations (5–25 \( \mu \text{mol/L} \)) of EM for 48 h. Microtubules (red) stained with anti-\( \alpha \)-tubulin antibody and DNA (blue) stained with DAPI were analyzed as described in Materials and Methods. Scale bar, 10 \( \mu \text{m} \). A, effect of EM on the interphase microtubules. B, effect of EM on the spindle microtubules. C, MCF-7 cells were treated without or with different concentrations (5, 10, and 25 \( \mu \text{mol/L} \)) of EM for 48 h. Polymeric and soluble tubulin fraction were isolated as explained in Materials and Methods, and equal amounts of polymer and soluble tubulin fractions were resolved by SDS-PAGE followed by immunoblotting with anti-\( \alpha \)-tubulin antibody. D, polymer fraction of tubulin was measured from the intensity of the blot, which was plotted against the EM concentration. Columns, mean; bars, SD.](image-url)
and late apoptosis, respectively. About 2.6% of the cells had undergone death. EM treatment increased the percentage of cells that were apoptotic/dead. For example, at 10 μmol/L EM, there was an increase in the percentage of cells that were in late apoptosis (17.3%) and the dead cells (16.8%). At 25 μmol/L EM, 22.5% of the cells were dead.

**EM strongly suppressed the dynamic instability of individual microtubules in MCF-7 cells.** Consistent with the previous studies (21, 27), control microtubules (vehicle treated) were highly dynamic, alternating between phases of growth, shortening, and pause (Fig. 3A). EM clearly suppressed the dynamics of the microtubules (Fig. 3B and C); it altered each of the dynamic instability variables in a concentration-dependent manner (Table 1). EM (2 μmol/L), which is less than half of its IC50 value in MCF-7 cells, suppressed the mean growth rate by 23% (from 14.7 ± 5.1 to 11.3 ± 2.2 μm/min) and the mean shortening rate by 25% (from 20.1 ± 5.1 to 15 ± 4.3 μm/min). The rescue frequency, which was calculated based on time, was increased by 20%, whereas the time-based catastrophe frequency was reduced by 15% by 2 μmol/L EM. Notably, 2 μmol/L EM increased the length based rescue and catastrophe frequencies significantly. Dynamically, which is the total length grown and shortened during the measured life span of the microtubules, was reduced by 46% in the presence of 2 μmol/L EM (Table 1).

EM (5 μmol/L, IC50) strongly stabilized the dynamics of the microtubules. EM (5 μmol/L) reduced the rates of growing and shortening by 25% and 32%, respectively. The mean growth length and the mean shortening length were also strongly reduced (>60%) at this concentration. Interestingly, 5 μmol/L EM showed a high increase in the length-based catastrophe and rescue frequencies. EM (5 μmol/L) also greatly increased the percentage of time microtubules spent in the pause state (72%, which was twice that of the control microtubules) and reduced the overall dynamicity by 72% (Table 1).

**EM increased the level of acetylated tubulin in MCF-7 cells.** Tubulin acetylation is considered to be a marker of microtubule stabilization (28, 29). We performed three different experiments to show that EM increases microtubule acetylation. An indirect immunofluorescence experiment using an antibody against acetylated α-tubulin showed an increase in the level of acetylated tubulin in microtubules of EM-treated cells compared with control cells, which had very low levels of acetylated tubulin (Fig. 4A). The colocalization of microtubules and acetylated tubulin was examined using MCF-7 cells transfected with GFP-tubulin and an antibody against acetylated α-tubulin (Fig. 4B). In control cells, only a few microtubules were acetylated, whereas in the EM-treated cells there was a concentration-dependent increase in the level of acetylated tubulin. The ratio of acetylated tubulin to the GFP-tubulin for the cells treated in the absence and presence of 10 and 25 μmol/L EM were 0.92 ± 0.1, 2.2 ± 0.8, and 2.5 ± 0.5, respectively (Supplementary Fig. S3). The level of tubulin acetylation was also determined by Western blot analysis in MCF-7 cell extracts containing the polymerized tubulin fraction (Fig. 4C). When probed with antibody against α-tubulin, the percentage of polymerized tubulin in cells treated with EM was found to decrease in a concentration-dependent manner. To examine whether the polymerized microtubules that remained after EM treatment had been stabilized by acetylation, we quantified the intensity of the two blots (Supplementary Fig. S4). The ratio of acetylated polymerized tubulin to total polymerized tubulin in the absence and presence of 5, 10, and 25 μmol/L EM were 0.9 ± 0.1, 1.4 ± 0.2, 1.9 ± 0.3, and 2.9 ± 0.3, respectively. The relative acetyl tubulin intensity was increased by 46%, 77%, and 138% when the cells were treated with 5, 10, and 25 μmol/L EM, indicating that the majority of the tubulin in the polymerized fraction of the EM-treated cells were acetylated. Together, the results showed that EM treatment caused an increase in the acetylation levels of microtubules in MCF-7 cells and indicated that the microtubules in EM-treated cells had been stabilized by the drug.

**EM induced multipolarity in MCF-7 cells.** Control mitotic cells had two centrosomes at the poles of the bipolar spindle. Cells treated with 10 and 25 μmol/L EM showed large abnormalities in
EM was used at its IC50 concentration (5 μmol/L) or greater than its IC50 concentration, the arrangement of the kinetochores was perturbed (Fig. 5B). In the case of cells treated with 5, 10, and 25 μmol/L EM, kinetochore organization was completely disrupted resulting in misalignment of chromosomes at the metaphase plate. At these concentrations of EM, microtubules were completely depolymerized and the centromeres were scattered in the cell so that the sister kinetochores were not identifiable. The results suggest that EM perturbs microtubule-kinetochore interactions and reduces the tension exerted by microtubules on kinetochores.

EM perturbed the microtubule-kinetochore attachment in mitotic MCF-7 cells. The kinetochores of control metaphase spindles were attached to the spindle microtubules and the chromosomes were properly aligned at the metaphase plate (Fig. 5B). When EM was used at its IC50 concentration (5 μmol/L) or greater than its IC50 concentration, the arrangement of the kinetochores was perturbed (Fig. 5B). In the case of cells treated with 5, 10, and 25 μmol/L EM, kinetochore organization was completely disrupted resulting in misalignment of chromosomes at the metaphase plate. At these concentrations of EM, microtubules were completely depolymerized and the centromeres were scattered in the cell so that the sister kinetochores were not identifiable. The results suggest that EM perturbs microtubule-kinetochore interactions and reduces the tension exerted by microtubules on kinetochores.

Table 1. EM suppresses dynamic instability of interphase microtubules in live MCF-7 cells

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2 μmol/L EM</th>
<th>5 μmol/L EM</th>
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<tbody>
<tr>
<td>Growth rate (μm/min)</td>
<td>14.7 ± 5.1</td>
<td>11.3 ± 2.2a</td>
<td>11.0 ± 2.7†</td>
</tr>
<tr>
<td>Growth length (μm)</td>
<td>3.0 ± 1.8</td>
<td>1.4 ± 0.6†</td>
<td>1.0 ± 0.3†</td>
</tr>
<tr>
<td>Shortening rate (μm/min)</td>
<td>20.1 ± 5.1</td>
<td>15.0 ± 4.3†</td>
<td>13.8 ± 4.6†</td>
</tr>
<tr>
<td>Shortening length (μm)</td>
<td>4.8 ± 3.7</td>
<td>3.0 ± 2.0†</td>
<td>1.8 ± 0.9†</td>
</tr>
<tr>
<td>% Time spent in growing</td>
<td>38.2 ± 1.7</td>
<td>25.4 ± 1.3†</td>
<td>14.3 ± 6.1†</td>
</tr>
<tr>
<td>% Time spent in shortening</td>
<td>21.8 ± 7.8</td>
<td>18.3 ± 8.6†</td>
<td>13.0 ± 5.8†</td>
</tr>
<tr>
<td>% Time spent in pause</td>
<td>36.0 ± 2.6</td>
<td>56.0 ± 5.4†</td>
<td>72.3 ± 9.4†</td>
</tr>
<tr>
<td>Dynamicity (μm/min)</td>
<td>11.2 ± 3.8</td>
<td>6.0 ± 2.7†</td>
<td>3.1 ± 1.4†</td>
</tr>
<tr>
<td>Rescue frequency (events/min)</td>
<td>5.6 ± 3.0</td>
<td>6.7 ± 3.6†</td>
<td>8.7 ± 3.0†</td>
</tr>
<tr>
<td>Catastrophe frequency (events/min)</td>
<td>1.7 ± 0.8</td>
<td>1.4 ± 0.8†</td>
<td>1.2 ± 0.4a</td>
</tr>
<tr>
<td>Rescue frequency (events/μm)</td>
<td>0.3 ± 0.2</td>
<td>0.5 ± 0.3†</td>
<td>0.7 ± 0.4†</td>
</tr>
<tr>
<td>Catastrophe frequency (events/μm)</td>
<td>0.2 ± 0.1</td>
<td>0.5 ± 0.3†</td>
<td>0.8 ± 0.4†</td>
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NOTE: Data are the mean ± SD.

*aP < 0.01.

†P < 0.001.

bStatistically not significant.

EM alone or in combination with other anticancer agents is clinically used for the treatment of HRPC. In addition, EM was also proved to be highly effective in metastatic breast carcinoma (6, 7). We found that low concentrations of EM strongly suppressed the dynamic instability of microtubules in human breast cancer (MCF-7) cells without detectably altering the mass of polymerized microtubules. The microtubules, which became stabilized upon EM treatment, were highly acetylated. The stabilization of microtubule dynamics by EM inflicted spindle abnormalities, such as the perturbation of microtubule-kinetochore attachment, centrosomal organization, and multipolarity, which in turn activated the spindle checkpoint protein, BubR1. Mitotic defects induced by EM ultimately forced the cell to undergo apoptosis.
depolymerized them (Fig. 2). In contrast to its weaker depolymerizing effects on the interphase microtubules, EM showed relatively stronger depolymerizing effects on the spindle microtubules. A pronounced effect was the formation of multipolar spindles at higher concentrations of EM. A normal bipolar spindle has two centrosomes and the centrosome duplication is considered to be a tightly regulated process (31). A failure to regulate the centrosome number leads to the formation of multipolar spindles and defects in chromosome distribution during mitosis (32). EM treatment resulted in the formation of multipolar spindles containing centrosomes that stained with γ-tubulin. Multiple centrosomes can be formed either by fragmentation of centrosomes into multiple microtubule organizing centers or by amplification of centrosomes (33, 34). Separation of pericentriolar material without centriolar duplication may also cause multipolar spindle formation (35). Interference with the functions of the spindle protein NUMA and/or the motor protein dynein involved in the coalescence of the centrosomes can also lead to multipolarity (34, 36). It is possible that EM treatment might alter any of these processes required to produce a normal bipolar spindle.

Monopolar spindles were also observed in the EM-treated cells. Monopolar spindles may be formed by the failure of centrosome separation so that the microtubules nucleate from a single nucleation center. Alternatively, monopolar spindles may be formed if EM directly or indirectly interferes with the function or the expression of the kinesin Eg5 (37) or Kif2A (38), which are crucial for the

Figure 4. EM increased microtubule acetylation. A, MCF-7 cells stained with antibody against acetylated tubulin. B, GFP-tubulin transfected MCF-7 cells were incubated with EM (10 and 25 μmol/L for 48 h and stained for acetylated microtubules (red) and chromosomes (blue), as described in Materials and Methods. Scale bar, 10 μm. C, EM increases acetylation of polymer fraction of cellular tubulin. MCF-7 cells were treated with the vehicle control (0), 5, 10, and 25 μmol/L EM for 48 h. Polymeric and soluble tubulin fraction were isolated as explained in Materials and Methods and immunoblotted with anti-γ-tubulin antibody or anti–acetyl tubulin antibody.
organization of centrosomes. Monastrol is known to produce unipolar spindles by inhibiting Eg5 (39). Normally cancerous mitotic cells having multipolar spindles continue to progress through the cell cycle due to a failure to arrest at the mitotic checkpoint and form multinucleated interphase cells. In the EM-treated interphase cells, multinucleated cells were not observed, indicating that there was no mitotic exit. EM treatment resulted in a robust mitotic arrest, which activated the mitotic checkpoint and induced apoptotic cell death.

EM at its low effective concentration (IC50) disrupted microtubule-kinetochore attachment and relatively higher concentrations of EM completely disrupted kinetochore organization, thereby generating pronounced effects on the important mitotic functions dependent upon proper association of microtubules with kinetochores (Fig. 5B). It is believed that proper kinetochore-microtubule interaction causes a reduction in the concentration of spindle checkpoint proteins (40). A single unattached or improperly attached kinetochore can cause an accumulation of the spindle checkpoint protein BubR1, preventing the cell cycle progression to anaphase (41). BubR1 accumulation was seen in all the mitotic cells treated with EM (Fig. 5C). The disorganization of kinetochores together with the accumulation of BubR1 strongly suggested that the microtubules were not properly attached to the kinetochores in the EM-treated cells and the tension at the kinetochores was not maintained properly. EM suppressed the dynamics of interphase microtubules. Therefore, it is logical to assume that the dynamics of spindle microtubules will be severely suppressed by EM treatment so that these microtubules are unable to attach properly to the kinetochores and generate sufficient tension so as to facilitate proper chromosome segregation. Many drugs that suppress microtubule dynamics are found to reduce the tension between the sister kinetochores and to activate the spindle assembly checkpoint (42).

EM strongly inhibited the dynamic instability of microtubules in the MCF-7 cells (Fig. 3; Table 1). EM also strongly suppressed the growing and shortening rates of reconstituted MAP-free microtubules in vitro (16). The suppressive effects of EM on the dynamics of individual microtubules of MCF-7 cells were found to be qualitatively similar to that of the reconstituted brain microtubules in vitro (16). However, EM exerted comparatively stronger effect on the overall dynamicity (72% by 5 μmol/L EM) and the percentage of time microtubules spent in the attenuation phase.

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**Figure 5.** Effects of EM on centrosomes, kinetochores, and BubR1 in MCF-7 cells. A, MCF-7 cells were treated without or with 5, 10, and 25 μmol/L EM and stained with γ-tubulin antibody (to visualize the centrosomes) and α-tubulin (to visualize the spindle). B, effects of EM on kinetochore-microtubule attachment. Immunofluorescent images of mitotic MCF-7 cells treated with 5, 10, and 25 μmol/L EM and compared with those of the vehicle-treated (control) cells. C, EM activated the checkpoint protein BubR1. MCF-7 cells were treated with different concentrations of EM (5–25 μmol/L) for 48 h. Scale bar, 10 μm.
Overexpression of both HDAC6 and SIRT2 are shown to destabilize microtubules (46, 47). Furthermore, microtubules are hyperacetylated and stabilized when HDAC6 and SIRT2 are knocked down in cells (46, 47). Both HDAC6 and SIRT2 deacetylate tubulin at the lysine-40 of the α-tubulin subunit. Additionally, North and colleagues reported that SIRT2 colocalizes and interacts with HDAC6 (47). Recently, it has been suggested tubulin does not bind to either HDAC6 or SIRT2, but it binds only to the SIRT2-HDAC6 complex (48). Although it is not yet clarified whether the acetylation is the cause or the result of the stabilization of microtubules, it has been suggested that tubulin acetylation is a consequence of microtubule stability (49). In addition, the finding that the overexpression of MAPs, such as MAP1B, MAP2, or γ-tubulin increases tubulin acetylation suggests the idea that tubulin acetylation is one of the consequences of microtubule stability (50). We believe that the kinetic stabilization of microtubule dynamics by EM caused an increase in the acetylation level of microtubules. However, it is not clear whether EM has any direct role in increasing the level of tubulin acetylation; EM might reduce the interaction of these deacetylases to tubulin by inducing conformational change in tubulin or it might inhibit the functional activity of the HDAC6 or SIRT2.

Collectively, the data reveal that the antiproliferative activity of EM results from its strong suppressive effects on microtubule dynamics. Modulation of microtubule dynamics by EM, in turn, affected the formation of a normal bipolar spindle, arresting the cells at mitosis. Most of the EM-blocked mitotic cells were either multipolar or unipolar. The study has provided a significant insight for the antimitotic mechanism of action of EM, which might help to develop a sophisticated chemotherapeutic strategy for the treatment of the HRPC and breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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