Lipopolysaccharide Antagonists Block Taxol-induced Signaling in Murine Macrophages

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Summary

Taxol is the prototype of a new class of microtubule stabilizing agents with promising anticancer activity. Several studies show that taxol mimics the actions of lipopolysaccharide (LPS) on murine macrophages. To investigate the mechanism of taxol-induced macrophage stimulation, we evaluated the ability of *Rhodobacter sphaeroides* diphosphoryl lipid A (RsDPLA) and SDZ 880.431 to block taxol-induced effects. RsDPLA and SDZ 880.431 are lipid A analogues that lack LPS-like activity, but inhibit the actions of LPS, presumably by blocking critical cellular binding sites. We report that RsDPLA and SDZ 880.431 potently inhibited taxol-induced TNF secretion, gene activation, and protein-tyrosine phosphorylation. The role of microtubules in taxol signaling was investigated. Taxol-induced microtubule bundling in primary and transformed RAW 264.7 macrophages was not blocked by RsDPLA or SDZ 880.431. Taxotere, a semisynthetic taxoid, was more potent than taxol as an inducer of microtubule bundling, but did not induce tumor necrosis factor α secretion and gene activation. These data dissociate the microtubule effects of taxol from macrophage stimulation and suggest that taxol stimulates macrophages through an LPS receptor–dependent mechanism. The results underscore the potential of taxol as a tool for studying LPS receptor activation and provide insights into possible therapeutic actions of this new class of drugs.

Mammalian host defense mechanisms have evolved to recognize trace amounts of Gram-negative bacterial outer membrane, namely, endotoxin, and to respond in an integrated fashion involving each major effector arm of the immune system (1). In extreme cases, autonomic systems involved in homeostasis are dysregulated, as occurs during septic shock (2). Despite the complexity and potential violence of this response, only recently has there been significant understanding of how endotoxin interacts with cells at the molecular level (3, 4). The major component of endotoxin, i.e., LPS, appears to exist in the extracellular milieu as a complex with one or more plasma proteins. Select proteins increase the efficiency of LPS binding to CD14, a phosphatidylinositol-anchored membrane protein expressed by macrophages and neutrophils (5–9). Delivery of LPS to CD14 is required (8, 9), but not sufficient, for LPS stimulation of macrophages. This is illustrated by the finding that the inactive lipid A analogue, lipid IVₐ, suppresses LPS signaling under conditions that do not block LPS binding to CD14 (9). Consequently, it has been speculated that CD14 serves to facilitate delivery of LPS to an as yet unidentified LPS “receptor” protein (4).

Identification of this putative receptor is an area of active investigation.

Taxol* is the first effective anticancer agent with a novel mechanism of action to be developed in over a decade, and it has generated considerable enthusiasm in the oncology community due to its favorable response rate in patients with advanced metastatic ovarian, breast, and lung cancer (10). Taxol is a complex diterpenoid isolated from the bark of the Pacific yew (Fig. 1). The antiproliferative effects of taxol appear to be related to its ability to bind β-tubulin and stabilize microtubules (11–13). The dynamic depolymerization necessary for microtubule function during mitosis (and other cellular processes) is blocked. Taxol-treated cells develop large bundles of microtubules and multiple mitotic asters and are arrested in the M phase of the cell cycle (14). Numerous taxol analogues have been generated by semisynthetic approaches (13, 15). One of these, taxotere‡, is approximately twice as po-
tent as taxol in cytotoxicity and microtubule depolymerization assays (15) and is also being tested in clinical trials (16).

Nature may have provided in taxol an invaluable tool for dissecting LPS-signaling pathways. Ding et al. (17) discovered that taxol exhibited profound cell cycle-independent effects on murine macrophages. Taxol activated the acute internalization of TNF-α receptors and initiated a slower induction of TNF-α protein, properties that were remarkable, in part, because they were shared by LPS. Further investigation revealed that this activity of taxol was restricted to macrophages from mice bearing the wild-type Lip A gene, i.e., responsiveness to taxol was genetically linked to responsiveness to LPS. Subsequently, taxol was shown to stimulate a panel of macrophage responses in a manner virtually identical to LPS (18–20). Taxol activated the expression of six of LPS-inducible genes with kinetics and amplitude indistinguishable from LPS, and taxol, like LPS, induced rapidly the tyrosine phosphorylation of several 41–45-kD proteins (18, 20). In toto, the overlapping activities of taxol and LPS suggest the two agents share signaling pathways. Recently, Ding et al. (21) have presented the provocative finding that LPS binds specifically and with high affinity to β-tubulin, providing a potential mechanism by which taxol and LPS could induce shared responses.

A second set of tools useful in studying LPS signaling include several synthetic/natural lipid A and lipid X analogues that lack LPS-like activity, but that retain the ability to block LPS stimulation of various cell types. It is believed that they represent competitive inhibitors of LPS binding to its critical receptor(s) due to their structural similarity to the active center of LPS, namely, lipid A, and because they are highly specific inhibitors of LPS (i.e., they do not block other macrophage activating agents such as Gram-positive cell walls, phorbol ester, or cytokines [9, 22, 22a]), and can be overridden in the presence of excess LPS (9, 22–25). Rhodobacter sphaeroides diphosphoryl lipid A (RsDPLA) is one of the best characterized LPS “antagonists” (Fig. 1). Initially purified by Takayama et al. (26) and chemically defined by Qureshi et al. (27), RsDPLA has been used to block LPS-induced TNF production in vitro (28) and by monocytes/macrophages in vitro (22, 26, 28, 29). Others have reported that RsDPLA blocks LPS-induced pre-B cell activation (23) and CD18 surface expression on human neutrophils (24). In addition to other lipid A analogues, several synthetic monosaccharide derivatives also have inhibitory activity (25, 30). SDZ 880.431 (3-aza-Lipid X 4-phosphate) (Fig. 1) has been well characterized and shown to inhibit LPS-induced macrophage TNF secretion (31), neutrophil CD18 expression (23), and expression of procoagulant activity in cultures of human PBMC (30). In general, disaccharide inhibitors are approximately an order of magnitude more potent than monosaccharide inhibitors.

In the present study, we investigated further the relationship between taxol- and LPS-signaling pathways. We have used RsDPLA and SDZ 880.431 as tools to suggest that taxol stimulates murine macrophages through an LPS receptor-dependent pathway, and to dissociate taxol-induced signaling from taxol-induced microtubule changes.

**Materials and Methods**

**Reagents.** Protein-free (<0.008%), phenol/water-extracted Escherichia coli K235 LPS was prepared by the method of McIntire et al. (32). RsDPLA was prepared as described previously (27). SDZ 880.431 was synthesized by Sandoz Research Institute (Vienna, Austria) as described (30). Taxotere was a kind gift of Rhône-Poulenc Rorer (Vitré Sur Seine, France). Taxol was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD), and was stored at −70°C as a 10-mM stock solution in DMSO. The highest concentration of DMSO (0.3%) used in these studies did not induce LPS-inducible genes or tyrosine phosphorylation. A 1-mM dilution of taxol contained <0.03 EU/ml by Limulus amoebocyte lysate assay, corresponding to approximately <90 fg/ml LPS in a 30-μM taxol solution; minimum concentrations of LPS required to induce gene expression and tyrosine phosphorylation were 0.1 and 1 ng/ml, respectively.

**Primary Macrophage Isolation and Culture.** Thioglycollate-elicited peritoneal macrophages from C3H/10t mice (The Jackson Laboratory, Bar Harbor, ME) were isolated and cultured (2 × 10⁶ cells/ml) in complete medium (RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes, 0.3% sodium bicarbonate, and 2% heat-inactivated FCS) as described previously (18). Cells were cultured at 37°C and 6% CO₂, and nonadherent cells were removed by washing with media 2–4 h after plating. For TNF secretion studies, 2 × 10⁶ cells/well were plated into Falcon 96-well microtiter plates (Becton Dickinson & Co., Lincoln Park, NJ). For Northern blot analysis, 4 × 10⁶ cells/well were cultured in six-well polystyrene tissue culture plates (Becton Dickinson & Co.). For analysis of protein tyrosine phosphorylation, 10⁶ cells/well were plated into 24-well culture plates (Costar Corp., Cambridge, MA). TNF-α Biosay. 4-h culture supernatants were assayed for TNF-α bioactivity in a cytotoxicity assay using actinomycin D–treated L929 cells as described previously (18).

**Northern Blot Analysis of Taxol-induced Genes.** Adherent macrophages were cultured for 4 h with the indicated stimuli, lysed in 1 ml of RNAzol B (Tel-Test, Inc., Friendswood, TX), and total RNA was isolated according to the manufacturer’s instructions. RNA (5–10 μg) was fractionated on 1% agarose gels containing formaldehyde, transferred via capillary action to a Nytran filter (Schleicher & Schuell, Inc., Keene, NH), UV crosslinked to the filters, and hybridized overnight with random-primed 32P-labeled cDNA probes. Probes specific for the transcripts encoding murine TNF-α, TNFR-2, IL-1β, IP-10, D3, D8, and β-actin were used in this study and have been described in detail previously (18, 33–35). Hybridized blots were washed three times for 10 min at 65°C in 0.1x SSC containing 0.1% SDS, and exposed to Kodak XAR-5 film with intensifier screens for 18–24 h at 70°C. The ratio of the signal between the probe of interest and β-actin was used as a measure of relative induction. Between successive hybridizations, Nytran filters were boiled in distilled water for 5 min.

**Antiphosphotyrosine Immunoblotting.** Macrophages were cultured with treatments as indicated. At the end of the culture period, the media was rapidly replaced with 100 μl/well ice-cold lysis buffer (100 mM TrisHCl, pH 8.0; 100 mM NaCl; 2 mM EDTA; 1% NP-
40; 1 mM Na2VO4; 50 mM NaF; 100 μM TPCK; 100 μM quercitin; 1 mM PMSF; 1 μg/ml leupeptin, and pepstatin). Culture plates were agitated in an ice water bath for 10 min, and cell lysates were transferred to microfuge tubes and centrifuged (12,000 g for 1 min) to remove detergent-insoluble material. Supernatants (80 μl) were boiled for 5 min with 28 μl of 4X loading buffer (200 mM Tris-HCl, pH 6.8, 10% SDS, 400 mM dithiothreitol, 40% glycerol, and 0.4% bromophenol blue), and 13 μl/lane was resolved by SDS-PAGE on 10% acrylamide gels (7 x 8 cm). The separating gel was buffered with 375 mM TrisHCl, pH 8.5. Resolved proteins were blotted onto Immobilon-P transfer membranes (Millipore Corp., Bedford, MA). Blots were blocked 1 h in wash buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) containing 3% gelatin and 5% milk. Blots were then incubated 1 h in wash buffer containing 1 μg/ml antiphosphotyrosine mAb (clone 4G10; Upstate Biotechnology, Inc., Lake Placid, NY), washed, and incubated with horseradish peroxidase-conjugated goat anti–mouse IgG (1:3,000 dilution; Bio-Rad Laboratories, Richmond, CA). Binding of secondary antibody was detected with the enhanced chemiluminescence (ECL) detection method (Amersham, UK). Molecular weights of phosphoproteins were determined by comparison with prestained markers (Bio-Rad Laboratories).

Immunofluorescent Detection of Microtubules. RAW 264.7 cells (American Type Culture Collection, Rockville, MD) (maintained in DMEM containing 5% FCS) or primary macrophages were plated (2 x 10^5 cells/well) onto Lab-Tek eight-well glass chamber slides (Nunc, Inc., Naperville, IL). RAW 264.7 cells were allowed to adhere and proliferate overnight. Cells were then pretreated 10 min with media alone or containing 2 μg/ml RsDPLA or 20 μg/ml SDZ 880.431. Where indicated, culture media was adjusted to contain 3 μM taxol, and the cells were cultured an additional 4 h before fixation for 30 min at room temperature in 10% buffered formalin. Microtubules were visualized in fixed cells by immunofluorescence as described previously (18) using a rabbit anti–sea urchin tubulin IgG fraction (Polysciences, Inc., Warrington, PA) and a fluoresceinated F(ab')2 fragment of goat anti–rabbit IgG (Cappel Laboratories, Malvern, PA).

Results

Taxol-induced TNF Secretion Is Blocked by Lipid A–based LPS Inhibitors. As a first approach to define the signaling mechanism of taxol, the ability of RsDPLA to inhibit taxol-induced TNF secretion was evaluated. C3H/OuJ macrophages were treated with medium alone or with medium containing 30 μM taxol with or without increasing concentrations of RsDPLA. 4-h supernatants were harvested and assayed for TNF activity (Fig. 2 A). RsDPLA (10–1,000 ng/ml) potently inhibited taxol-induced TNF secretion. We next determined if the ability of RsDPLA to block taxol was specific to RsDPLA or general to LPS antagonists. SDZ 880.431 was selected for testing due to its structural dissimilarity to RsDPLA. SDZ 880.431 effectively inhibited taxol-induced TNF over a concentration range of 0.3–10 μg/ml (Fig. 2 B). The concentrations of RsDPLA and SDZ 880.431 found to inhibit taxol-induced TNF were virtually identical to the concentrations required to block LPS (1 ng/ml)-induced macrophage TNF secretion and gene expression (22a). Although the taxol solutions contained no detectable activity in the Limulus amoebocyte lysate assay (see Materials and Methods), the ability of polymyxin B to block taxol-induced TNF was tested to control for the presence of endotoxin (Fig. 2 C). Polymyxin B inhibited the TNF-inducing activity of 1 ng/ml LPS, but had no effect on a submaximal concentration of taxol (16 μM).

Taxol-induced Gene Expression and Tyrosine Phosphorylation Is Blocked by Lipid A–based LPS Inhibitors. Taxol induces the expression of TNF-α and at least five other LPS-inducible genes (18). We next sought to determine if RsDPLA blocks taxol-induced TNF-α at the level of mRNA induction, and, if so, whether blockade of taxol-induced responses is selective or extends to the other inducible genes. Fig. 3 shows that RsDPLA inhibited taxol-induced expression of TNF-α and IL-1β mRNAs. In addition, RsDPLA inhibited the taxol-induced expression of transcripts encoding IP-10, TNFR-2, D3, and D8, and taxol-induced expression of this entire panel of genes was similarly blocked by 0.3–10 μg/ml SDZ 880.431 (data not shown). Inhibition of gene expression by RsDPLA appeared to be of a competitive nature since it could be overridden by increasing the concentration of taxol (Fig. 4).

One of the earliest biochemical events measurable during taxol and LPS signaling is tyrosine phosphorylation of 41.5-, 43-, and 47-kD proteins (Fig. 5, solid lines between the two panels). This is accompanied by the apparent dephosphory-
Figure 2. LPS inhibitors block taxol-induced TNF secretion in murine macrophages. C3H/HeJ macrophages were cultured 4 h in the presence of the indicated stimuli and inhibitors, and supernatants were harvested and tested for TNF activity as described in Materials and Methods. RsDPLA and SDZ 880.431 were added to taxol solutions simultaneously with cell stimulation. Polymyxin B was added to LPS and taxol solutions 15 min before cell stimulation. (A) RsDPLA concentration–dependent inhibition of taxol (30 μM)-induced TNF secretion. (B) SDZ 880.431 concentration–dependent inhibition of taxol (30 μM)-induced TNF secretion. (C) Polymyxin B concentration–dependent effects on TNF secretion induced by 1 ng/ml LPS or 16 μM taxol. Values in A and B are mean values and SEM of three separate experiments. Values in C are from a single experiment.

Figure 3. LPS inhibitors block taxol-induced gene expression in murine macrophages. C3H/OuJ macrophages were cultured 4 h with 30 μM taxol with and without increasing concentrations of RsDPLA. Cellular RNA was harvested and subjected to Northern blot analysis of TNF-α, IL-1β, and β-actin gene expression as described in Materials and Methods. Results are representative of two separate experiments.

Taxol Signaling Blocked by Lipopolysaccharide Antagonists

Polymyxin B (μg/ml)
Figure 4. Raising the concentration of taxol overrides RsDPLA inhibition of TNF-α mRNA. C3H/OuJ macrophages were cultured 4 h with media alone or containing 20 ng/ml RsDPLA and increasing concentrations of taxol. Cellular RNA was harvested and subjected to Northern blot analysis of TNF-α and β-actin gene expression. Relative gene expression was determined by Phosphorimaging as described in Materials and Methods.

The "mitotic index," can be determined by counting the percentage of cells that contain mitotic spindles. By stabilizing microtubules, taxol induces the formation of multiple, dysfunctional mitotic asters and blocks progression through mitosis. Consequently, RAW 264.7 cells that contain mitotic spindles or asters accumulated sevenfold when cultured 4 h in the presence of taxol. As shown in Table 1, excess RsDPLA or SDZ 880.431 did not reduce the percentage of cells blocked in mitosis by taxol. These results confirm that RsDPLA or SDZ 880.431 do not inhibit taxol-mediated microtubule stabilization.

**Taxotere Stabilizes Microtubules but Fails to Induce TNF Secretion or Gene Activation.** Taxotere is a semisynthetic taxoid that is two- to threefold more potent than taxol as a microtubule stabilizing agent (15). In our experiments, bundling and aster formation in RAW 264.7 cells was induced by as little as 0.075 μM taxotere, while 0.3 μM taxol was required to achieve a comparable effect. To investigate further the relationship between microtubule stabilization and cell signaling, the ability of taxotere to induce murine macrophages to secrete TNF and express TNF-α mRNA was examined (Fig. 6). Taxotere did not induce detectable TNF secretion or mRNA expression.

### Table 1. Taxol-induced Mitotic Arrest Is Not Blocked by LPS Inhibitors

<table>
<thead>
<tr>
<th>Taxol (3 μM)</th>
<th>Inhibitor</th>
<th>Mitotic index*</th>
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<td>-</td>
<td>-</td>
<td>2.2</td>
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<tr>
<td>-</td>
<td>RsDPLA (2 μg/ml)</td>
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<td>-</td>
<td>SDZ 880.431 (40 μg/ml)</td>
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<td>+</td>
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<td>+</td>
<td>RsDPLA (2 μg/ml)</td>
<td>15.7</td>
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<tr>
<td>+</td>
<td>SDZ 880.431 (40 μg/ml)</td>
<td>17.6</td>
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RAW 264.7 macrophages were pretreated 10 min with indicated lipid A-based inhibitor and an additional 4 h with media alone or adjusted to contain 3 μM taxol. Cells were stained for microtubules as described in Materials and Methods.

* Mitotic index was determined as the percentage of cells exhibiting mitotic spindles or mitotic asters. At least 500 cells per group were examined.

**Discussion**

Taxol-induced TNF secretion, gene activation, and protein-tyrosine phosphorylation were blocked by 10–1,000 ng/ml RsDPLA or 0.3–10 μg/ml SDZ 880.431. These concentrations of RsDPLA and SDZ 880.431 inhibit LPS responses.
inhibition of taxol by LPS inhibitors (C. L. Manthey, unpublished). The role of CD14 and 73-kD protein in taxol signaling is not likely to be involved in taxol stimulation of macrophages. The role of CD14 and 73-kD protein in taxol signaling is unknown and under investigation. LPS binds polymerized tubulin (21), and this provocative data suggest microtubules may be important binding sites for LPS and taxol stimulation of macrophages. However, the microtubule effects of taxol occur independently of macrophage stimulation. As shown herein, RsDPLA and SDZ 880.431 blocked taxol stimulation of normal C3H/OuJ macrophages, but had no effect on microtubule bundling, and, conversely, taxotere induced microtubule bundling but did not induce LPS-like signaling. Moreover, taxol induces normal microtubule bundling in macrophages from C3H/HeJ (Lps+) mice, but fails to stimulate these cells (17, 18). Thus, microtubule stabilization induced by taxol and taxotere is not sufficient for stimulation of macrophages.

It is our view that taxol signals through an as yet unrecognized protein, and identification of this molecule will provide needed insights into the nature of the LPS-signaling receptor. Early binding studies with radiolabeled taxol identified polymerized tubulin as the primary cellular target of taxol (11). More recently, direct photoaffinity labeling has demonstrated selective binding of taxol to β-tubulin (12). Progress is being made in the development of taxol derivatives with photoactivatable crosslinking moieties to map more efficiently the β-tubulin binding site (13). It will be important to determine if crosslinking derivatives of taxol retain LPS-mimetic activity, and to apply these derivatives to the identification of novel proteins that may also bind LPS and lipid A-based LPS inhibitors.

Taxol represents the first of a class of new anticancer drugs, and much may be learned regarding tumor biology and treatment as the pharmacology of these agents is more completely understood. The mechanism of action of this group of agents is felt to reside in their ability to stabilize microtubules and prevent cell progression through mitosis. Our results suggest taxol may also activate putative LPS receptors on murine macrophages. For decades investigators have known that LPS can cure rodents of tumors (38, 39), but the use of LPS in humans is confounded by its unacceptable toxicity. Antitumor activity of LPS arises, in part, from the ability of LPS to induce TNF and other cytokines and to serve as a second signal in the activation of macrophage nitric oxide synthase and tumoricidal activity (39, 40). LPS substructures have been identified that are relatively nontoxic, but that retain the antitumor activity of LPS (39). Two such compounds, monophosphoryl lipid A and SDZ MRL 953, activate murine macrophages in an LPS-like fashion and are blocked by lipid A-based inhibitors (29, 31). We propose that taxol, by analogy to these nontoxic LPS mimetics, may activate host antitumor activity. However, induction of TNF may not be required for the therapeutic efficacy of other taxoids. Although taxotere did not induce TNF in murine macrophages, recent phase II clinical trials indicate that taxotere is a highly effective drug against breast cancer and other solid tumors (16). More work is required to determine if taxol can act as a second signal for induction of tumoricidal activity and to determine the cell specificity of the LPS-mimetic actions of taxol, e.g., it will be important to determine if taxol stimulates cell types other than macrophages, and if taxol activates genes in human immune or neoplastic tissues.
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