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Advances in Brief

Relationship between the Structure of Taxol and Other Taxanes on Induction of Tumor Necrosis Factor-α Gene Expression and Cytotoxicity[1]

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Abstract

Taxol is an antitumor drug with cytotoxic properties that correlate with its microtubule-stabilizing activities. It has been reported that taxol parallels lipopolysaccharide in its effects on the induction of tumor necrosis factor-α (TNF-α) gene expression in macrophages (C. Bogdan and A. Ding, J. Leukocyte Biol., *52:* 119–121, 1992; C. L. Manthey, M. E. Brandes, P. Y. Perera, and S. Vogel, J. Immunol., *149:* 2459–2465, 1992; J. M. Carboni, C. Singh, and M. A. Tepper, Natl. Cancer Inst. Monogr., *15:* 95–101, 1993). Structure-activity studies using taxol and related taxanes have been done to determine the relationship between the effects of taxol on TNF-α gene expression and its cytotoxic and microtubule-stabilizing activities. Using Northern blot analysis, it was found that changes in the structure of taxol that did not alter cytotoxicity did prevent the induction of TNF-α gene expression. The data presented in this paper demonstrate that the effects of taxol on TNF-α gene expression are distinct from its known cytotoxic properties.

Introduction

Taxol is a clinically effective antitumor drug that demonstrates encouraging activity in human ovarian and breast carcinomas (1). It has a novel chemical structure and an unusual mechanism of action (2). The drug is an antimitotic agent whose action results in the formation of stable bundles of microtubules within cells. The hallmark activity of the drug *in vitro* is its ability to enhance the assembly of stable microtubules in the absence of GTP, which is normally required for assembly. Taxol is unusual in its propensity to polymerize tubulin into microtubules, even under conditions that normally depolymerize microtubules, such as low temperature and high Ca^{2+} concentrations. These *in vitro* activities are facilitated by the binding of taxol within the NH_2-terminal 31 amino acids of β-tubulin (3). In the past few years, new activities have been assigned to taxol that relate to the ability of the drug to mimic the effects of bacterial LPS.[4] Like LPS, taxol induces expression of the genes for the cytokines TNF-α and interleukin 1β (4–6) and the "early" LPS-inducible genes, *IP-10, D3, D7,* and *D8* in macrophages (5). In addition, taxol causes a reduction in the surface expression of TNF-α receptors (7); induces tyrosine phosphorylation of microtubule-associated protein kinases, also known as mitogen-activated protein kinases (5, 6, 8, 9); and enhances γ-interferon induction of nitric oxide synthase and secretion of nitric oxide, a macrophage tumoricidal factor (10). All of these LPS-like responses initiated by taxol can be observed in macrophages isolated from LPS-responsive mice but not in macrophages from LPS-hyporesponsive mice, suggesting that there exists a common signaling pathway for both taxol and LPS (5, 7–10). In addition, LPS interacts with β-tubulin and microtubule-associated protein 2 (11).

TNF-α is a multifunctional polypeptide produced primarily by activated macrophages (12). With regard to cancer, the activities of TNF-α are complex and include cytotoxicity to some tumor cells, induction of cachexia, and the stimulation of tumor growth. The possible involvement of taxol in the secretion of TNF-α from macrophages during chemotherapy brings into question whether TNF-α could be responsible for mediating some of the antitumor properties of the drug. This question is of particular interest since taxol has activity against solid tumors (1), a rather unusual but very important aspect of the antitumor activity of the drug. To begin to explore these questions, we compared the structure-activity relationships of taxol and related taxanes for TNF-α gene expression in light of previous studies from our laboratory and other laboratories on cytotoxicity and the assembly of stable microtubules. The cytotoxicity of the taxanes used in this study correlates with their ability to stabilize microtubules (13–16). The results reported in this paper suggest that the expression of the gene for TNF-α and its secretion are unrelated to the microtubule assembly and cytotoxic properties of taxol.

Materials and Methods

Drugs and Cell Culture. Taxol, baccatin III, and cephalomannine were obtained from the Drug Development Branch of the National Cancer Institute. Taxotere was a kind gift from Rhône-Poulenc Rorer; 7-acetyltaxol was received from Dr. David Kingston of Virginia Polytechnic Institute and State University; 10-deacetyltaxol and 10-acetyltaxotere were gifts from Bristol Myers Squibb; baccatin III 13-(*R*-3-phenyllactate), baccatin III 13-(*S*-3-phenyllactate), baccatin III 13-(*R-N*-benzoyl-isoserinate), and baccatin III 13-(*S-N*-benzoyl-isoserinate) were synthesized as described (16). All drugs were prepared as 45 mM stock solutions in 100% dimethyl sulfoxide except for 10-acetyltaxotere which was dissolved in 100% ethanol at a final concentration of 25 mM. L929, a murine fibrosarcoma cell line that is used as a biological assay for TNF-α cytotoxicity, was maintained in Dulbecco's modified Eagle's media with 10% fetal calf serum, 1% nonessential amino acids, 1% penicillin/streptomycin, and 1% glutamine. RAW 264.7, a murine macrophage cell line, was maintained in the same media as the L929 cells with the addition of 10% NCTC-109 medium. Both cell lines were grown at 37°C in 6% CO_2. All cell culture supplies were from GIBCO. The *Limulus* amebocyte lysate kit, used for the detection of LPS contamination, was purchased from Sigma.

Measurement of TNF-α Secretion and mRNA Expression. RAW 264.7 cells were incubated with each drug (1–30 μM) or with 10 ng/ml of *Escherichia coli* LPS 055:B5 (Sigma) at 37°C for 4 h. Media were removed from the cells prior to RNA isolation and assayed for TNF-α using the L929 cytotoxicity assay (17). Serial dilutions of the media, each done in triplicate, were used to determine the presence of TNF-α. Serial dilutions (1:2) of a solution (400 units/ml) of recombinant murine TNF-α (R&D Systems, Inc.) were used as a standard.

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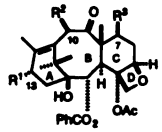
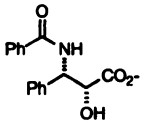
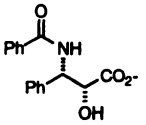
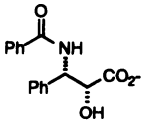
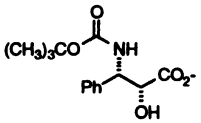
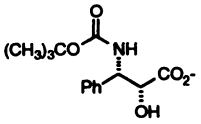
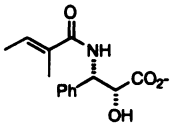
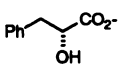
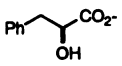
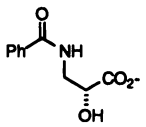
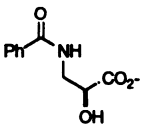
[4] The abbreviations used are: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

Table 1 *Chemical structures of taxol and related taxanes*



Name	R^1	R^2	R^3
(1) Baccatin III	HO—	AcO—	—OH
(2) Taxol		AcO—	—OH
(3) 10-Deacetyltaxol		HO—	—OH
(4) 7-Acetyltaxol		AcO—	—OAc
(5) Taxotere		HO—	—OH
(6) 10-Acetyltaxotere		AcO—	—OH
(7) Cephalomannine		AcO—	—OH
(8) Baccatin III 13-(*R*)-3-phenyllactate		AcO—	—OH
(9) Baccatin III 13-(*S*)-3-phenyllactate		AcO—	—OH
(10) Baccatin III 13-(*R*)-*N*-benzoyl-isoserinate		AcO—	—OH
(11) Baccatin III 13-(*S*)-*N*-benzoyl-isoserinate		AcO—	—OH

Total RNA was isolated from the cells using Tri Reagent (Molecular Research Center, Inc.) following the protocol of the manufacturer (18). Ten μg of total RNA were separated on a 1.5% agarose gel containing formaldehyde and transferred to a Genescreen Plus nylon membrane (DuPont New England Nuclear, Boston, MA). Northern blots were probed at 65°C with a 1.1-kilobase murine TNF-α complementary DNA insert (19) in a hybridization buffer [10% dextran sulfate, 50 mM Tris (pH 7.8), 1 M NaCl, and 1% sodium dodecyl sulfate] and quantitated using the Phosphorimager (Molecular Dynamics). To control for variations in sample loading, the blots were reprobed with a *Xenopus* histone H4 probe. Both probes were randomly labeled with [α-^{32}P]dCTP. Each compound was tested for its ability to induce TNF-α gene expression a minimum of three times, except for 10-acetyltaxotere, which was tested twice.

Results and Discussion

Taxol and related taxanes (Table 1) were assayed for their ability to induce the expression of the gene for TNF-α. A representative

Northern blot of total RNA prepared from RAW 264.7 cells that had been incubated with each of the compounds listed in Table 1 is presented in Fig. 1 along with its quantitative data. There was a clear dose response with taxol beginning at 1 μM with maximum effects observed with 20–30 μM concentrations of drug (data not shown). Of the 11 compounds tested, only taxol and 7-acetyltaxol induced the expression of TNF-α mRNA. Taxol treatment resulted in a 50–70-fold increase in TNF-α mRNA levels, whereas 7-acetyltaxol caused a 10–17-fold increase. There was no expression of TNF-α evident when media or media with dimethyl sulfoxide were tested. LPS served as a positive control. Both TNF-α-inducing drugs were free of endotoxin contamination as determined by the *Limulus* amebocyte assay (sensitivity, <0.05–0.1 endotoxin unit/ml). Our results for taxotere are consistent with those of Manthey *et al.* (20) who noted the inability of taxotere to induce the expression of TNF-α.

Data on the effects of taxol and 7-acetyltaxol on the secretion of biologically active TNF-α are illustrated in Fig. 2. As with TNF-α gene expression, taxol induced the maximum secretion of TNF-α; 7-acetyltaxol also had an effect. None of the other drugs tested demonstrated any observable effect on TNF-α secretion. Under the conditions of the assay, taxol was not cytotoxic to L929 cells. However, the effect of recombinant murine TNF-α on L929 cells was diminished in the presence of 30 μM taxol. This effect was not discernible at high concentrations of TNF-α, but it was detectable at dilutions of 1:256 and greater. At a dilution of 1:256, there was a 2–4-fold decrease in cytotoxicity (data not shown). This finding is consistent with results demonstrating a decrease in the number of TNF-α receptors on the surface of murine macrophages in response to taxol (7).

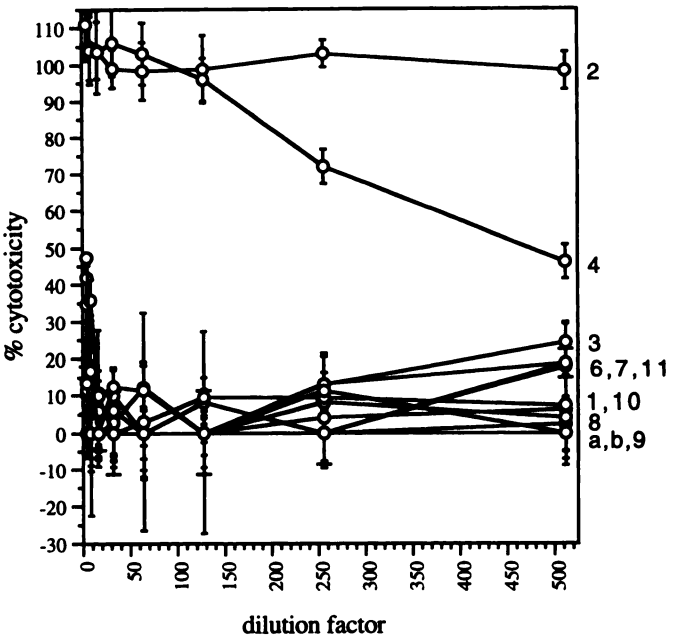


Fig. 2. TNF-α cytotoxicity of media from drug-treated RAW 264.7 cells as measured with L929 cells. Serial dilutions were used to determine the presence of biologically active TNF-α in the media. *Points*, mean; *bars*, SD.

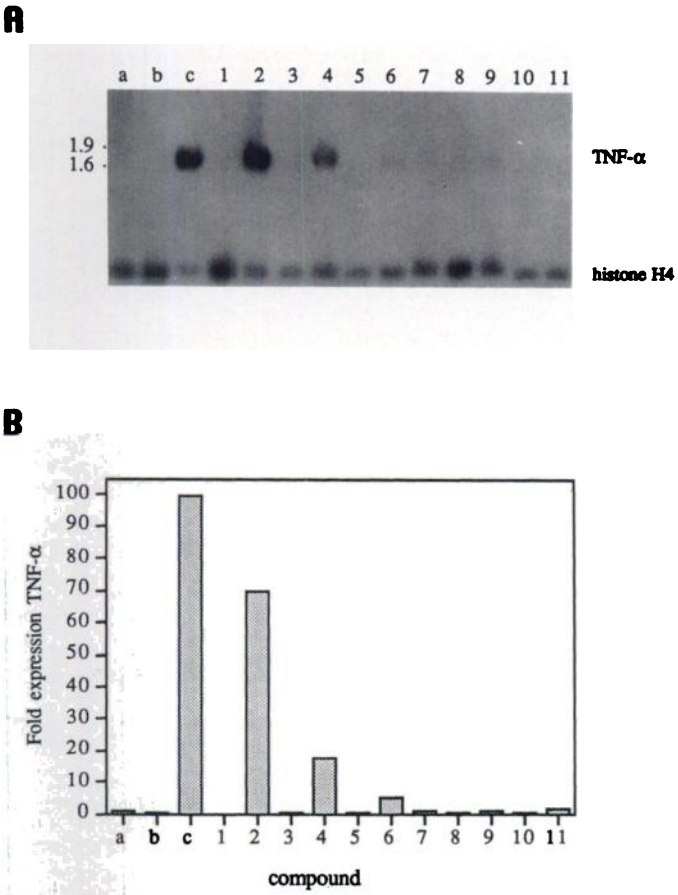


Fig. 1. Effects of taxol and related taxanes on the expression of TNF-α in RAW 264.7 cells following a 4-h incubation with 30 μM drug or 10 ng/ml LPS. *A*, representative autoradiogram of a Northern blot probed with a 1.1-kilobase murine TNF-α probe and reprobed with a *Xenopus* histone H4 probe. *B*, quantitation of the level of TNF-α mRNA normalized to histone H4. *a*, *b*, and *c* correspond to samples treated with media from untreated cells, media plus dimethyl sulfoxide, and LPS, respectively. *1-11*, numbers assigned to the compounds in Table 1.

Due to the positive therapeutic effects of taxol that have been reported for human malignancies and, until recently, to the scarcity of the drug, extensive research has been done to define the structure-activity profile of taxol to determine the elements of the taxol molecule that are required for activity. In addition, the hydrophobic nature of taxol has made it very difficult to formulate the drug, thereby encouraging chemists to design taxol analogues that would be more water soluble in addition to being more potent and better therapeutic agents. Taxol is a complex diterpenoid with a taxane ring system fused to a 4-membered oxetane ring and an ester side chain at C-13 of the A-ring (Ref. 21; Table 1). The two main assays that have been used to evaluate taxol analogues are cytotoxicity against tumor cells growing in tissue culture and the assembly of stable microtubules *in vitro* in the absence of GTP, the hallmark of taxol activity. Structure-activity studies revealed that the A-ring side chain is crucial for biological activity (15, 22). For example, baccatin III is inactive in terms of both cytotoxicity and microtubule assembly, thereby indicating a definite requirement for the side chain at C-13. However, the side chain can be modified and retain its activity as it does in cephalomannine (15) and in taxotere (13, 23). In this latter compound, the benzamide group is replaced by a *tert*-butylcarbamate [$NHCO_2(CH_3)_3$] moiety. Taxotere is of particular interest because, like taxol, it demonstrates positive activity in human malignancies (1). In the laboratory it has been shown to be slightly more active than taxol as a cytotoxic agent and as an enhancer of microtubule assembly (13, 23). These characteristics may be related to the somewhat better water solubility of taxotere as compared to taxol. The 3′-amide may play an important function in the preorganization of the taxol side-chain in binding to microtubules, although it is not required for activity. Taxol and taxotere both have the 2′-*R*/3′-*S* configuration. When the 3′-amide substituent was removed in a series of taxol analogues, the configuration at C-2′ was no longer important as a determinant of microtubule assembly (16). Alterations at C-7 and

Table 2 *Comparison of the cytotoxicity, microtubule assembly properties, and the induction of TNF-α by taxol and related taxanes*

Compound[a]	ED_{50}[b] (μM)	% of MT assembly	TNF-α expression
1[c]	>20	<0.01	–
2[d–f]	0.05–0.09	100	+++
3[c]	0.30	105	–
4[e]	0.08	95	+
5[d]	0.02	101	–
6[g]	0.012	118	–
7[c]	0.15	101	–
8[f]	3.5	68	–
9[f]	3.4	69	–
10[f]	20.0	60	–
11[f]	>45.0	<0.01	–

[a] Sample number corresponds to the number of each compound in Table 1.
[b] ED_{50}, drug concentration that inhibits cell division by 50% after 72 h in J774.2 cells; MT, microtubule.
[c] ED_{50} and MT assembly data from the report of Parness *et al.* (15).
[d] ED_{50} and MT assembly data from the report of Ringel and Horwitz (13).
[e] ED_{50} and MT assembly data from the report of Mellado *et al.* (14).
[f] ED_{50} and MT assembly data from the report of Swindell *et al.* (16).
[g] ED_{50} and MT assembly data from the report of Burkhart *et al.* (unpublished data, 1994).

C-10 in the taxol molecule resulted in only small changes in cytotoxicity (14, 15, 22).

The information available on the structure-activity relationships of taxol and related compounds in terms of cytotoxicity and microtubule stability has been compared to the results obtained for TNF-α gene expression (Table 2). Although 9 of the 11 compounds tested were known to have activity in the tubulin/microtubule system and were cytotoxic, only taxol and 7-acetyltaxol demonstrated significant effects on TNF-α gene expression and secretion. 7-Acetyltaxol induced a level of TNF-α mRNA that was 75–80% less than that of taxol, indicating that the replacement of a hydroxyl group for an acetoxy group at position 7 has important consequences. Even the replacement of an acetoxy group with a hydroxyl group at position 10 (10-deacetyltaxol) resulted in a compound with no activity and emphasized that the induction of TNF-α gene expression is extremely sensitive to small changes in the taxol molecule. Of particular interest were the results seen with taxotere, since this compound has clearly been observed to be active as an antitumor agent in humans (1). The inactivity of taxotere is not due to the hydroxyl group at C-10, which is absent in taxol, since 10-acetyltaxotere was also inactive. It is of interest that the majority of microtubules assembled with taxol have a 12-protofilament lattice, whereas microtubules induced with taxotere have 13 protofilaments, as is true with typical non-drug-induced microtubules (24).

These results raise the issue of what role TNF-α plays in the antitumor activity of taxol. The structure-activity studies reported in this paper on TNF-α gene expression are different from the known taxane structure-activity profile for microtubule assembly and cytotoxicity. The results indicate that cytotoxicity and induction of TNF-α gene expression are sensitive to different parts of the taxane structure and suggest that these activities are clearly separate. For example, taxotere has demonstrated antitumor activity comparable to taxol in human tumors, but it does not induce TNF-α mRNA expression or secretion. Moreover, the taxol C-10 substituent appears to play a more important role in TNF-α mRNA expression than it does in microtubule assembly or cytotoxicity. How is taxol, but not several of its otherwise biologically active analogues, able to induce TNF-α gene expression? TNF-α gene expression may very well be a unique characteristic of taxol and an important component of its overall cytotoxicity. The induction of a cytokine by an antitumor agent could have meaningful therapeutic consequences. It would not necessarily be expected that unrelated receptors should recognize the same structural features of a common drug ligand. However, the possibility also exists that the induction of TNF-α gene expression by taxol is mediated by microtubules which interact with one portion of the taxol structure but present another portion of microtubule-bound taxol for interactions with the factor(s) responsible for modulating TNF-α gene expression.

Recent reports on the toxic side effects of taxotere on patients have indicated unexpected pleural thickening and effusions, a problem that has not been reported with taxol (25). The latter may be true because taxol-treated patients are premedicated with steroids in contrast to taxotere-treated patients who are not premedicated. However, the idea must be considered that TNF-α could be altering the toxicities associated with taxol in the absence of, or in addition to, its modulating efficacy of the antitumor drug.

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