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Identification of the structural region of taxol that may be responsible for cytokine gene induction and cytotoxicity in human ovarian cancer cells

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Abstract *Purpose*: Interleukin-8 (IL-8) is a pleiotropic chemokine with both chemoattractant and angiogenic properties. In addition to its cytotoxic effects on ovarian cancer cells, taxol can transcriptionally activate genes such as IL-8 that may play a role in tumorigenesis. Utilizing IL-8 as a prototypic marker of tumor-derived modulators of growth, we undertook a systematic study of taxol and 11 structurally modified taxol analogs to identify the region of the taxane skeleton responsible for IL-8 gene induction. *Methods*: The human ovarian cancer cell line OVCA-420 was exposed to taxol or taxol analogs. IL-8 gene induction was assessed by Northern blot analysis after 6 h and cytotoxicity after 72 h. *Results*: Changes in the southern hemisphere (C-1 to C-4) of the taxane skeleton had greater effects on IL-8 induction than changes in the northern hemisphere (C-7 to C-11). Some of the taxol analogs modified at positions C-1 and/or C-2 with increased hydrophobicity induced IL-8 expression more than threefold over that induced by taxol or taxotere and more than 20-fold over control cells. Cells that failed to induce IL-8 gene expression in response to taxol were only marginally responsive to the analogs unless first primed with IL-1β. Modifications to the northern hemisphere did not alter taxol's effect on IL-8 expression in human cells, but did influence TNFα expression in murine macrophage cells, suggesting species and/or gene specificity. We found a direct correlation between IL-8 induction and cytotoxicity, in that analogs that dramatically upregulated IL-8 expression proved to be the most cytotoxic, inhibiting cell survival by >90%. *Conclusion*: Taken together our results demonstrate that changes in the southern hemisphere of the taxane skeleton influence both the gene induction and cytotoxic potential of taxol in human ovarian cancer cells.

Key words Taxol · Analogs · Gene induction · IL-8 · Human ovarian cancer · Cytotoxicity

Abbreviations *IL-8* interleukin 8 · *TNF*α tumor necrosis factor α · *DMSO* dimethylsulfoxide · *LPS* lipopolysaccharide · *MTT* (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide:thiazole blue)

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Introduction

Interleukin-8 (IL-8) is a pleiotropic chemokine originally identified as a potent chemoattractant [1]. In addition to its chemoattractant properties, IL-8 can modulate adhesion receptors and adhesive interactions between neutrophils and endothelial cells [2]. Many diverse cell types produce IL-8 either constitutively or in response to proinflammatory stimuli [1]. IL-8 can function as a tumor modulator since it is produced constitutively by some colon cancer cell lines and melanoma cells [3–5], functioning as an autocrine growth factor [4]. IL-8 expression correlates directly with tumorigenicity and metastatic potential in vivo [4, 5] possibly reflecting IL-8-induced angiogenesis [6, 7].

Taxol is one of the few recent chemotherapeutic drugs that has improved the progression-free interval for human ovarian cancer patients with advanced disease [8]. The in vivo efficacy of taxol is greater than that of other antimitotic agents suggesting that mitotic inhibition is not solely responsible for its clinical response. This has led investigators to pursue other mechanisms of action for taxol. One such mechanism may be through the induction of cytokines via signal transduction pathways. Recently taxol has been shown to transcriptionally activate IL-8 gene expression in a subset of human ovarian cancer cell lines and to increase IL-8 secretion from several primary cultures of ovarian tumors [9]. Gately et al. have also demonstrated taxol-induced transcriptional activation of the GADD153 promoter in ovarian cancer cells [10]. Taxol alters a wide variety of regulatory activities including activation of microtubule-associated protein kinase, induction of protein-tyrosine phosphorylation, tumor necrosis factor (TNF) and IL-1, modulation of TNF receptor levels in murine macrophages [11, 12], augmentation of LPS-induced pro-IL-1β production, and inhibition of colchicine-induced IL-1α secretion in human monocytes [13, 14]. Additionally, taxol activates mitogen-activated protein (MAP) kinase and induces expression of the cell cycle regulatory protein p21, through the c-*raf*-1 pathway in both MCF-7 breast cancer cells and PC3M human prostate cancer cells [15].

Taxol (Fig. 1A) is a natural product derived from the bark of the Pacific Northwest Yew tree, *Taxus brevifolia* [16]. A number of taxane compounds have been isolated from other *Taxus* species, most notably the taxotere precursor, 10-deacetyl baccatin III, from the needles of *Taxus baccata* [17]. Some of these taxanes are as potent as taxol, exhibiting comparable cytotoxicity and microtubule stabilization properties [11, 18, 19]. Little is known about the structural elements of the taxol molecule that are required for gene induction although modifications to the northern hemisphere of taxol have been shown to influence TNF gene induction in murine macrophages [11]. Using IL-8 as a prototypic marker for tumor-derived modulators of growth induced by taxol, we evaluated a series of taxol analogs modified at different positions on the taxane ring skeleton to identify the region of the molecule responsible for IL-8 gene induction in the human ovarian cancer cell lines OVCA 420 and 429. We found that some analogs modified at positions C-1 and C-2 resulting in a loss of polarity, dramatically enhanced IL-8 gene induction and that this had a direct correlation with cytotoxicity.

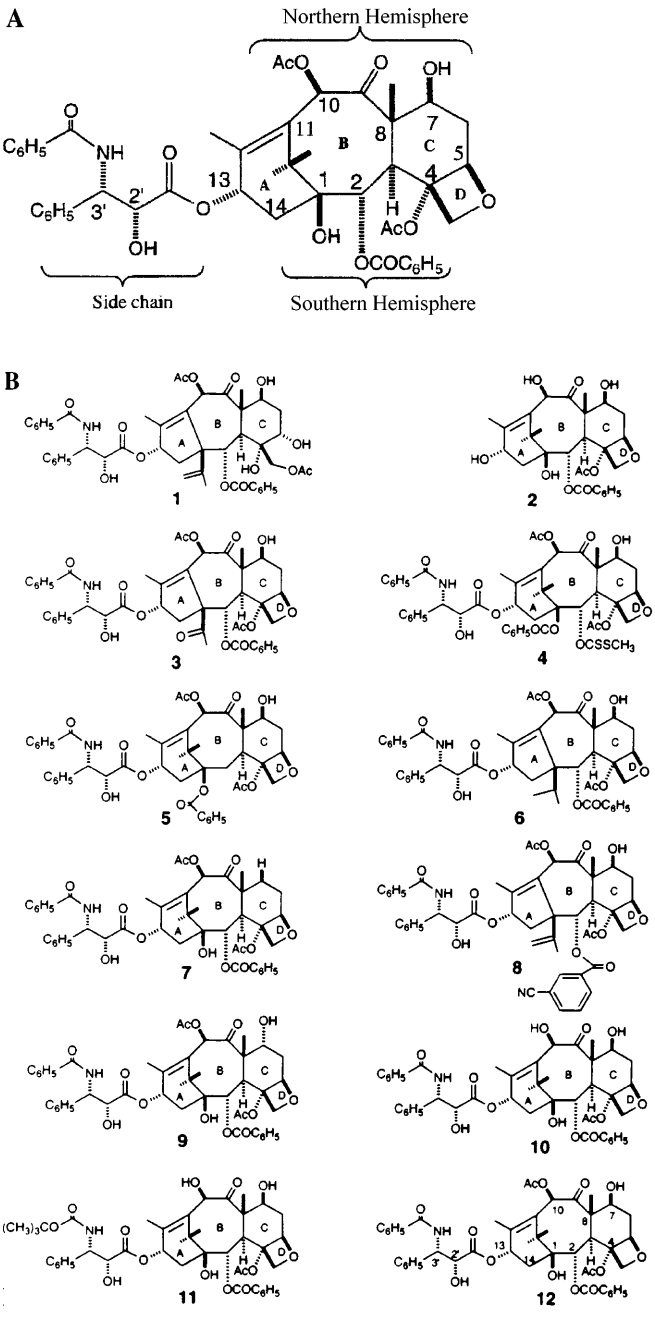

Fig. 1A,B Structure of taxol analogs. **A** Taxol. Positions of all carbons, rings, and hemispheres are noted. **B** Taxol analogs and arbitrarily assigned numbers

Materials and methods

Reagents

Taxol was purchased from Sigma Chemicals (St. Louis, Mo.). The synthesis and characterization of the analogs has been described previously [16, 17, 20–25]. Structurally related taxol analogs (designated analogs 1–11) are described in Table 1 and Fig. 1B. All drugs were resuspended in sterile dimethylsulfoxide (DMSO) to a concentration of 20 m*M*. All drugs were stored at 4 °C and warmed to room temperature prior to use.

Cells

The human ovarian cancer cell lines OVCA 420, OVCA 429 (kindly provided by Dr. Robert Bast, Jr., MD Anderson, Houston) and OC-194 (kindly provided by Dr. Otoniel Martínez-Maza, UCLA) were maintained as monolayer cultures in DMEM/F12 medium supplemented with 5% fetal bovine serum. RAW 264.7 cells were obtained from and maintained according to recommendations from the American Type Culture Collection (Rockville, Md.).

Table 1 Structurally related taxol analogs (*N* nortaxol, *T* taxol)

No.	Compound	Basic ring structure	Position modified	Reference
1	4-Deacetyl-5-hydroxy-20-acetyl-A-nortaxol	N	C-1[a], C-5	20
2	10-Deacetylbaccatin	T	C-10[a], C-13[b]	21
3	16-Demethyl-1-keto-A-nortaxol	N	C-1[a]	21
4	2-*S*-Methylxanthyl-1-benzoyltaxol	T	C-1[a], C-2[a]	22
5	1-Benzoyl-2-debenzoyloxytaxol	T	C-1[a], C-2[b]	22
6	15,16-Dihydro-A-nortaxol	N	C-1[a]	20
7	7-Deoxytaxol	T	C-7[b]	23
8	2-(*m*-Cyanbenzoyl)-A-nortaxol	N	C-1[a], C-2[b]	21
9	7-Epitaxol	T	C-7[c]	23
10	10-Deacetyltaxol	T	C-10[a]	20
11	Taxotere	T	C-10[a], C-13[a]	24,25
12	Taxol	T		16

[a] Substitution
[b] Deletion
[c] Steric change

RNA analysis

Proliferating, subconfluent cells, were exposed to the analogs at a concentration of 20 μ*M*, a concentration previously shown to maximally induce IL-8 expression [9]. After 6 h, total RNA was isolated using guanidium isothiocyanate/cesium chloride ultracentrifugation. Northern analysis was performed on 5 μg total RNA per sample separated on 1% agarose/formaldehyde gels. Blots were prehybridized, hybridized and washed as described previously [9]. All experiments were performed at least twice. Representative Northern blots are shown. Densitometry was performed using the NIH Image 1.49 software for Macintosh. Where appropriate data are expressed as fold induction for each analog compared to the untreated control.

Cytotoxicity assays

Cells, 5000 per well, were plated in 96-well microtiter plates. After adhering overnight, cells were exposed to 20 μ*M* taxol or taxol analog for 72 h. Cytotoxicity was determined using the MTT metabolic assay [26]. MTT (Sigma) was added to a final concentration of 50 μg/ml during the last 4 h of incubation. Formazan product was solubilized in 100% DMSO and the optical density measured at 595 nm. The results are expressed as percent survival relative to control cells after subtraction of the background values. Assays were performed in triplicate. All experiments were performed at least twice. Representative data are shown.

Results

Taxol (Fig. 1A) transcriptionally activates IL-8 gene expression and induces IL-8 secretion in the ovarian cancer cell line OVCA 420 [9]. We made chemical modifications to the taxol molecule to determine which region of the molecular skeleton is important for IL-8 gene induction (analogs 1–11; Table 1, Fig. 1B). OVCA 420 cells were treated with these structurally related taxol analogs and the resultant changes in IL-8 gene expression were assessed by Northern analysis (Fig. 2A). A low level of constitutive IL-8 mRNA was detected in unstimulated cells when the blot was overexposed (lane 1). IL-8 mRNA was not upregulated by exposure to DMSO (Fig. 2A, lane 2). Analogs 1 and 2 did not induce IL-8 mRNA expression (lanes 3 and 4). Analogs 4, 5 and

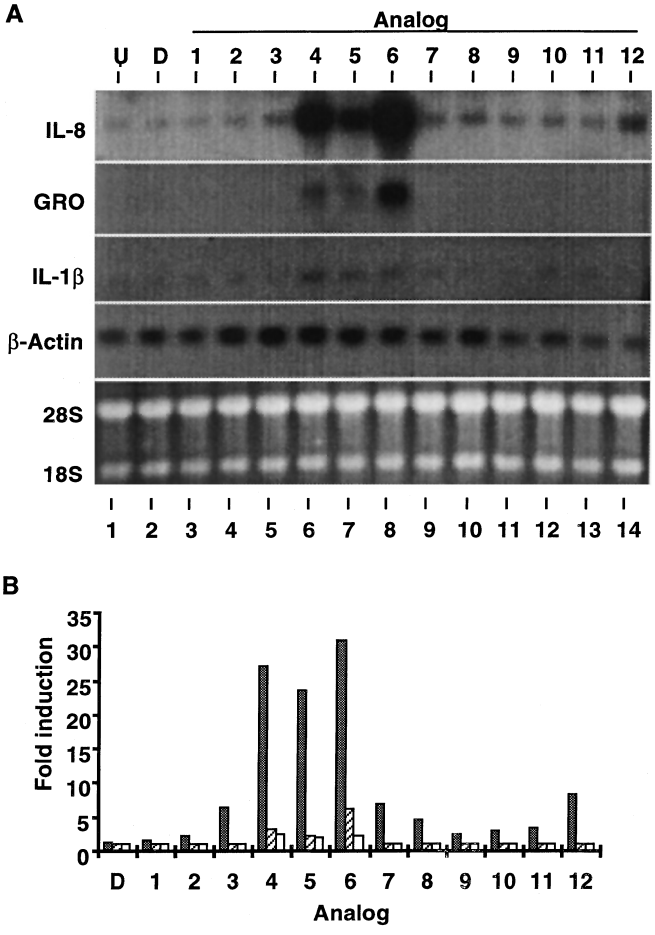

Fig. 2A,B Taxol analogs induce cytokine gene expression in OVCA 420 cells. **A** OVCA 420 cells were treated with 20 μ*M* taxol analogs for 6 h. Total RNA was isolated as described in Materials and methods with 5 μg per lane separated on 1% agarose/formaldehyde gels. Blots were probed sequentially for IL-8, GRO, IL-1β and β-actin. Ethidium bromide-stained ribosomal RNA is shown for loading comparison (*U* untreated cells, *D* DMSO-treated cells). Analog numbers refer to those in Table 1 and Fig. 1. **B** Graphical representation of densitometry of Northern blot. Data are expressed as fold induction for each analog compared with the untreated control (*Filled bars* IL-8, *hatched bars* GRO, *open bars* IL-1β)

6 (lanes 6–8) dramatically upregulated IL-8 mRNA expression compared to taxotere (analog 11, lane 13) and taxol (analog 12, lane 14). When compared with untreated control cells, the induction of IL-8 by taxol was approximately 8-fold, while IL-8 mRNA induced by analogs 4, 5 and 6 was 27-, 23- and >30-fold, respectively (Fig. 2B). The remaining analogs (analogs 3, 7–10) induced levels of IL-8 mRNA comparable to that induced by taxol, ranging from 2 to 7-fold over the untreated control (Fig. 2B).

We further screened the taxol analogs 1–11 for the induction of other cytokine genes to determine if the response was specific only for IL-8. We probed the Northern blots for the related chemokine GRO, and for a nonrelated cytokine, IL-1β. In a pattern similar to that seen for IL-8, analogs 4, 5 and 6 were more effective than taxol at inducing GRO (Fig. 2A). In agreement with previous results [9], taxol did not induce IL-1β mRNA. However, analogs 4, 5 and 6 did induce a small increase (2-fold) in IL-1β mRNA expression. Taxol and several analogs also affected the basal expression of β-actin (Fig. 2A).

We extended the studies to include additional ovarian cancer cell lines (Fig. 3A). OVCA 429 cells, previously shown to express IL-8 mRNA in response to taxol [9], also were responsive to analogs 4 and 5 (Fig. 3A) with a marked enhancement of IL-8 gene induction (approximately 15-fold over control DMSO-treated cells). Exposure to the other analogs induced IL-8 mRNA expression comparable to that seen with taxol (approximately 2–3-fold over controls). An exception was analog 8 which induced IL-8 mRNA approximately 7-fold over its induction in control DMSO-treated cells. OC-194 cells, which do not express IL-8 in response to taxol, showed marginal gene induction following exposure to analogs 4 and 5 (data not shown) and analog 6 (Fig. 3B). These cells can express IL-8 in response to IL-1β stimulation [27]. We first primed OC-194 cells with 0.5 ng/ml IL-1β and then exposed them to drug in the absence of additional cosignals, and assessed IL-8 mRNA levels by Northern analysis. Figure 3B shows that following such treatment there was an enhanced induction of IL-8 in cells treated with analog but not in cells treated with taxol (Fig. 3B). OC-194 cells exposed to IL-1β and then analogs 1, 2, 3, 9, 10 and 11 did not show enhanced induction of IL-8 mRNA (data not shown).

We further analyzed the ability of the structural analogs 1–11 to induce TNFα expression from the murine macrophage cell line RAW 264.7 (Fig. 3C). In contrast to the studies with the human ovarian OVCA 420 cell line, TNFα expression was induced most prominently by taxol, analog 7 and analog 9 (Fig. 3C, lanes 14, 9 and 11, respectively). Analogs 4, 5 and 6 induced only marginal TNFα gene expression. Stimulation by LPS was used as a positive control (Fig. 3C, lane 2).

Finally, we attempted to correlate IL-8 gene induction with cytotoxicity. OVCA 420, OVCA 429 and OC-194 cells were continuously exposed to 20 μ*M* taxol or taxol analogs for 72 h and cytotoxicity assessed by the

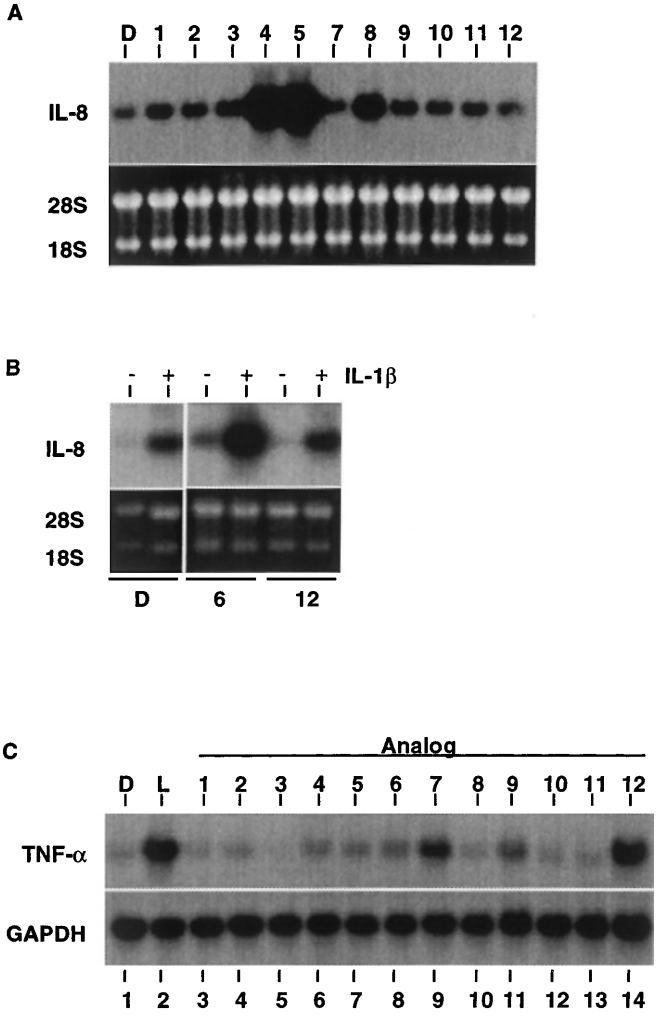

Fig. 3A–C Analog-induced IL-8 gene expression in OVCA 429 and OC-194 cells. **A** OVCA 429 cells were exposed to 20 μ*M* taxol or analogs for 5 h. **B** OC-194 cells were exposed to 0.5 ng/ml IL-1β for 10 min at 4 °C prior to a 5-h to exposure to 20 μ*M* taxol or analog 6 in the absence IL-1β. **C** TNFα expression induced in murine RAW 264.7 cells following exposure to 20 μ*M* analogs or LPS (*L*) 10 ng/ml for 4 h. GAPDH expression is shown for loading comparison. Ethidium bromide-stained gels showing ribosomal RNA are shown for loading comparison in **A** and **B**

MTT assay (Fig. 4A). The cytotoxicity induced by the analogs mirrored the pattern of IL-8 gene induction in all cell lines. Treatment with analog 1 failed to significantly induce cytotoxicity, while exposure to analog 2 resulted in approximately 30% cytotoxicity. The analogs 3, 7, 8, 9, 10, 11 induced cytotoxicity comparable to that seen with taxol. These analogs significantly inhibited cell survival by approximately 50–70%. Analogs 4, 5 and 6 exhibited the greatest cellular cytotoxicity, approximately 85–98%. Similar results were obtained when the cells were exposed to taxol or taxol analogs for 3 h and then maintained in the absence of drug for 72 h (data not shown).

Figure 4B shows the correlation between cytotoxicity and IL-8 gene induction. Analogs that demonstrated the highest cytotoxicity (poorest cell survival) produced the

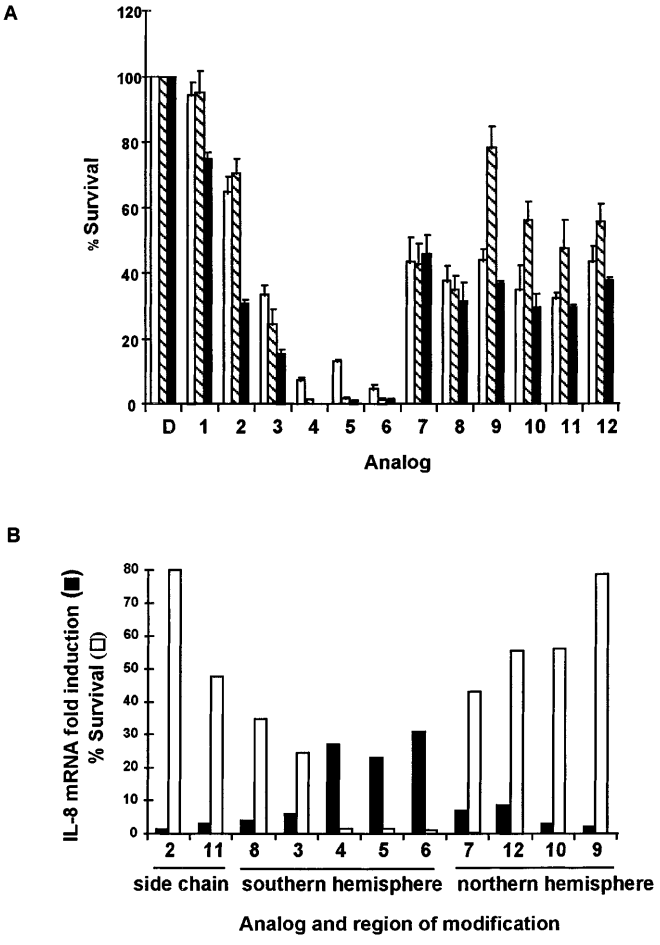

Fig. 4A,B Analog-induced cytotoxicity and correlation with IL-8 gene induction. **A** Cells were exposed to 20 μ*M* taxol or analog for 72 h and cytotoxicity determined using the MTT assay. **A** representative of three experiments is shown (*open bars* OC-194, *slashed bars* OVCA 420, *solid bars* OVCA 429). **B** Correlation between IL-8 gene induction (fold induction, *solid bars*) and cytotoxicity (percent survival, *open bars*) for OVCA 420 cells. Data are shown only for analogs that induce IL-8 gene expression

highest gene induction (analogs 4, 5 and 6). Analogs (analogs 3 and 8) modified in the same southern hemisphere were more cytotoxic than those that were modified in the northern hemisphere including taxol and taxotere.

Discussion

The in vivo efficacy of taxol is greater than that of other antimitotic agents, suggesting additional mechanisms of action. As taxol has been shown to transcriptionally activate a number of genes including IL-8 and *GADD153* [9, 10], we hypothesized that taxol could alter the expression of factors that either directly or indirectly modulate tumor growth. While the signaling pathways responsible for taxol-induced transcriptional activation are under much investigation, little is known about the structural elements of the taxol molecule itself that are required for optimum gene induction. To address this question we tested a series of structurally related taxol analogs modified at the core structure and/or functional group disposition and assessed changes in IL-8 gene expression in two human ovarian cancer cell lines.

Our results indicate that the southern hemisphere of the taxol molecule, C-1 to C-4, plays a dominant role in both IL-8 gene induction and cytotoxicity. In particular, modification at C-1 and C-2 atoms, as in analogs 4, 5 and 6, enhanced the ability to induce IL-8 gene expression (Figs. 2A and 3A), IL-8 protein secretion (data not shown) and cytotoxicity (Fig. 4). In analog 4, 2-*S*-methylxanthyl-1-benzoyltaxol, the C-1 hydroxyl group is replaced by a benzoate ester and the C-2 benzoate ester is replaced by an *S*-methyl xanthate functional group. In analog 5, 1-benzoyl-2-debenzoyloxytaxol, the C-1 hydroxy group is protected as a benzoate group and the C-2 benzoate group has been reduced to a methylene group [22]. These modifications in the southern hemisphere of the molecule are functional group transformations which cause a loss in the polarity and increase the hydrophobicity in this region by removing the C-1 hydroxyl. It is thus possible that the loss in hydrophilicity and the gain in hydrophobicity in the southern hemisphere of the taxane molecule, specifically at the C-1 and C-2 positions, enhances both IL-8 gene induction and cytotoxicity in OVCA 420 and OVCA 429 cells.

In analog 6, dihydro-A-nortaxol, the taxane ring skeleton is modified by a contraction from 6:8 AB ring fusion with the loss of C-1 hydroxy group (loss of hydrophilicity) and the gain of a pendant isopropyl group (gain in hydrophobicity). It is noteworthy that despite a change in the ring skeleton of analog 6 as compared to taxol, the overall conformation of both these molecules remains the same [21]. Analogs 3 and 8 also contain the same modified skeleton as in analog 6, but in these analogs the C-1 carbon is attached to an sp^2 carbon, the effect of which is not clearly understood. The addition of a cyano group at the meta position of the C-2 aroyl ring in analog 8, 2-(*m*-cyanbenzoyl)-A-nortaxol, reduced the IL-8-inductive capacity of analog 6. Movement of this group to other positions on the 2-benzoyl ring also resulted in a loss of IL-8 induction (data not shown), inferring specificity of this region for gene induction. This region is also important in cytotoxicity as analogs 3 and 8 were less effective than analog 6 at cell killing.

In parallel to IL-8 gene induction, the cytotoxicity assays show similar trends, with analogs 4, 5 and 6 being the most effective (Fig. 4B). Previously, these analogs have been reported to be less effective than taxol at inducing cytotoxicity against P-388 cells and several human cancer cell lines with ED_{50} values being approximately 10–50-fold higher than that of taxol [21, 22]. In this study, we showed that these analogs were more effective than taxol at cell killing when cells were exposed to a single concentration that induced IL-8 gene expression. While the results reported here do not reflect the ED_{50} values for these analogs against the cell

lines used in this study, our results are in agreement with those of earlier studies confirming the importance of the C-2 benzoyl group for cytotoxicity [21, 22].

In the case of analog 1, 4-deacetyl-5-hydroxy-20-acetyl-A-nortaxol, not only is the basic ring skeleton modified as in analog 6, but also the oxetane ring (ring D) is opened. When compared with analog 6, it appears that the overall conformation of the taxol molecule is also required for IL-8 induction. It is known that opening the oxetane ring causes the molecule to loose its rigid conformation and this results in a loss of bioactivity as shown previously [16, 19, 20, 28].

Both cytotoxicity and IL-8 induction in human ovarian cancer cells appear sensitive to modifications in the southern hemisphere of the taxane molecule. This is in contrast to studies analyzing TNFα production in murine macrophages where changes in the northern hemisphere, specifically in the C-7 carbon substituent on the C-ring, have profound effects on the TNFα gene response. Burkhart et al. [18] have reported that the induction of TNFα by C-7 acetyl taxol, where the C-7 hydroxy group is protected as an ester, is 70–80% less than that induced by taxol. In agreement with these observations, we found that changes in the northern hemisphere, specifically a stereochemical change in the C-7 hydroxyl group (analog 9, 7-epitaxol), resulted in a diminution of TNFα expression from murine macrophages compared to taxol. Interestingly, we found that deletion of the C-7 hydroxyl group (analog 7, 7-deoxytaxol) induced TNFα mRNA.

Of interest was the finding that in contrast to taxol, the analogs that markedly induced IL-8 (analogs 4, 5 and 6) were capable of augmenting IL-8 expression in IL-1-primed cells (Fig. 3B). While this observation suggests that the analogs and taxol utilize separate pathways for inducing IL-8 gene expression in taxol-unresponsive cell lines (those that fail to induce IL-8 in response to taxol), it does not negate the possibility of a common mechanism in taxol-responsive cell lines (those that do induce IL-8 in response to taxol). We have determined that the induction of gene expression follows similar kinetics in cells exposed to taxol or taxol analogs. However, while the transcriptional response to taxol is transient, the analogs cause sustained transcriptional activation (J.M.W. and J.S.H., unpublished observation) resulting in the increased mRNA levels seen 6 h after exposure. These data support the utilization of a common signal pathway by both taxol and analogs to elicit the response, possibly through the c-*raf*-1/MAP kinase pathway or the activation of the transcription factors AP-1 and NF-κB [15, 29], but an additional unknown pathway may be used by the analogs for maintenance of IL-8 gene expression.

Although the importance of IL-8 induction in the pathogenesis of ovarian cancer remains unclear, we observed a direct correlation between IL-8 induction and cytotoxicity. The analogs that were most potent at inducing IL-8 gene expression were also the most cytotoxic (Fig. 4B). IL-8-neutralizing antibodies failed to block taxol or analog-induced cytotoxicity (data not shown) indicating that IL-8 does not mediate taxol-induced cytotoxicity. Furthermore cells that failed to express IL-8 mRNA (Fig. 3B) were as sensitive to the cytotoxic effects of taxol or analogs (Fig. 4) as those cells that did express IL-8 mRNA (Figs. 2 and 3A). However, there are several important biological functions of this chemokine that could be of significance to the pathology of ovarian cancer and the in vivo effectiveness of taxol. As a potent chemoattractant [1], taxol-induced IL-8 may enhance the infiltrate of inflammatory cells into the tumor microenvironment. Patients with ovarian cancers that present with increased percentages of inflammatory cells have a better prognosis than those with few inflammatory cells [30]. IL-8 also has the potential to modulate cell adhesion molecules and cell adhesive properties of both inflammatory and presumably tumor cells, ultimately affecting cell metastasis [2]. It is unclear whether this would have beneficial or detrimental effects for the patient. In a murine model of melanoma, the expression of IL-8 directly correlates with the ability of the tumor to metastasize [4, 5], possibly through enhanced angiogenesis induced by IL-8 [6, 7]. Finally, it is unlikely that IL-8 is the only cytokine or protein induced by taxol that could modulate the tumor microenvironment. Although taxol induces IL-8 in the human colon cancer cell line T84 [9], it is unclear whether this response is unique to these cells. Future studies aimed at identifying other taxol-induced factors will be aided by understanding which structural regions of taxol contribute to the various biological functions. This knowledge may improve understanding of taxol's therapeutic efficacy in vivo and may lead to enhanced therapeutics.

We assessed IL-8 gene induction and cytotoxicity of a limited number of taxol analogs. We primarily restricted our study to the investigation of analogs with modifications on the ring skeleton, although we did include two analogs that were modified on the C-13 sidechain, taxotere (analog 11) and 10-deactylbaccatin (analog 2). Other studies have shown that modifications to the C-13 sidechain can increase drug solubility [31–33] or can increase or decrease cytotoxicity [18, 28, 34]. The effects of other analogs modified at sidechain positions 3′ or 2′ on IL-8 gene induction are unknown but would be worthy of investigation. We did find that taxotere (analog 11) could induce IL-8 expression but to a lesser extent than taxol. In our system, taxotere and taxol showed comparable cytotoxicity. In conclusion, we have demonstrated that changes in the southern hemisphere of the taxol molecular structure have prominent effects on IL-8 gene induction in two human ovarian cancer cell lines. This induction of IL-8 mRNA is in parallel with the observed cytotoxicities for these analogs and may thus be of significant clinical importance.

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