Epothilone B Stabilizes Microtubuli of Macrophages Like Taxol without Showing Taxol-like Endotoxin Activity

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Abstract

Epothilones are a new class of potential antitumor compounds that were isolated from the myxobacterium Sorangium cellulosum. Epothilones have effects on the cytoskeleton similar to those of the antineoplastic drug Taxol. Both compounds inhibit cell proliferation by stabilizing microtubuli, and they compete for the same binding site. In addition, Taxol displays endotoxin-like properties in that it activates macrophages to synthesize proinflammatory cytokines and nitric oxide. We measured nitric oxide release by IFN-γ-treated murine macrophages as an indicator of macrophage activation by epothilone B. Although epothilone B showed the expected effects on the microtubuli, there was no indication of macrophage stimulatory activity by epothilone B, nor did epothilone B inhibit lipopolysaccharide-mediated nitric oxide release. We conclude that, unlike Taxol, epothilone-mediated microtubulin stabilization does not trigger endotoxin-signaling pathways. Moreover, because the endotoxin-like activity of Taxol may be the cause of some nonhematological clinical side effects, it is to be expected that such effects may not occur with epothilones.

Introduction

The epothilones, a new class of cytotoxic compounds (1) isolated from the myxobacterium Sorangium cellulosum (2) and recently synthesized by three groups in a hotly competitive race (3) are potential antineoplastic agents. There are only vague similarities in structure between the epothilones and the antineoplastic agent Taxol (Fig. 1), but both classes of compounds act on the cytoskeleton by stabilization of cellular microtubuli that eventually leads to arrest of cell proliferation and apoptosis (4). Moreover, epothilones and Taxol compete for the same binding site (5). Taxol, like endotoxin, activates macrophages to synthesize proinflammatory cytokines (6) and nitric oxide (7), and a major endotoxin-signaling pathway in macrophages may involve activation of microtubule-associated protein kinases (8). As has been suggested, proinflammatory cytokines (9) or nitric oxide (7) may contribute to the antitumor activity of Taxol. We therefore assayed the putative macrophage stimulatory activity of epothilone B, which is the most potent antiproliferative epothilone species.

Materials and Methods

Peritoneal exudate cells from NMRI outbred mice (Bomholtgard, Ry, Denmark) were obtained by lavage of the peritoneal cavity immediately after killing the animals with carbon dioxide gas. The cells were suspended at 10⁶ cells/ml in DMEM, substituted with 5% FCS (Life Technologies, Inc.) and 25 μM 2-mercaptoethanol, and incubated in 0.1-ml volumes in 96-well flat-bottomed microtiter plates. Putative stimulants were added at the beginning of the cultivation period as indicated: 60 units/ml recombinant mouse IFN-γ, and various amounts of phenol-extracted Salmonella typhimurium LPS² (10), Taxol (Fluka, Neu-Ulm, Germany; Taxol is a trade name of paclitaxel), or epothilone B. For testing vitality in the presence of epothilone B, the macrophages among the peritoneal exudate cells were allowed to adhere for 2 h before removal of nonadherent cells by repeated rinsing of the microtiter plates with fresh medium. The tetrazolium salt reduction assay was done with 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) (Boehringer Mannheim, Mannheim, Germany) according to the supplier's instructions. Alternatively, 6 × 10⁵ cells were seeded in 1-ml volumes in 24-well multiculture plates with coverslips at the bottom of the wells, so that the adherent macrophages could be inspected for tubulin condensation. After a 45-h incubation at 37°C in a moist atmosphere containing 7.5% CO₂, culture medium was removed, and nitric oxide release was determined from the sum of its decay products nitrate and nitrite. Nitrate was reduced with nitrate reductase, and nitrite was measured with Griess reagent as described previously (11). The absorbance at 550 nm of 0.720 corresponds to 100 μM nitrite. For inspection of microtubuli, the cells were rinsed with PBS (Life Technologies, Inc.) at 37°C, fixed with 3.7% paraformaldehyde in PBS for 10 min, and permeabilized in 0.1% Triton X-100 in PBS for 5 min. The cells were immunostained with a primary anti-α-tubulin (1:500; Sigma Chemical Co.) and a secondary antimouse IgG antibody conjugated with tetramethylrhoda-mine isothiocyanate (1:100; Sigma).

Results and Discussion

Upon stimulation, macrophages and monocytes produce the cytokines interleukin-1, interleukin-6, and tumor necrosis factor. Stimulated murine macrophages, when primed or simultaneously treated with IFN-γ, also produce nitric oxide (12). This highly reactive gas constitutes one of the cytoidal mechanisms of macrophages (13). It rapidly decays to nitrite and nitrate, products that can be quantitatively determined after conversion of nitrite into nitrite in a diazocoupling reaction (11). Nitric oxide release from IFN-γ-primed macrophages is conveniently used to quantify the macrophage stimulatory capacity of various compounds, including that of Taxol (7). We chose this assay to examine the putative macrophage stimulatory potential of epothilone B, using peritoneal exudate cells from NMRI mice as a source of macrophages. S. typhimurium LPS and Taxol were used as positive controls. Surprisingly, epothilone B proved to be inactive in this assay over a wide concentration range, whereas the other macrophage activators stimulated nitric oxide release at the expected concentrations (Fig. 2).

The experiment was repeated in 24-well culture plates with round microscope coverslips at the bottom of each well to be able to inspect the adherent macrophages for microtubule condensation as an intrinsic control of epothilone activity. To this end, culture medium was removed from the cells after a 45-h incubation period with putative macrophage activators and/or IFN-γ and assayed for nitric oxide release, whereas the adherent macrophages from these same cultures were fixed for subsequent indirect immunostaining with α-tubulin-specific antibodies. The nitric oxide release data are shown in Table

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² The abbreviation used is: LPS, lipopolysaccharide.
Table 1. Effects of Taxol, LPS, and epothilone B on mouse macrophage activation and condensation of microtubuli

<table>
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<th>No.</th>
<th>IFN-γ</th>
<th>Taxol (µg/ml)</th>
<th>LPS (ng/ml)</th>
<th>Epothilone B (ng/ml)</th>
<th>Effect on microtubuli</th>
<th>Nitric oxide release (AΔ550 nm)</th>
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<td></td>
<td></td>
<td></td>
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We conclude that although epothilone B shows Taxol-like activity on the microtubuli of macrophages, it is not capable of activating these cells like Taxol does. That means that in the case of epothilone B, microtubule stabilization and activation of the LPS signaling pathway are independent events.

Our results may be pertinent to a potential application of epothilone as an antitumor drug. It is presently not clear whether endotoxin-like effects of Taxol are therapeutically beneficial (7, 9) or may rather be the cause of nonhematological side effects (rarely severe), such as myalgia/arthralgia (14, 15). It is to be expected that such side effects, which have typically been noted in trials with endotoxin (16) and with the proinflammatory cytokines interleukin-1 (17), interleukin-6 (18), and tumor necrosis factor (19) may be less pronounced with epothilones than with Taxol. This must, of course, await clinical studies. Our results are also of interest to those who try to understand the endotoxin- and Taxol-mediated signaling mechanisms in macrophages, which, as shown with nonstimulatory analogues of LPS, may both be triggered by an LPS receptor-dependent mechanism (20).
with IFN-\(\gamma\), B, epothilone-treated cells from well 10 (12.5 ng/ml). Bar, 10 \(\mu\)m.

After removal of the culture medium for determination of nitric oxide release, cells were immunostained with anti-\(\alpha\)-tubulin-specific antibodies. A, control cells from well 1, incubated

Acknowledgments

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References


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