Mutations in β-Tubulin Map to Domains Involved in Regulation of Microtubule Stability in Epothilone-resistant Cell Lines

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Abstract

The epothilones (Epos) are a group of natural products isolated from the myxobacterium, Sorangium cellulosum. They have a mechanism of action similar to that of Taxol, i.e., they stabilize microtubules and induce the formation of microtubule bundles in cells. Because they are simpler in structure than Taxol and preserve their activity in P-glycoprotein-expressing cells, they are being studied as potential antitumor drugs. In this work, a series of Epo-resistant A549 and HeLa cell lines have been selected and analyzed. Class I β-tubulin, the major isotype of β-tubulin in these Epo-resistant cell lines, has been sequenced in a search for mutations. In the Epo B-resistant A549 cells, there is a mutation at β292 from Gln to Glu, in the Epo A-resistant HeLa cell line there is a mutation at β173 from Pro to Ala, and in the Epo B-resistant HeLa cell line there is a heterozygous mutation at β422 from Tyr to a mixture of Tyr and Cys. These mutations are close to the M-loop, the nucleotide-binding site, and the microtubule-associated protein binding sites, respectively. It is likely that these mutations in β-tubulin provide cells with a mechanism of resistance to the Epos and taxanes. Among these resistant cell lines, A549.EpoB40 is hypersensitive to microtubule-destabilizing drugs, such as vinblastine and colchicine, and HeLa.EpoB1.8 is dependent on the Epos or taxanes for growth. Our studies provide evidence that the M-loop, the GTP binding site, and the microtubule-associated protein binding sites at the COOH terminus in β-tubulin are critical for the regulation of microtubule stability.

Introduction

Microtubules are a validated target for antitumor drugs. During the interphase portion of the cell cycle, microtubules are involved in the maintenance of cell shape, the movement of cells, and the intracellular transportation of vesicles and organelles. At the mitotic phase of the cell cycle, microtubules play a critical role. Through quick transitions between assembly and disassembly processes, the interphase microtubule network is reassembled into spindle-like microtubule arrays with their origins at the opposite poles of a dividing cell. The movement of microtubule spindles enables the separation of duplicated chromosomes into daughter cells. Drugs targeting microtubules disrupt microtubule spindle-mediated chromosome segregation as well as other processes that rely on the integration of the microtubule network. Microtubule-interacting drugs can be classified into two groups, microtubule-stabilizing agents such as Taxol (Fig. 1; Ref. 1) and microtubule-destabilizing drugs such as the Vinca alkaloids and colchicine (2). Taxol binds to and hyperstabilizes microtubules, and cells treated with Taxol are blocked at metaphase in mitosis. However, at low nanomolar concentrations, both Taxol and the Vinca alkaloids inhibit microtubule dynamics in cells (3–5).

Taxol has been approved by the Food and Drug Administration for the treatment of ovarian, breast, and lung carcinomas (6), and until recently, the taxanes were the only microtubule-stabilizing drugs with antitumor activity that had been described. Three new microtubule-stabilizing natural products, the Epos (7–9), discodermolide (10–12), and eleutherobin (13, 14), have been discovered. Epos A and B are natural products, isolated from the myxobacterium Sorangium cellulosum, with simpler chemical structures and better water solubility compared to Taxol (Fig. 1). Furthermore, the Epos maintain their cytotoxic activity in drug-resistant cells that overexpress P-glycoprotein (7, 15), a membrane transporter that significantly reduces the intracellular concentration of antitumor agents such as Taxol or vinblastine (16–18), and is associated with drug resistance. Because of these advantages, the Epos are undergoing extensive preclinical trials as candidates for antitumor drugs.

Here we have selected cancer cell lines resistant to the Epos and studied the alterations in the resistant cell lines. Our results provide us with information on the mechanisms of resistance to the Epos and a further understanding of those sites in β-tubulin that are required for normal microtubule function.

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2 The abbreviations used are: Epo, epothilone; RT-PCR, reverse transcription-PCR; Pgp, P-glycoprotein; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; MAPs, microtubule-associated proteins; MTP, microtubule protein.
Materials and Methods
Taxol was obtained from the Drug Development Branch, National Cancer Institute. The Epos were kindly provided by Professor Samuel J. Danishefsky, Memorial Sloan Kettering Cancer Center and Columbia University, New York, NY. All compounds were prepared in DMSO. GTP, anti-α-tubulin antibody, methylene blue, and SDS were purchased from Sigma Chemical Co.

Cell Culture. A549, a human non-small cell lung cancer line, was grown in RPMI 1640 with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, Inc.) at 37°C in 7% CO₂. HeLa cells were maintained in MEM with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, Inc.) at 37°C in 7% CO₂. Epo-resistant cell lines were maintained in medium containing the indicated concentrations of Epo A or Epo B.

Selection of Epo-resistant Cell Lines. Three Epo-resistant cell lines were developed over a 1-year period by growing A549 in Epo B and HeLa cells in Epo B and Epo A independently. These cells were selected by stepwise increases in the concentrations of the drug. A series of Epo B-resistant A549 cell lines were selected, and A549.EpoB40, the most resistant line, is maintained in 40 nM Epo B. Two Epo-resistant HeLa cell lines, HeLa.EpoA9 and HeLa.EpoB1.8, were maintained in 9 nM Epo A and 1.8 nM Epo B, respectively.

Cytotoxicity Assay. A methylene blue-based cytotoxicity assay was developed from previous research (19, 20) to study the drug resistance profiles of the Epo-resistant cell lines. Approximately 30,000 cells (1 ml) were seeded into each well in a 24-well plate and allowed to settle for 6 h, and drugs were added to the first well of the plate and serial diluted to subsequent wells. After 72 h of incubation, the medium was discarded, and 200 μl of a methylene blue solution (0.5% in ethanol:water; 50%, v/v) were added to each well. One h later, unbound methylene blue was washed off with water and bound stain was solubilized by the addition of 1 ml of 1% SDS solution. The plates were stirred gently on a rotator for 1 h at room temperature, and the absorbance in each suspension was read at 630 nm in a spectrophotometer. The staining in the control well was taken as 100%, and the IC₅₀s were defined as the drug concentrations that inhibited the cell number by 50% after 72 h.

Doubling Time. Cells (40,000; 2 ml) were seeded into each well of a six-well plate (40,000 cells/well) and incubated until the cell population reached ~80% confluency. Cells were harvested and counted on a Coulter counter. The doubling time was calculated from the total incubation time divided by the number of times that the cells replicated.

Sequencing of Class I β-Tubulin. Total RNA was prepared from cells as described (21) and reverse transcribed to cDNA. Human class I β-tubulin was amplified by RT-PCR and sequenced using four pairs of primers developed in our laboratory. Results obtained with Epo-resistant cell lines were compared with the sequence from drug-sensitive parental cell lines as well as from the class I β-tubulin sequence published in GenBank.

PCR of MDR1 and MRP Genes. Total RNA was prepared and reverse transcribed to cDNA. Primers for MDR1 or MRP were used to detect their expression by PCR. SKVLB1, a cell line derived from SKOV3 that expresses the MDR1 gene, was used as a positive control for MDR1 expression, and the β₂M gene was also included in the study as a control for the quality of the samples (21).

Determination of Soluble Tubulin Dimers after Epo B Treatment. Cells were grown to 70–80% confluency and lysed at 37°C for 5 min with 500 μl of hypotonic buffer (0.5% NP40, 0.1 M 2-(N-morpholino)ethanesulfonic acid, 1 mM EGTA, and 0.5 mM MgCl₂, pH 6.6) containing various concentrations of Epo B. In this study, the final concentration of Epo B in cell lysates was 0, 1, 10, 100, 1,000, and 10,000 nM. The lysates were transferred to 1.5-ml Eppendorf tubes and after 30 min of incubation at 37°C, centrifuged at 14,000 rpm for 10 min at room temperature. Three hundred μl of the supernatant were transferred to a fresh Eppendorf tube and mixed with 100 μl of 4× SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotted with an anti-α-tubulin antibody. ECL exposure was quantitated by densitometry. To eliminate experimental variations, all of the samples were prepared and analyzed under identical conditions at the same time.

Molecular Modeling. Molecular modeling was done with the Insight II software (Molecular Simulations, Inc.). Tubulin structure was taken from Nogales et al. (Refs. 22, 23; Protein Data Base file code: 1TUB). The X-ray structure of Taxol was
Resistance Profiles of Epo-resistant A549 and HeLa Cell Lines. A series of Epo-B-resistant A549 cell lines were selected by stepwise increases in drug concentration and maintained in various concentrations of Epo B: 0.2, 0.6, 1.2, 2.4, 4.8, 9.6, and 40 nM. A549.EpoB40, the cell line that is most resistant to Epo B in this series, was used in this study. Two Epo-resistant HeLa cell lines were selected, HeLa.EpoA9 and HeLa.EpoB1.8. They are maintained in 9 nM Epo A and 1.8 nM Epo B, respectively.

To characterize the drug resistance profiles, the resistance cell lines were tested with Epo A, Epo B, Taxol, Taxotere, discodermolide, vinblastine, and colchicine in cytotoxicity assays. IC50s were determined, and the extent of drug resistance was indicated by the fold-increase of IC50 (Tables 1 and 2).

A549.EpoB40 is ~95-fold resistant to Epo B, 72-fold resistant to Epo A, 22-fold resistant to Taxol, and 13-fold resistant to Taxotere (Table 1). The IC50 for discodermolide is increased only by 1-fold. It is of interest to note that A549.EpoB40 cells are sensitized to microtubule-destabilizing drugs. The IC50 for vinblastine and colchicine in A549.EpoB40 cells decreased by ~50%.

The Epo-resistant HeLa cells are of very low resistance, and it has been difficult to increase their level of resistance (Table 2). HeLa.EpoA9 cells are 2.5-fold resistant to Epo A and are 6.4-, 5.5-, 1.7- and 2.1-fold resistant to Taxol, Taxotere, Epo B, and discodermolide, respectively. This cell line is more sensitive to microtubule-destabilizing drugs, and the IC50 for vinblastine and colchicine are lower when compared with the wild-type HeLa cells.

The HeLa.EpoB1.8 cell line is 2.3-fold resistant to Epo B and is 2.8-, 3.4- and 1.5-fold resistant to Taxol, Taxotere, and Epo A, respectively. There is no cross resistance to discodermolide, and as a matter of fact, the IC50 for discodermolide is decreased slightly. The sensitivity to microtubule-destabilizing drugs is mixed; the IC50 for vinblastine and colchicine are 1.6- and 0.4-fold, respectively, of that in wild-type HeLa cells.

Epo-resistant Cells Proliferate at a Slower Rate Than Their Drug-sensitive Parental Cells. The growth rate of resistance cells was determined by measuring the doubling time of each cell line (Table 3). The doubling times for A549.EpoB40, HeLa.EpoA9, and HeLa.EpoB1.8 cells were 35, 10, and 122% longer, respectively, than their drug-sensitive parental cells. This increase in generation time could contribute to the resistance phenotype.

HeLa.EpoB1.8 Cells Are Dependent on Epo B and Other Microtubule-stabilizing Drugs for Normal Growth. It was noted during the cytotoxicity studies that the growth of HeLa.EpoB1.8 cells was dependent on Epo B or other microtubule-stabilizing drugs such as Taxol (Fig. 2). There is a 50–100% increase in the growth of HeLa.EpoB1.8 cells in the presence of 1–2 nM Epo A, Epo B, Taxol, or Taxotere. However, discodermolide, among all of the microtubule-stabilizing agents studied, was the only compound that could not rescue the growth of HeLa.EpoB1.8 cells. A549.EpoB and HeLa.EpoA9 cells exhibited a small (10–20%) increase in cell growth in the presence of nanomolar concentrations of microtubule-stabilizing agents (data not shown).
Mutations in β-Tubulin

Table 2 Drug resistance profile of HeLa.EpoA9 and HeLa.EpoB1.8 cells

IC₅₀ values were determined after 72 h of incubation with the indicated drugs. The IC₅₀ of HeLa.EpoA9 and HeLa.EpoB1.8 cells were divided by the IC₅₀ of HeLa cells to indicate the fold decrease or increase in drug sensitivity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>HeLa IC₅₀ (nM)</th>
<th>HeLa.EpoA9 IC₅₀ (nM)</th>
<th>Fold</th>
<th>HeLa.EpoB1.8 IC₅₀ (nM)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epo A</td>
<td>4.02 ± 0.01</td>
<td>9.95 ± 0.19</td>
<td>2.5</td>
<td>6.18 ± 0.04</td>
<td>1.5</td>
</tr>
<tr>
<td>Epo B</td>
<td>1.96 ± 0.48</td>
<td>3.40 ± 0.08</td>
<td>1.7</td>
<td>4.41 ± 0.16</td>
<td>2.3</td>
</tr>
<tr>
<td>Taxol</td>
<td>1.68 ± 0.01</td>
<td>10.70 ± 0.84</td>
<td>6.4</td>
<td>4.75 ± 0.03</td>
<td>2.8</td>
</tr>
<tr>
<td>Taxotere</td>
<td>1.42 ± 0.05</td>
<td>7.84 ± 0.15</td>
<td>5.5</td>
<td>4.89 ± 0.06</td>
<td>3.4</td>
</tr>
<tr>
<td>Disco*</td>
<td>7.54 ± 0.19</td>
<td>16.01 ± 0.46</td>
<td>2.1</td>
<td>6.70 ± 0.92</td>
<td>0.9</td>
</tr>
<tr>
<td>VBL</td>
<td>0.95 ± 0.08</td>
<td>0.81 ± 0.05</td>
<td>0.9</td>
<td>1.55 ± 0.07</td>
<td>1.6</td>
</tr>
<tr>
<td>CLC</td>
<td>10.80 ± 0.78</td>
<td>6.24 ± 0.10</td>
<td>0.6</td>
<td>4.52 ± 0.18</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* Disco, discodermolide; VBL, vinblastine; CLC, colchicine.

Table 3 Doubling time for Epo-sensitive and Epo-resistant cell lines

Cells (40,000 cells/well) were seeded in a six-well plate and allowed to grow to 80% confluency. The total number of cells in each well was counted, and the length of incubation was used to obtain the doubling time.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (h)</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>23.5</td>
<td></td>
</tr>
<tr>
<td>A549.EpoB40</td>
<td>31.8</td>
<td>35</td>
</tr>
<tr>
<td>HeLa</td>
<td>19.5</td>
<td></td>
</tr>
<tr>
<td>HeLa.EpoA9</td>
<td>17.3</td>
<td>10</td>
</tr>
<tr>
<td>HeLa.EpoB1.8</td>
<td>43.3</td>
<td>122</td>
</tr>
</tbody>
</table>

Fig. 2. HeLa.EpoB1.8 cells are dependent on either the Epos or the taxanes for growth. In the presence of low nanomolar concentrations of Taxol, Taxotere, Epo A, or Epo B, there is an approximate 1.5- to 2-fold increase in cell growth compared with cells grown in the absence of drug. Discodermolide does not rescue the growth of HeLa.EpoB1.8 cells. Error bars were omitted for clarity. SD did not exceed 5%.

Epo B was used as an indication of an increase in microtubule assembly (Fig. 4). In A549 cells, the amount of soluble MTP decreased by 20, 30, and 40% at 10, 100, and 1,000 nM Epo B, respectively. This indicated that the assembly of microtubule polymers increased by 20, 30, and 40%. In A549.EpoB40 cells, the amount of soluble MTP protein began to decrease linearly at concentrations greater than 100 nM Epo B. The MTP in A549.EpoB40 cells demonstrated a lag phase in responding to low concentrations of Epo B (0–100 nM). The total amount of MTP in Epo-resistant A549 and HeLa cells was similar to that present in the parental cell lines (data not shown).

Fig. 3. Epo-resistant cell lines do not express the MDR1 gene. Total RNA was prepared from Epo-resistant cells and examined for the expression of the MDR1 gene by RT-PCR. SKVLB1, a cell line that overexpresses the MDR1 gene, was used as a positive control, and the βM gene was used as a control for the quality of the samples. Lane 1, A549; Lane 2, A549.EpoB40; Lane 3, HeLa; Lane 4, HeLa.EpoA9; Lane 5, HeLa.EpoB1.8; Lane 6, SKOV3; Lane 7, SKVLB1.

Table 4 Class I β-tubulin mutations in Epo-resistant cell lines

Total RNA was prepared from Epo-resistant cell lines and the parental drug-sensitive cell lines. RNA was reverse transcribed to cDNA. The class I β-tubulin gene was amplified by PCR and sequenced with four pairs of primers. A mutation is noted after comparison to both the published sequence and the class I β-tubulin in the drug-sensitive parental cell line.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Amino acid</th>
<th>Codon</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549.EpoB40</td>
<td>B292Gln → Glu</td>
<td>CAG → GAG</td>
</tr>
<tr>
<td>HeLa.EpoA9</td>
<td>B173Pro → Ala</td>
<td>CCC → GCC</td>
</tr>
<tr>
<td>HeLa.EpoB1.8</td>
<td>B422Tyr → Tyr/Cys</td>
<td>TAT → TAT/TGT</td>
</tr>
</tbody>
</table>

β-Tubulin Mutations Map to Domains Near the M-Loop, Nucleotide Binding Site, and the COOH Terminal. Molecular modeling studies revealed the location of β292, β173, and β422 mutations in β-tubulin (Fig. 5A). β292 is near both the M-loop, which is essential for the interactions between neighboring protofilaments and Thr-274, an important amino acid residue in the Taxol/Epo binding pocket (Fig. 5B). The distance between β292Gln and β274Thr is approximately 6 Å. β292Gln is also at the COOH-terminal end of the M-loop. β173Pro is on the T5-loop forming part of the nucleotide-binding pocket. β171 is also a Pro, and it is very likely that these two Pro residues determine the conformation of this loop (Fig. 5C). β422Tyr is on helix H12 at the COOH terminus of β-tubulin, the binding site for MAPs and motor proteins (Fig. 5D).
Discussion

Microtubules are regulated by sensitive mechanisms to accommodate the critical roles that these organelles play at different phases of the cell cycle. Their assembly and disassembly are driven by the binding and hydrolysis of GTP at the plus end of β-tubulin. Microtubule binding proteins such as MAP4, MAP2, and tau bind and promote the assembly and stabilization of microtubules, thereby playing a role in the regulation of microtubules (35, 36). Recently, a group of microtubule-destabilizing proteins has been discovered (37, 38). For example, stathmin, a Mr 19,000 phosphoprotein, binds to α,β-tubulin heterodimers and induces microtubule depolymerization. The integrity and proper function of the microtubule network are further regulated by cell cycle progression signaling molecules and checkpoint mechanisms.

Natural products that mimic the activity of these two classes of microtubule regulatory proteins induce imbalance between microtubule-stabilizing and -destabilizing forces. This kind of imbalance/dysfunction leads to mitotic arrest or aberrant mitosis and eventually apoptosis of cancer cells. Taxol and the Epos, although there are certain overlapping functions, such as the stabilization of microtubules.

Sequencing of β-tubulin in these resistance cell lines revealed point mutations at β292 (Gln to Glu), β173 (Pro to Ala), and β422 (Tyr to Tyr/Cys) in A549.EpoB40, HeLa.EpoA9, and HeLa.EpoB 1.8 cells, respectively (Table 1). Less resistant A549 cells have been sequenced, and it was found that the mutation at β292 was present in cells maintained in 9.6 and 4.8 nM Epo B but absent in cells maintained in 0.3 and 0.2 nM Epo B (data not shown). In A549.EpoB1.2 that is maintained in 1.2 nM Epo B, there is a heterozygous expression of both the wild-type allele (Gln) and the mutation (Glu; data not shown). The positive correlation between the increase in drug resistance and the presence of the β-tubulin mutation strongly suggests that the β292 mutation is involved in the development of drug resistance to Epo B.

Molecular modeling studies have indicated that the β-tubulin mutations described in this study are located at/near domains involved in the regulation of microtubule stability. β292 is near the M-loop as well as Thr-274, a residue forming part of the binding pocket for Taxol/Epo (Fig. 5B). This is the region regulating lateral interactions between adjacent protofilaments (23). Previous studies of Taxol- and Epo-resistant cells pointed to mutations at β270 (33), β274, and β282 (28), residues also located near the M-loop and the Taxol binding pocket.

β173Pro is on the T5-loop forming the ribose binding part of the nucleotide-binding pocket (Fig. 5C), and it is very likely
that β173Pro and β171Pro determine the conformation of this loop. Therefore, mutation of β173 from Pro to Ala would alter the conformation of this loop. Because GTP binding and hydrolysis is an essential regulatory mechanism of microtubule stability, changes in the nucleotide binding pocket may lead to alterations in microtubule stability. Studies of mutations in yeast β-tubulin have revealed that mutations at β174K and β177D to Ala lead to cold sensitivity and super-sensitivity to benomyl, a microtubule-destabilizing agent in yeast (40). Mutations near the nucleotide-binding pocket in β-tubulin account for the majority of mutations associated with poor response to Taxol treatment in non-small cell lung cancer patients. Of the 19 mutations identified, 16 were located near the nucleotide-binding pocket (34).

β422 is located on helix H12, on the external surface of the microtubule (Fig. 5D). This is the binding site for MAPs and motor proteins that constitute another mechanism for regulating microtubule stability. A mutation at β422 could result in altered binding of these factors to the COOH terminus of β-tubulin and thus lead to less stable microtubules. There are at least two possible reasons why the mutation is heterozygous: (a) a homozygous mutation at this site may not be compatible with life. In yeast β-tubulin, it has been shown that homozygous mutations, in this region at β417D and β421E to Ala, are recessive lethal; and (b) a homozygous mutation may require further selection with higher concentrations of Epo B.

One idea is that cancer cells develop mechanisms that result in microtubules with decreased stability to compensate for the presence of microtubule-stabilizing agents. Point mutations in β-tubulin are one of these mechanisms. Mutations in our Epo-resistant cells map to domains involved in the regulation of microtubule stability, the M-loop, the nucleotide-binding pocket, and the binding sites for MAPs. Cells carrying these mutations are not just resistant to the Epos but are also resistant to other microtubule-stabilizing agents, such as Taxol and Taxotere.

Consistent with this hypothesis, we have observed that there is a lag period before microtubule assembly occurs in A549.EpoB40 cells, compared with A549 cells, in the presence of Epo B. Although this difference is subtle and is only significant at 100 nM drug, the result is very repeatable. Furthermore, A549.EpoB40 cells are hypersensitive to microtubule-destabilizing drugs. The IC₅₀s for vinblastine and colchicine are decreased by 0.5- and 0.6-fold, respectively. This indicates that the microtubules in A549.EpoB40 cells are probably less stable than those in A549 cells and, therefore, more sensitive to destabilizing agents.

β-Tubulin mutations have been identified as a mechanism associated with Epo resistance (28). Most of the mutations have been located either near the Taxol/Epo binding pocket or near the M-loop (28, 41). In this study, an association has been noted between Epo resistance and mutations at other regions, such as the nucleotide-binding site and the binding site for MAPs on the COOH terminus of β-tubulin. This strongly suggests that alterations in microtubule stability, in addition to a decreased affinity between a drug molecule and its binding pocket in β-tubulin, can contribute to drug resistance to the Epos. Resistant cells carrying these mutations...
are cross-resistant to other microtubule-stabilizing agents and may become hypersensitive to microtubule-destabilizing drugs.

The results obtained from this study may also shed some light on the use of the Epos in treating human carcinomas. For example, cancer patients harboring mutations in β-tubulin, who were treated previously with the taxanes (34), may have poor responses to the Epos. Patients with special polymorphisms in their β-tubulin sequence may be predisposed to being either hypersensitive or resistant to microtubule-interacting agents such as the taxanes, the Epos, or the Vinca alkaloids.

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References


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