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Structural Requirements of Taxoids for Nitric Oxide and Tumor Necrosis Factor Production by Murine Macrophages

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Taxol (paclitaxel), a microtubule stabilizer with antitumor activity, mimics the actions of lipopolysaccharide (LPS) on murine macrophages (Mϕ). In the present study, a variety of synthetic analogs of paclitaxel were examined for their potencies to induce nitric oxide (NO) and tumor necrosis factor (TNF) production by peritoneal Mϕ from LPS-responsive C3H/HeN, and LPS-hyporesponsive C3H/HeJ mice, and by Mϕ-like LPS-responsive J774.1 and its mutant LPS-hyporesponsive J7.DEF3 cells. In this structure-activity relationship study, we found that (i) the benzoyl group at the C-3′ position of paclitaxel is the most important site to activate C3H/HeN Mϕ; (ii) the phenyl group at C-3′ is not a requisite for the activity; (iii) there is good correlation between NO and TNF production by the Mϕ in response to compounds, except for the analogs having a *tert*-butoxycarbonyl (10-acetyldocetaxel) or a thiophene-2-carbonyl group at C-3′-N instead of a benzoyl group, which is more dominant in TNF than in NO production; (iv) the compounds tested induce neither NO nor TNF production by C3H/HeJ Mϕ; (v) active compounds to C3H/He Mϕ induce TNF production by J7.DEF3 cells as well as J774.1 cells; and (vi) there is no correlation between the NO/TNF inducibility to C3H/HeN Mϕ and growth inhibitory activity against Mϕ-like J774.1 and J7.DEF3 cells. These data also suggest that the binding of taxoid/LPS to tubulin is not essential for the Mϕ activation.

Taxol (paclitaxel), a complex diterpene isolated from the stem bark of *Taxus brevifolia*, has antiproliferative activity against various cultured cells as well as antitumor activity *in vivo* (1). The activity is believed to reside in its ability to bind β-tubulin, to promote microtubule assembly, and to stabilize microtubules by bundle formation (2, 3). Ding et al. (4) found that paclitaxel mimics certain effects of bacterial lipopolysaccharide (LPS) on murine macrophages (Mϕ), in spite of the lack of similarities in their chemical structures. Mϕ stimulated with LPS produce various active mediators, including nitric oxide (NO) and tumor necrosis factor (TNF), and it causes endotoxic shock (5, 6). Peritoneal Mϕ taken from LPS-responsive C3H/He mice with *Lps*^{n} gene secrete the mediators when stimulated with paclitaxel as well as LPS *in vitro*, whereas the LPS-hyporesponsive C3H/HeJ Mϕ with *Lps*^{d} gene (7, 8) are unable to secrete these mediators in response to either LPS or paclitaxel (4, 9, 10). Genetic analyses have indicated that the gene which regulates the responsiveness and hyporesponsiveness to paclitaxel is genetically linked to *Lps* allele (4). These data have provided strong evidence that paclitaxel may share the actions of LPS for Mϕ activation, i.e., paclitaxel is an LPS-agonist.

Here we report structure-activity relationships of a set of synthetic paclitaxel analogs (taxoids) for their potencies to induce NO and TNF production by murine C3H/HeN and C3H/HeJ Mϕ and to inhibit the growth of Mϕ-like cell lines, LPS-responsive J774.1 and its LPS-hyporesponsive mutant J7.DEF3 cells. The data obtained reveal that there are different struc-

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Abbreviations: IFNγ, interferon γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; Mϕ, macrophages; NO, nitric oxide; TNF, tumor necrosis factor.

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tural requirements, among taxoids, for macrophage activation as compared to those for inhibition of the cell-proliferation.

METHODS

Mice. C3H/HeN and C3H/HeJ mice were bred and maintained in the Animal Facility of the Jichi Medical School. Female mice were used at 10 to 15 weeks of age.

Taxoids. Paclitaxel was obtained from Sigma Chemical Co., St. Louis, MO, and paclitaxel analogs (taxoids) were synthesized (11-17) by means of the β-Lactam Synthon Method (18-24). The structures are given in Table I. Paclitaxel and its analogs were stored at -80°C as a 10 mM stock solution in dimethylsulfoxide (DMSO), and dissolved in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) containing 2% heat-inactivated fetal bovine serum (Summit Biotechnology, Ft. Collins, CO) at a concentration of 300 μM before adding it to the cells.

Reagents. Murine recombinant interferon γ (IFNγ) was donated from Shionogi Pharmaceutical Co., Osaka, Japan. Rabbit anti-inducible NO synthase (iNOS) antibody was purchased from Affinity Bioreagent Inc., Neshanic Station, NJ.

Cell preparations. Mouse Mϕ were isolated by peritoneal lavage 4 days after i.p. injection of 1.5 ml of 3% thioglycolate broth, exactly as described previously (25). Mouse Mϕ-like J774.1 cells and TNF sensitive L929 cells were kindly provided by Dr. T. Suzuki and Dr. M. J. Parmely, the University of Kansas Medical Center, respectively. A mutant J7.DEF.3 cell line derived from J774.1 cells has been characterized previously (26). Although the J7.DEF3 cells are defective in binding of ^{125}I-LPS (26) and in expression of CD14 antigen (unpublished observation), which serves as a receptor for the complex of LPS and LPS-binding proteins (reviewed in 6). These cells were grown at 37°C with 5% CO_2 in RPMI 1640 medium containing 8% FBS, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (8% FBS-RPMI 1640). The Mϕ and Mϕ-like cells were plated at 2×10^5 per well in 96-well plates for NO release and, if necessary, at 2×10^6 per well in 6-well plates for Western blot analysis.

Stimulation of NO and TNF production by Mϕ. C3H/He Mϕ were incubated with paclitaxel analogs in 2% FBS-RPMI1640. C3H/HeJ Mϕ were incubated with paclitaxel analogs or LPS in 2% FBS-RPMI 1640 containing 0.5 U/ml IFNγ for NO production, or 5 U/ml IFNγ for TNF production. Stimulation times were 4 h for TNF assay, 48 h for NO assay, 16 h for Western blot.

NO assay. NO formation was measured as the stable end product nitrite (NO_2^-) in culture supernatants using the Griess reagent (25, 27).

TNF assay. TNF activity was determined by a functional cytotoxic assay using actinomycin D-treated L929, as described previously (10). TNF activity is expressed in units per milliliter, with 1 U being the amount of TNF causing 50% lysis of L929 cells.

Detection of inducible NO synthase (iNOS) protein in Mϕ. iNOS in the Mϕ was detected by Western blot with anti-iNOS antibody as described previously (26).

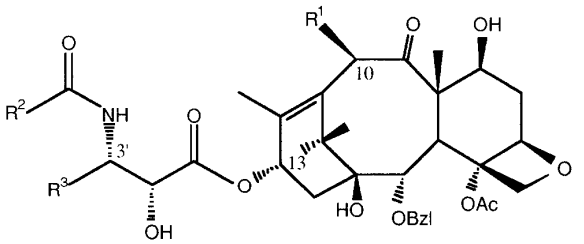
Cell growth inhibition assay. The J774.1 cells or J7.DEF3 cells were plated at 5×10^4 per well in 96-well plates, and cultured for 3 days in the presence of serial diluted taxoids. At the end of culture, the number of viable cells were determined by a quantitative calorimetric staining assay using a tetrazorium salt (MTT, Sigma). The doubling time was 13.1 h and 18.3 h, respectively.

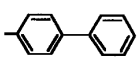
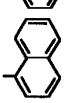
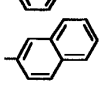
RESULTS

NO and TNF production by peritoneal Mϕ stimulated with paclitaxel analogs. Nineteen compounds (Table I) were examined for their ability to induce NO/TNF production by the C3H/HeN and C3H/HeJ peritoneal Mϕ. Compounds **1, 4, 5, 9, 10, 16** and **17** with the range of 1 μM to 30 μM did not show any ability to induce NO/TNF production by these Mϕ. Compound **7** induced weak but significant NO production, however, it did not induce any detectable TNF production. The other 11 compounds (paclitaxel, 10-acetyldocetaxel, **2, 3, 6, 8, 11, 12, 13, 14** and **15**) definitely induce both NO/TNF production by C3H/HeN Mϕ (Figs. 1A and 1B). Especially, paclitaxel, **2, 6, 8, 11** and **12** showed strong activity to induce NO/TNF production. Dose-range of the compounds approximately from 7 to 30 μM seemed to be effective. LPS-hyporesponsive C3H/HeJ Mϕ could not produce NO/TNF in response to the active taxoids, even in the presence of IFNγ (2 U/ml). NO and TNF inducibilities of these compounds to C3H/HeN and C3H/HeJ Mϕ are summarized in Table II. The results suggest that NO and TNF inducibility by these compounds is fundamentally correlated.

Expression of iNOS molecules in the C3H/HeN peritoneal Mϕ in response to active compounds. The iNOS expression in the C3H/HeN Mϕ in response to compounds was also

TABLE I
Chemical Structures of Taxoids



compound	R^1	R^2	R^3
paclitaxel	OAc		
1	OAc	$OC(CH_3)_3$	$-CH=C(CH_3)_2$
10-Ac-docetaxel	OAc	$OC(CH_3)_3$	
2	OAc		$-CH=C(CH_3)_2$
3	OAc		
4	OAc		
5	OAc		
6	OAc		
7	OAc		
8	OAc	$-CH_2$ 	
9	OAc	$-CH=CH$ 	
10	OAc		$-CH=C(CH_3)_2$
11	OAc		
12	OAc		
13	OAc		$-CH=C(CH_3)_2$
14	OAc		
15	OAc	$OC(CH_3)_3$	
16	OH	$OC(CH_3)_3$	
17	$OCOCH_2CH_3$	$OC(CH_3)_3$	$-CH=C(CH_3)_2$

examined (Fig. 2). Paclitaxel and compounds **2, 6** and **8** induced the strong expression. The expression induced by compound **11, 12** and **13** were weak but significant, while those by 10-acetyldocetaxel and compounds **3, 7, 10** and **14** were very weak or negligible. NO formation and iNOS expression should be closely correlated. However, the dissociation between iNOS

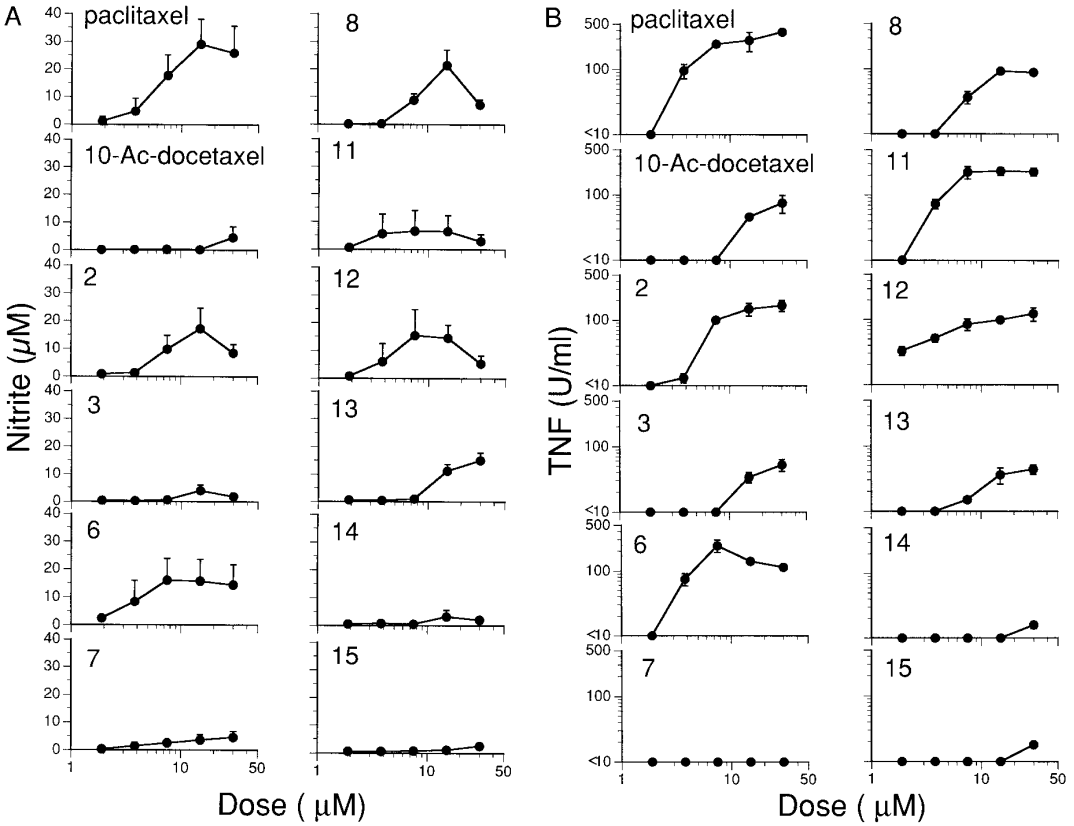


FIG. 1. (A) Taxoid-induced NO production by C3H/He Mϕ. C3H/He Mϕ were incubated with various doses of taxoids for 48 h. NO production was determined by measuring nitrite accumulation in the supernatants in triplicate with Griess reagent. Each point represents the mean ± standard error of the mean of three or four experiments. (B) Taxoid-induced TNF production by C3H/He Mϕ. C3H/He Mϕ were incubated with various doses of taxoids for 4 h. TNF activity in the supernatants in triplicate was determined by the cytotoxic assay using L929 cells. The data are from one of two experiments with similar results.

expression and NO production in response to some of the compounds was seen. It may cause the different sensitivities between iNOS and NO detection methods.

Structural requirement of active compounds to induce NO/TNF production by C3H/HeN Mϕ. Some special structures or groups are required for the induction of NO/TNF production. The aromatic group (R^2) attached to the N-acylamino group at position C-3′ is especially the most important structure to induce NO/TNF production by C3H/He Mϕ, i.e., replacement of the benzoyl group at position C-3′ (paclitaxel) with a *tert*-butoxycarbonyl (10-acetyldocetaxel), 2-furoyl (**4**), biphenyl-4-carbonyl (**5**), 2-naphthoyl (**7**), cinnamoyl (**9**) or cyclohexylcarbonyl (**14** vs. **11**) group results in a substantial loss of activity, whereas the introduction of a 1-naphthoyl (**6**), phenethoyl (**8**) or fluorobenzoyl (**12** vs. **11**) virtually does not affect the activity (Table II). The phenyl group (R^3) at position C-3′ is not required for the induction of NO/TNF, i.e., **2** and **13** retain the activity nearly equivalent to that of paclitaxel and **12**, respectively. The acetyl group (R^1) at position C-10 has a distinct effect on the activity (**15** vs. **16**). Interestingly, 10-acetyldocetaxel and **4** distinguished the potency for NO production from that of TNF production. In comparison with paclitaxel, both compounds revealed less NO formation, but induced the same level of TNF secretion (Fig. 1A and 1B). These two compounds did not induce iNOS expression (Fig. 2).

TABLE II
Macrophage Activation and Cell-Growth Inhibition by Taxol Analogues

	Macrophage activation				Growth inhibition (IC_{50}, nM)[b]	
	C3H/He		C3H/HeJ			
Compounds	NO	TNF	NO	TNF	J774.1	J7.DEF3
paclitaxel	++++[a]	++++	−	−	18	24
1	−	−	−	−	0.045	0.088
10-Ac-docetaxel	+	++	−	−	5.8	7.3
2	+++	+++	−	−	0.042	0.061
3	+	++	−	−	2.0	16
4	−	−	−	−	5.0	200
5	−	−	−	−	49	140
6	++++	++++	−	−	67	370
7	+	−	−	−	21	55
8	+++	+++	−	−	77	48
9	−	−	−	−	30	77
10	−	−	−	−	2.3	9.5
11	++++	++++	−	−	56	19
12	++++	+++	−	−	100	58
13	++	++	−	−	2.5	16
14	+	+	−	−	8.6	24
15	+	+	−	−	0.025	0.085
16	−	−	−	−	0.049	0.18
17	−	−	−	−	0.035	0.049

[a] The minimal concentration of compounds to induce 4 μM of NO formation or 20 U/ml of TNF secretion which were semiquantitatively estimated from those dose-response curves from three independent experiments for NO formation and two independent experiments as followed; ++++: $\leqslant$3.8 μM, +++: $\geqslant$7.5 μM, ++: $\geqslant$15 μM, +: 30 μM or compounds which induced less but significant amounts of NO formation (1−4 μM) or TNF secretion (10−20 U/ml) at any concentrations, −: $>$30 μM.

[b] The concentration of compounds which inhibited 50% (IC_{50}) of the growth of J774.1 cells and J7.DEF3 cells, after 72 h incubation with compounds. The data represent the mean values of three independent experiments.

TNF production by J774.1 cells and J7.DEF.3 cells stimulated with taxol analogs. Paclitaxel induced NO/TNF production by J774.1 cells and J7.DEF.3 cells. However, the NO production by J7.DEF3 cells was very weak and unreliable. Therefore, we examined the ability of paclitaxel and taxoids to induce TNF, but not NO (Fig. 3). Among 5 compounds tested, paclitaxel, compounds **2** and **6** induced the production by these cells. Compound **7** induced weak production of TNF by J774.1 cells, but did not induce the production by J7.DEF3 cells. Compound **1** was inactive.

Growth inhibition of cells lines by paclitaxel analogs. Growth inhibitory potency of the compounds (Table I) was examined against Mϕ-like cell lines, J774.1 and J7.DEF3 cells, and the results are summarized in Table II. All compounds effectively inhibited the cell growth, although slight differences were observed between the two cell lines. J774.1 cells seemed to be more sensitive than J7.DEF3 to these compounds, except **8, 11** and **12**. Compounds **1, 2** and **17**, bearing isobutenyl group instead of a phenyl group (R^3) at position C-3′, showed strong inhibitory activity (IC_{50} = 20 - 80 pM).

Correlation between the cell growth inhibitory activity and NO/TNF inducibility. The antiproliferative activities of taxoids against murine Mϕ-like J774.1 and J7.DEF3 cells were not correlated to their ability to induce NO/TNF production by the C3H/HeN Mϕ (Table II). Paclitaxel and compounds **6, 8, 11** and **12** showed strong activity to induce NO/TNF production,

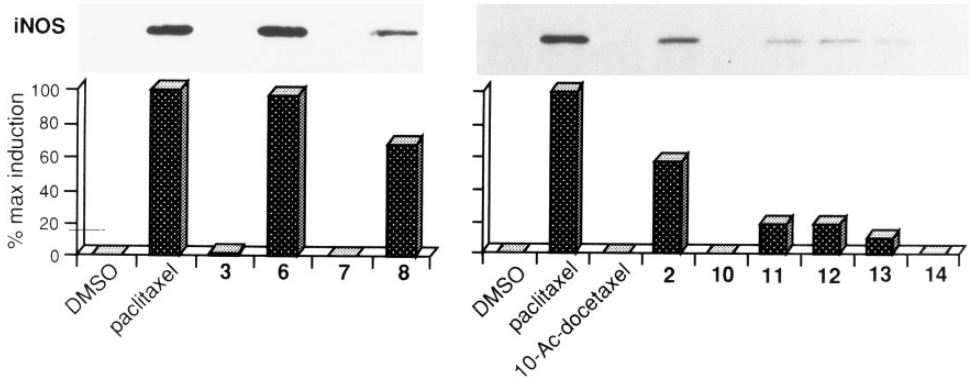


FIG. 2. Taxoid-induced iNOS protein expression. C3H/He Mϕ were incubated with taxoids (15 μM) for 16 h. The expression of iNOS protein was detected by Western-blot analysis with rabbit anti-iNOS antibody. iNOS protein expression were quantified by Quantity One (Huntington Station, NY). Relative induction values were calculated and given as percent relative to induction by taxoids. The data are from one of two experiments with similar results.

although the compounds exhibited weak inhibitory activity for cell growth. On the contrary, compounds **1, 16** and **17** did not show no inducibility for NO/TNF production, but strongly suppressed the cell growth. 10-Acetyldocetaxel and compounds **3, 7, 13** and **14** showed mild

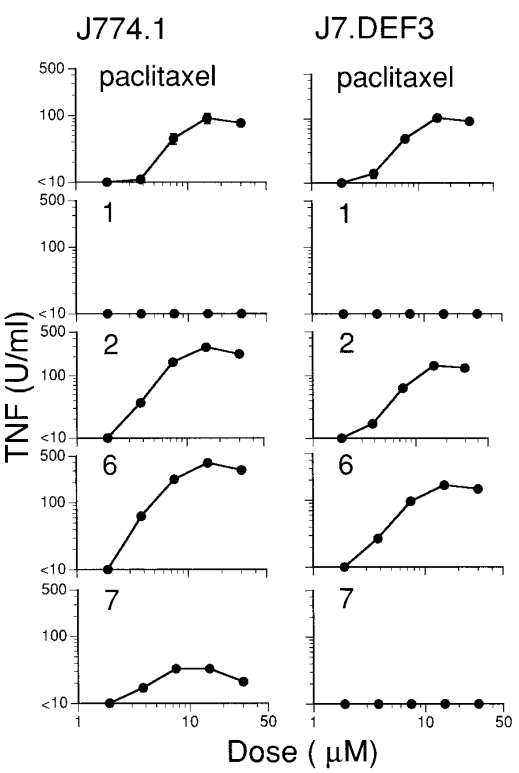


FIG. 3. Taxoid-induced TNF production by Mϕ-like cell lines. J774.1 and J7.DEF3 cells were incubated with various doses of taxoids for 4 h. TNF activity in the supernatants in triplicate was determined by the cytotoxic assay using L929 cells. The data are from one of two experiments with similar results.

anti-proliferative activity and weak NO/TNF inducibility. The anti-proliferative activities of taxoids were also not correlated to their ability to induce TNF production by J774.1 cells and J7.DEF3 cells. Paclitaxel and compounds **2** and **6** were strong active to induce TNF production, although the compounds exhibited weak inhibitory activity for cell-growth. Whereas, compounds **1** and **7** did not show weak inducibility for TNF production, but strong inhibitory activity for cell-growth. These findings indicate that the inducibility of taxoids for NO/TNF production by the murine Mϕ and the murine Mϕ cell lines is not correlated to their growth-inhibitory effect on the cell lines.

DISCUSSION

We examined the effects of paclitaxel and its 19 analogs with modification of two phenyl groups at C-3′ and C-3′-N and/or the acetyl group at C-10 (R^1, R^2 and/or R^3 in Table I) on the inducibility of NO/TNF production in vitro by the peritoneal Mϕ of LPS-responsive C3H/HeN and LPS-hyposensitive C3H/HeJ mouse strains. None of compounds induced the production by C3H/HeJ Mϕ, even if the Mϕ were primed with IFN-γ. However, C3H/HeN Mϕ produced both NO/TNF in response to some of the compounds (Table II). The LPS-mimicking activity of paclitaxel, which can activate C3H/HeN Mϕ, but not C3H/HeJ Mϕ, is retained in these active compounds. The findings presented here also clearly demonstrated the strict structural requirement of an aromatic group attached to the N-acylamino group at C-3′(R^2) for the NO/TNF production by Mϕ; A phenyl group at C-3′(R^3) is not essential for the activation of Mϕ (Tables I and II). Manthey and Vogel (28) reported that taxotere (docetaxel: R^1=OH and R^2=$OC(CH_3)_3$) neither elevated the expression of LPS-inducible genes nor secreted TNF by Mϕ, suggesting that the substituent R_1 and/or R^2 of docetaxel might be responsible for the loss of Mϕ activation ability. Our findings that (i) 10-acetyldocetaxel is less active than paclitaxel, but still active for NO/TNF production (Table II) and (ii) the deacylation of R^1 at C-10 results in a decrease of the ability to activate Mϕ (**15** vs. **16**), indicate that the acetyl group at C-10 (R^1) has a distinct effect on the activity.

All taxoids used in the experiments inhibited the growth of murine Mϕ-like J774.1 and J7.DEF3 cells. We have already reported that CD14-negative J7.DEF3 cells partially lack the ability to respond LPS (26), while the cells are capable of producing TNF in response to paclitaxel (10), indicating that paclitaxel induces TNF production in a CD14-independent manner. The present finding that the anti-proliferative ability against J774.1 and J7.DEF3 cells is independent of their ability to induce NO/TNF production by C3H/HeN Mϕ and TNF production by the cell lines suggests that taxoids possess two different mechanisms of action. We have demonstrated the presence of an aromatic acyl group at C′-3-N and an acyl group at C-10 (R^1) is important to induce NO/TNF production. Paclitaxel promotes the assembly of microtubules and inhibits the disassembly process of microtubules to tubulin (2, 3). It has been shown by a structure-cytotoxicity relationship study that inhibition of microtubule disassembly is quite sensitive to the substituents at C-2′ and C-3′ (29). Accordingly, the structural requirements for taxoids to induce NO/TNF production are different from those for inhibition of tumor growth.

Paclitaxel is an LPS surrogate for murine Mϕ (10, 28, 30). Paclitaxel can produce the same stimulative effects as LPS does on LPS-responsive murine Mϕ, whereas paclitaxel and LPS can hardly activate LPS-hyporesponsive *Lps*^{d} Mϕ. LPS as well as paclitaxel binds microtubules (30, 31). Microtubules participate in the regulation of TNFα receptor expression on murine Mϕ (4, 32), the activation of membrane-bound GTPase (33), LPS-induced interleukin-1 production by human monocytes (34), and LPS and paclitaxel-induced NO synthesis (25). These functions of microtubules may be affected by the binding of LPS or paclitaxel. However, we have already demonstrated (10) that microtubules do not serve as functional receptors for either LPS or paclitaxel (as a LPS agonist), since LPS did not inhibit the binding of isotopically

labeled paclitaxel in cultured Mϕ and vice versa. The putative receptor for LPS or paclitaxel as an LPS-agonist might be a microtubule-associated protein like MAP-2, as suggested by Ding et al. (30). Two paclitaxel analogs, 10-acetyldocetaxel and compound **3** induce TNF production quite well, although they poorly induce NO production (Figs 1A and 2B, and Table II). This may suggest the existence of slightly different mechanisms between NO and TNF production.

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