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Taxane-mediated gene induction is independent of microtubule stabilization: Induction of transcription regulators and enzymes that modulate inflammation and apoptosis

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ABSTRACT **Pharmacological traits of the antineoplastic agent taxol may originate in part from its effects on gene expression and not simply from its effects on microtubule assembly. This prompts three questions. First, how extensive is gene induction by taxol? Second, is gene induction confined to taxol itself, or does it occur with other taxane analogs? Third, do the functions of any induced genes correspond with known attributes of taxol or taxane analogs? We report that taxol induces numerous early-response genes, not just cytokine genes. Previously unidentified taxol-induced genes include genes coding transcription factors with tumor suppressor effects (*krox-24*) and enzymes that govern proliferation, apoptosis, and inflammation (*2′5′-oligoadenylate synthase*, *cyclooxygenase-2*, and an IκB kinase termed *chuk*). Taxotere, a potent analog of taxol, did not induce any of these genes, implying that taxol modulates gene expression by a mechanism that is distinct from microtubule stabilization and cell cycle arrest. Other taxane analogs induce some of the same genes as taxol, indicating that this process is not unique to taxol. Functional changes coincided with changes in gene expression. For instance, induction of tumor necrosis factor α (TNFα) accentuated apoptosis in cells treated with taxol compared with corresponding cells treated with taxotere. The functions of several induced genes (e.g., *krox-24* and *cyclooxygenase-2*) are self-consistent with beneficial and adverse effects encountered during taxol administration. These results may be relevant to the safe and effective use of taxol or its analogs in oncology and other areas of medicine.**

Taxol (paclitaxel) and taxotere (docetaxel) are clinically effective antineoplastic agents (1–3). Both taxanes bind to tubulin at the same site, retard microtubule depolymerization (4–6), impair mitosis, block progression through the cell cycle, and facilitate apoptosis (7–9). Two observations suggest that taxanes act by additional mechanisms. First, tubulin binding and microtubule stabilization alone do not account for the cytotoxic efficacy of taxanes, relative to other antimitotic agents. Both taxanes work in tumors containing defective, allelic variants of the p53 tumor suppressor (10–12) and in certain tumors resistant to chemotherapy or radiation therapy (1–3). Second, taxol, taxotere, and taxane homologs exhibit distinctive pharmacological traits, even though they share a common primary mechanism. For instance, the structure–activity relationships for tubulin binding and microtubule stabilization (13) diverge from the structure–activity relationships for induction of tumor necrosis factor α (TNFα) (14, 15). Modulation of gene expression is an attractive hypothesis to account for these observations; however, the scope of this hypothesis, and its potential pharmacological significance, are uncertain. To date, investigators have focused on cytokine genes induced by taxol (14–18). This prompted us to investigate differential expression of genes in cells treated with taxol, taxotere, or related microtubule stabilizing agents. We found that taxol modulated the expression of numerous genes. These included genes encoding transcription factors with tumor-suppressor effects; enzymes that participate in proliferation, apoptosis, and inflammation; and membrane proteins. Gene induction was readily dissociated from microtubule stabilization. Changes in gene expression appear relevant to the pharmacological and toxicological traits of the individual taxanes.

METHODS

Cell Culture. We maintained RAW 264.7 murine macrophages (American Type Culture Collection) in DMEM with high glucose, 2 mM L-glutamine, pyridoxine hydrochloride, 1 mM MEM sodium pyruvate solution, and 10% vol/vol fetal bovine serum (GIBCO/BRL). For mRNA isolation we used 1×10^7 cells. For *in situ* RNase protection assays we used 4×10^3 cells/200 μl dispensed into 96-well Cytostar-T plates (Amersham). For quantitation of caspase activity and TNFα secretion we used 1×10^6 cells/4 ml dispensed into 6-well plates (Costar).

cDNA Library Construction, Clone Selection, and Expressed Sequence Tag (EST) Sequencing. We used oligo(dT) cellulose (Stratagene mRNA isolation kit 200347) to isolate poly(A)+ mRNA from RAW 264.7 cells incubated with 10 μM taxol or taxotere for 6 hr. We used 2 μg of mRNA to construct normalized cDNA libraries and subtracted cDNA libraries (CLONTECH PCR-select cDNA subtraction kit no. K1804–1). We prepared separate cDNA libraries from cells incubated with taxol or taxotere. We prepared the corresponding, subtracted libraries of taxol cDNA minus taxotere cDNA and vice versa. We cleaved cDNA with *Rsa*I and pBluescript II-SK(+) with *Sma*I endonuclease for ligation of cDNA fragments into pBluescript plasmids. We transformed DH5α cells with these plasmids, plated them on 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal)-Amp-isopropyl β-D-thiogalactoside (IPTG) agar plates, and grew them at 37°C for 18 hr. We selected, randomly, approximately 25 transformed clones derived from the taxol and taxotere cDNA libraries, respectively. We purified the plasmids and sequenced their DNA by using Applied Biosystems PRISM Ready Dye-Deoxy Terminator kit with *Taq* FS polymerase. Generally, we sequenced 200–700 bp for single-pass sequencing of ESTs. We analyzed the nucleotide sequences of approximately 45 ESTs via BLAST analysis of

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Abbreviations: ATF-4, transcription factor 4; CHUK, conserved helix–loop–helix ubiquitous kinase; COX-2, cyclooxygenase-2; IL, interleukin; IκB, inhibitor of κB; NFκB, nuclear factor κB; 2′5′ OAS, 2′5′-oligoadenylate synthase; TNFα, tumor necrosis factor α; EST, expressed sequence tag.

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the public database (19). Designation of a gene identity for an EST indicates that the misassignment probability was less than 10^{-35} and the sequences aligned with 98–100% identity.

Quantitative PCR. We amplified the cDNA encoding genes identified by BLAST analysis. The sense and antisense oligonucleotide primers represent regions within the EST or the corresponding full-length cDNA. Using cDNA from two libraries designated taxol-enriched and taxotere-enriched, we conducted PCRs with: 25 ng cDNA template (1 μl), 100 pmol oligonucleotide primers (1 μl) (Genosys, The Woodlands, TX), 200 μM deoxynucleotidetriphosphates (0.6 μl), and KlenTaq polymerase mix (0.6 μl) (CLONTECH Advantage cDNA PCR kit no. K1905–1). We amplified samples in a total volume of 30 μl for 33 cycles (denature at 94°C for 30 sec, anneal at 60°C for 30 sec, and extend at 68°C for 2 min). We removed 5-μl aliquots after 18, 23, 28, and 33 cycles; fractionated the oligonucleotides via electrophoresis on a 1.2% agarose gel; stained the gels with 0.0001% vol/vol Syber Green I, and quantified the oligonucleotide products by image analysis with a fluorescent spectrophotometer and IMAGE QUANT software (Molecular Dynamics). The relative abundance of cDNA in each library is proportional to the rate of formation of the respective PCR product.

Preparation of Riboprobes. We generated cDNAs encoding approximately 400 bp of *cox-2* and *tnf*α with RNA isolated from RAW 264.7 cells incubated with 10 μM taxol and 10 units/ml recombinant murine interferon γ (Genzyme) for 12 hr. Primers to both *cox-2* and *tnf*α were designed with an *Xba*I restriction site in the sense primer and a *Hin*dIII restriction site in the antisense primer to ensure directional cloning of the PCR products into pBluescript II-SK(+) (Stratagene). PCR products were ligated into pBluescript II-SK(+) by using standard procedures, and cloned fragments were identified by restriction analysis with *Apa*I, *Hin*dIII, *Pst*I, and *Xba*I endonucleases. Plasmids containing *cox-2* and *tnf*α fragments were linearized with *Not*I before riboprobe synthesis. All other riboprobe templates (Table 1) were generated by PCR from cloned products or by reverse transcriptase–PCR from cells incubated with taxanes if the EST was shorter than 300 bp. These cDNA templates for riboprobe synthesis contained a T7 phage RNA polymerase promoter in the antisense primer. Riboprobes were synthesized by using the MAXIscript *in vitro* transcription kit (Ambion, Austin, TX) with the T7 phage RNA polymerase according to the manufacturer's instructions. A 316-ribonucleotide *gapdh* riboprobe was synthesized from the pTRI-GAPDH mouse antisense control template (Ambion, accession no. M32599) by using the T3 phage RNA polymerase. One nonhomologous, nonmammalian riboprobe (263 ribonucleotides) was made from a *Pvu*II digestion of pBluescript II-SK(+) and synthesized with T3 phage polymerase as a background control.

RNase Protection Assay. We performed RNase protection assays (20) on cells treated with taxol or taxotere for 0, 1.5, 3, and 6 hr. For RNase protection assays of certain immediate early-response genes we incubated cells with taxol, taxotere, or other agents in medium with 0.5% FCS to reduce the background associated with serum-dependent mRNA induction. For *krox-24* we performed RNase protection assays on cells incubated with taxol, taxotere, a 12,13-isotaxane designated PNU-105319 (21) (Pharmacia and Upjohn), and epothilone A (22) (Gesselschaft für Biotechnologische Forschung, Braunschweig, Germany).

TNFα Immunoassay. We incubated RAW 264.7 macrophages (2.5×10^5 cells/ml) with 10 μM taxol or taxotere and removed 1 ml aliquots at 0–48 hr for quantitation of TNFα by ELISA (Genzyme Factor-Test-X mouse TNFα ELISA kit no. 80–2802-00). In certain experiments we quantified TNFα secretion from cells incubated for 48 hr with combinations of: (*i*) 10 ng/ml murine recombinant IL-10 (R & D Systems) plus 10 μM taxol or (*ii*) 2.5 ng/ml murine recombinant TNFα (R & D Systems) plus 10 μM taxotere.

Table 1. Primers for riboprobe templates

Primer	Sequence	PCR, bp	GenBank accession no.
	ttatac**tctaga**tgggggcttccag		
TNFα	ttatg**aagctt**tgggtgaggagcacgtag	409	M38296
	ttgcat**tctaga**cccagcacttcacccatc		
COX-2	gtttgg**aagctt**tgctcatcaccccactc	377	M64291
	ctgttctttgaagttgacgg		
IL-1β	*gagcacgaggctttttgttg	316	M15131
	tggatggctagtcctaattg		
Crg-2	*ataacccctttgggaagatg	382	M86829
	ttgccccctttacattcttg		
ATF-4	*tttctagctccttacactcg	480	X61507
	attacctccttcccgacacc		
2′-5′ OAS	*ccaccatgaactctggac	329	X04958
	aagaaagcaaagggagagg		
Krox-24	*tacaaagatgcagggcagg	414	M19643
	tttgcatgtgaagagatgac		
CHUK	*acactgagaggctggtttcc	479	U12473
	cttcgagaggcattctacc		
Sec61	*ccagatgatgtatccgac	303	M96630
	gcccagtcacaggcatcg		
ABC2	*tctcagggacagacacgc	346	X75927
	cagagaaccaggacattgac		
L-plastin	*accttctgtccacctcc	398	D37837
	atccgctaccaggaggaaag		
SLP-76	*tgttcaaaatctcatgtctg	394	U20159

Primers are listed in 5′ to 3′ orientation with the sense first followed by the antisense primer. *Xba*I and *Hin*dIII restriction sites are shown in bold.

*Primers synthesized with the universal promoter, ggatcctaatacgactcactatagggag, at the 5′ end.

Caspase Enzyme Assay. We quantified caspase proteolytic activity to reflect the initiation of apoptosis. Cleavage of the caspase substrates acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid α-(4-methyl-coumaryl-7-amide) (DEVD-MCA) and acetyl-L-tyrosyl-L-valyl-L-alanyl-L-aspartic acid α-(4-methyl-coumaryl-7-amide) (YVAD-MCA) (Peptides International) reflect caspase-3 and caspase-1 activity, respectively. We incubated RAW 264.7 cells (10^6 cells per well) with 10 μM taxol and taxotere for 0–48 hr. We washed the cells with PBS at 4°C and then scraped them off the plates in 250 μl of lysis buffer (25 mM Hepes, pH 7.5/5 mM EDTA/2 mM DTT/0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). We homogenized the cell debris and lysate mixture (10 pulses at 40% duty cycle). We dispensed 45 μl of lysate and 5 μl of 500 μM DEVD-MCA or YVAD-MCA into 96-well microtiter plates. At intervals for 4–6 hr we excited samples at λ360 nm and measured fluorescence emission at λ460 nm with a microtiter plate spectrofluorometer. Caspase activity (pmol/min per mg protein) is proportional to the rate of MCA fluorescence. We determined protein concentrations spectrophotometrically. In certain experiments we quantified caspase activity in cells incubated for 48 hr with combinations of: (*i*) 10 ng/ml murine recombinant interleukin IL-10 (R & D Systems) plus 10 μM taxol, (*ii*) 2.5 ng/ml murine recombinant TNFα (R & D Systems) plus 10 μM taxotere, or (*iii*) 20 μl TNFα neutralizing polyclonal antibody (Genzyme) plus 10 μM taxol. This is sufficient to neutralize 2,000 units of TNFα.

RESULTS

Analysis of ESTs. We prepared two cDNA libraries from RAW 264.7 cells incubated with 10 μM taxol or 10 μM taxotere. Taxol and taxotere have comparable effects on microtubule stability and the cell cycle in these cells. Thus, they

will have comparable effects on expression of many genes in these cells. To investigate their unique effects on gene expression we used the two primary cDNA libraries to prepare two "subtracted" libraries consisting of taxol cDNA minus taxotere cDNA and vice versa. These "subtracted" libraries are depleted of common genes and genes induced by both taxol and taxotere. Conversely, these "subtracted" libraries are enriched in genes induced preferentially by taxol or taxotere, respectively. Table 2 summarizes the BLAST analyses on 45 ESTs derived from these two "subtracted" libraries. We designated an EST as a particular gene if the nucleotide sequence homology exceeded 98% and the probability from the BLAST analysis was 10^{-35} (19). Forty-five of 50 ESTs clustered into four categories based on functional or cellular location. Four ESTs (9%) were transcription factors and proliferation regulators including *krox-24*, *atf-4*, *2′-5′-oligoadenylate synthetase*, and *chuk*, a conserved helix–loop–helix ubiquitous kinase. Three ESTs (7%) were proinflammatory proteins including; *interleukin-1β* (*il-1β*), *cyclooxygenase-2* (*cox-2*), and *crg-2*. Ten ESTs (22%) were cytoplasm and membrane proteins including *sec61*, *abc2*, *l-plastin*, *slp-76*, *cathepsin D*, *rap*, and other transmembrane-spanning proteins. Eighteen ESTs (40%) were "housekeeping" genes including: *major histocompatibility complex* (*mhc*), *tetrahydrofolate dehydrogenase* (*thfd*), *lactate dehydrogenase* (*ldh*), *phosphogluconate dehydrogenase*, ribosomal proteins, and ribosomal RNAs. Ten ESTs (22%) had insufficient homology to sequences in GenBank to designate a corresponding gene.

The sample of clones in Table 2 contained ESTs for three cytokine genes induced by taxol (16, 17), consistent with enrichment of taxol-induced genes in the "subtraction" library. Therefore, we used the ESTs/cDNA in Table 1 to guide an investigation on differential expression of genes induced by taxol or taxotere. Using cDNA from the "subtracted" library enriched for taxol-induced genes, quantitative PCR suggested that taxol preferentially increased cDNA encoding *il-1β*, *crg-2*, *cox-2*, *atf-4*, *krox-24*, *chuk,* and *2′-5′-oligoadenylate synthetase* (Fig. 1). The other "subtracted" library, enriched for taxotere-induced genes, contained less cDNA corresponding with these genes. Quantitative PCR suggested that cDNA encoding *abc2*, *l-plastin*, and *slp-76* was approximately equal in abundance in both "subtracted" libraries (Fig. 1).

Differential Induction of Genes by Taxanes. To substantiate the results in Fig. 1 we quantified the expression of representative mRNA from RAW 264.7 cells incubated with 10 μM taxol or taxotere. Using Table 1 we focused on genes not yet recognized as taxol-inducible genes and we used TNFα as a control (16, 17). Taxol preferentially induced the expression of mRNA for TNFα, COX-2, ATF-4, and Krox-24. Maximal expression occurred within 90 min. Taxol preferentially induced the expression of mRNA for 2′-5′-oligoadenylate synthetase and Sec61 within 3 hr. In contrast to taxol, taxotere did not modulate the expression of these six genes appreciably (Fig. 2). Constitutive expression of *gapdh* was essentially constant with a relative standard error of ±3% for all treatment conditions. Taxol and taxotere each altered the expression of mRNA for ABC2 to the same extent. We stress that taxotere and taxol each bind to tubulin and stabilize microtubules within 15 min and block cell cycle at the G_2/M interface. Thus, the results in Fig. 2 imply that: (*i*) taxol modulates cellular gene expression via a molecular mechanism that distinguishes between closely related taxane homologs and (*ii*) tubulin binding and microtubule stabilization cannot account for differential gene expression by taxol versus taxotere.

Table 2. BLAST analysis of ESTs

Category	Clones	Representative clones
Apparent differential induction	17	Transcription and proliferation regulation: Krox-24*, ATF-4*, 2′-5′-oligoadenylate Synthetase*, CHUK*
		Proinflammatory: IL-1β*, COX-2*, Crg-2*
		Secretory, membrane or cytoplasmic proteins: Sec61*, ABC2†, L-plastin†, SLP-76*, cathepsin D†, Rap†, TA p198*, LDL-R†, transmembrane segments that align with various proteins†
Housekeeping or rRNA	18	MHC, tetrahydrofolate dehydrogenase, lactate dehydrogenase, phosphogluconate dehydrogenase, 28S, 18S rRNA, riboproteins
Sequences not in GenBank	10	

*Taxol minus taxotere library.
†Taxotere minus taxol library.

Pharmacological and Toxicological Relevance of Differential Gene Expression by Taxanes. Sustained mitotic blockade by taxol is not always sufficient for apoptosis (23), implying that other molecular processes must contribute. Induction of prodeath genes is an attractive candidate in this context. To determine whether taxane-dependent changes in gene expression have tangible pharmacological consequences we examined the relationship between TNFα expression and apoptosis in cells treated with taxol or taxotere. Consistent with data on mRNA expression, cells incubated with 10 μM taxol secreted 10- to 100-fold more TNFα, compared with cells incubated with 10 μM taxotere. TNFα secretion approached an asymptote at 4 hr and it remained constant for 24 hr in cells treated with taxol (Fig. 3*A*). TNFα levels were undetectable (<0.1 μg/ml) from 0 to 24 hr in cells treated with taxotere (Fig. 3*A*). At 48 hr the media from cells incubated with taxotere contained detectable amounts of TNFα (1.7 ± 0.7 μg/ml). This was still 8-fold less compared with cells incubated with taxol: 13.8 ± 0.7 μg TNFα/ml. IL-10, an inhibitor of TNFα transcription, suppressed its formation by 57 ± 5%, to a level of 5.9 ± 0.3 μg TNFα/ml at 48 hr (Fig. 3*B*). Caspase-3 proteolytic activity, which reflects the initiation of apoptosis, was 2- to 3-fold greater in cells incubated with taxol, compared with cells incubated with taxotere (Fig. 3*C*). In cells treated with taxol, caspase-3 activity increased at an initial rate of ≅5 units/hr and reached 149 ± 7 pmol/min per mg at 48 hr. These values exceeded ($P < 0.01$) the corresponding values in cells treated with taxotere: caspase-3 activity increased at an initial rate of ≅1.5 units/hr and reached 85 ± 3 pmol/min per mg at 48 hr (Fig. 3*C*). IL-10, the inhibitor of TNFα transcription, reduced the caspase-3 activity to indistinguishable levels between taxol- and taxotere-treated cells (Fig. 3*D*). The combination of exogenous TNFα with taxotere increased caspase-3 activity relative to taxotere alone (Fig. 3*D*). The combination of an anti-TNFα serum with taxol also decreased caspase-3 activity relative to taxol alone (data not shown). These data fortify the conclusion that expression of "death" genes can augment apoptosis originating from microtubule bundling and mitotic arrest. In corresponding experiments neither taxol nor taxotere had an effect on caspase-1 activity. Thus, they specifically affected the caspase-3 subfamily of proteolytic enzymes associated with apoptosis.

Data from Figs. 1 and 2 can provide hypotheses to account for distinctive traits of taxane homologs. For example, Krox-24 is a transcription factor with an important role in neuronal and muscular development (24) and with tumor-suppressor activity (25–27). Differential expression of *krox-24* or related krox family members might contribute to neuropathies that accompany treatment with taxol (28) or to its efficacy. Therefore, we compared the effect of four separate microtubule-stabilizing agents on expression of *krox-24*. We examined taxol, taxotere, a 12,13-isotaxane (21), and epothilone, a chemically distinct microtubule-stabilizing agent (22). Taxol and PNU-105319 both induced the expression of *krox-24* within 90 min (Fig. 4*A*).

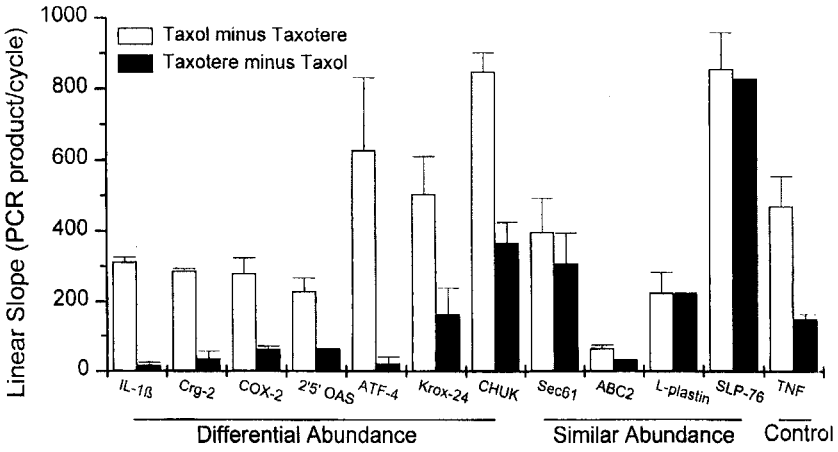

FIG. 1. Quantitative PCR of representative cDNA in "subtracted" libraries from RAW 264.7 cells incubated with 10 μM taxol or taxotere for 6 hr. Open bars, relative abundance in a "subtracted" library enriched for taxol-induced genes. Solid bars, relative abundance in a "subtracted" library enriched for taxotere-induced genes.

In contrast, neither taxotere nor epothilone-A altered *krox-24* expression (Fig. 4*A*). Among these only taxol and PNU-105319 caused a neurological toxicity, hind-limb paralysis, in mice (T. DeKoning, personal communication).

Taxotere and epothilone are equal or more potent than taxol and PNU-105319 as microtubule-stabilizing agents (21, 22). Thus, differential expression of Krox-24 mRNA is dissociated from microtubule stabilization. The potencies of taxol and PNU-105319 as microtubule-stabilizing agents also support this conclusion. Microtubule stabilization occurs with concentrations of 0.1–10 μM, but *krox-24* expression occurs only with 10 μM (Fig. 4*B*).

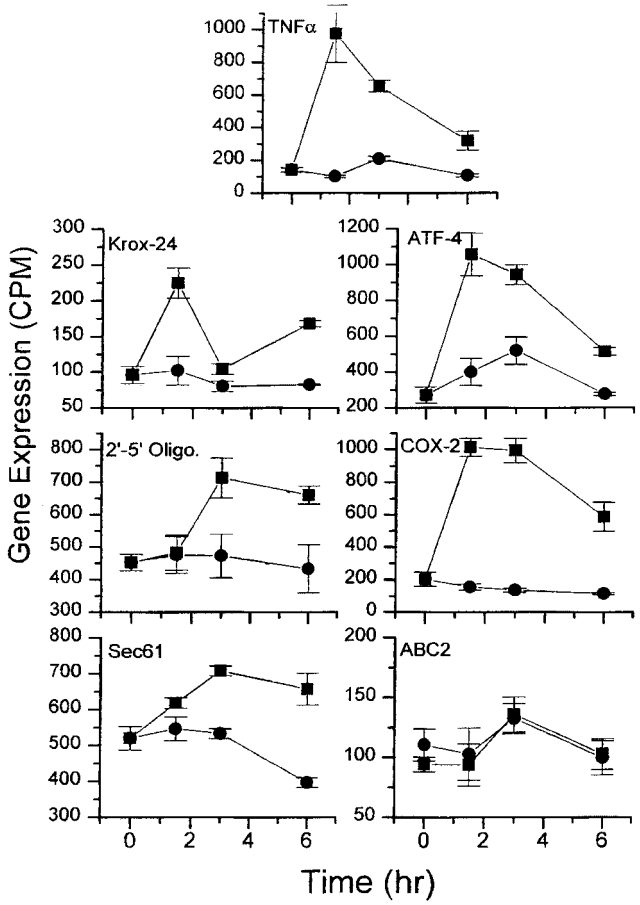

FIG. 2. Quantitative analysis of mRNA expression as a function of time in RAW 264.7 cells incubated with 10 μM taxol (■) or taxotere (●). At intervals of 0–6 hr cells were permeabilized and incubated with riboprobes that hybridized *in situ* to mRNA for genes selected from Fig. 1. Taxol increased the expression of mRNA for TNFα, a known taxol-inducible gene. Taxol also increased, by 2- to 5-fold, the expression of the mRNA for the enzymes COX-2 and 2′5′-oligoadenylate synthetase, the transcription factors ATF-4 and Krox-24, and the secretory protein Sec61. Taxotere did not alter expression of these same mRNA species. These species represent five new taxol-inducible genes outside the cytokine family.

DISCUSSION

We conclude that taxol and related taxanes typified by PNU-105319 can induce expression of genes via a mechanism that is dissociated from microtubule stabilization and mitotic blockade. We confirmed that taxol induced the expression of three genes, *crg-2*, *il-1β*, and *tnfα*, involved in apoptosis and inflammation (16–18). We also identified a new taxol-inducible gene, *cox-2*, that fits into this category. COX-2 (29) catalyzes the formation of prostaglandins, a family of biologically active lipid mediators. Induction of cytokines and COX-2 appears relevant to the pharmacology of taxol because they can modulate inflammation and apoptosis. By stimulating formation of cytoprotective prostaglandins COX-2 could either blunt apoptosis (30) or aggravate immediate hypersensitivity reactions associated with taxol administration (28). Most physicians attribute these anaphylactic reactions to cremaphor, the vehicle used for taxol administration. However, a recent report on successful parenteral desensitization to taxol implies that taxol itself contributes to the anaphylaxis (31).

Modulation of gene expression by taxol is not confined to cytokine genes. It also extends to genes that govern transcription. We identified four new taxol-inducible genes, *atf-4*, *krox-24*, *2′5′-oligoadenylate synthetase*, and *chuk* that fit into this category. ATF-4 and Krox-24 are transcription factors (24, 32). 2′-5′-Oligoadenylate synthetase catalyzes formation of purine oligomers that activate the nucleases necessary for the antiviral and cytopathic actions of interferons (33). CHUK is a conserved helix–loop–helix ubiquitous kinase (34), recently identified as an inhibitor of κB (IκB) kinase. Expression of these four genes seems relevant to the pharmacology and toxicology of taxanes. Krox-24, a zinc-finger transcription factor (also known as EGR-1, zif268, or NGF1A), is especially notable because its expression is deficient in several tumor cells and this defect correlates with tumor formation (25, 26). Enhanced expression of *krox-24* can prevent oncogenic transformation (27). Thus, induction of *krox-24* might augment the

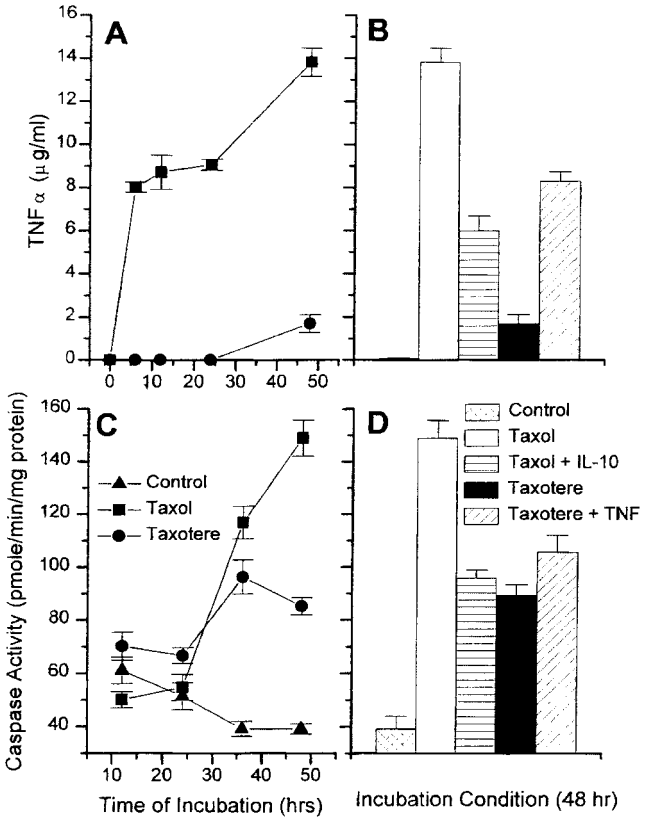

FIG. 3. Quantitative analysis of TNFα formation and caspase-3 activity as a function of time in RAW 264.7 cells incubated with taxol (■) or taxotere (●). In the media from cells incubated with taxol, TNFα levels increased by 4 hr and remained constant between 4 and 24 hr, consistent with the temporal profile of mRNA expression in Fig. 2*A*. TNFα was undetectable from 0 to 24 hr in media from cells incubated with taxotere, consistent with data in Fig. 2*A*. From 24 to 48 hr, TNFα levels in the media increased, modestly, for cells incubated with taxol and taxotere (*A*). IL-10 inhibited the formation of TNFα by cells incubated with taxol (*B*). Caspase-3 activity increased as a function of time in RAW 264.7 cells incubated with taxol (■) or taxotere (●) compared with vehicle control (▲) (*C*). Increased caspase-3 activity at 24–36 hr reflects the initiation of apoptosis in cells incubated with either taxol or taxotere. Caspase-3 activity continued to increase between 36 and 48 hr only in cells incubated with taxol, reflecting incrementally increased apoptosis compared with cells incubated with taxotere (*C*). IL-10, which inhibited TNFα formation, also inhibited casapase-3 activity in cells treated with taxol. Caspase-3 activity in cells treated with taxol plus IL-10 was indistinguishable from the caspase-3 activity in cells treated with taxotere (*D*).

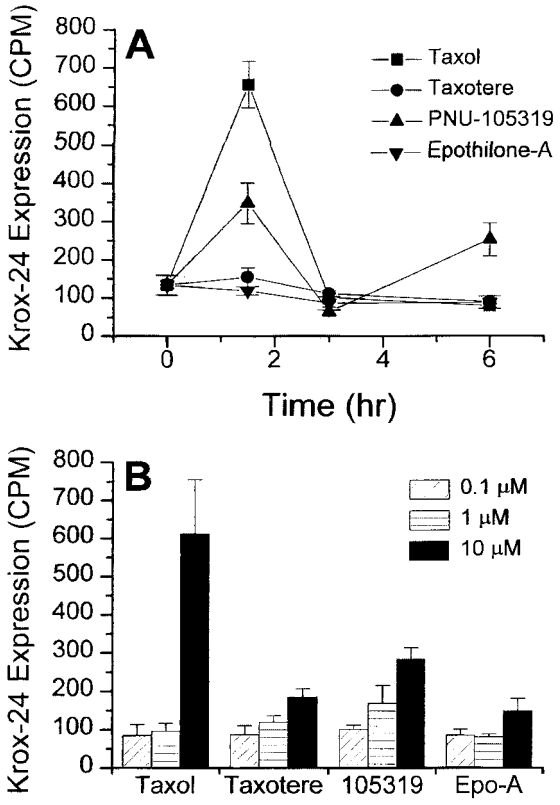

FIG. 4. Quantitative analysis of Krox-24 mRNA expression in RAW 264.7 cells incubated with taxol (■), taxotere (●), PNU-105319 (▲), or epothilone (▼). *A* shows the kinetics; *B* shows the concentration dependence of taxane-mediated Krox-24 expression. Among four different microtubule-stabilizing agents of comparable potency and efficacy, only taxol (■) and PNU-105319 (▲) increased the expression of *krox-24* mRNA by 6-fold and 3-fold, respectively. Taxol, taxotere, and PNU-105319 each have the fused-ring structure common among all taxanes. They differ in their substituents at C13 and C7. Changes in *krox-24* expression required taxol concentrations (10 μM) greater than those required for microtubule stabilization.

antineoplastic effects of taxol in some cases (2). It is interesting to note that prostate cancer deviates from this pattern. Namely, increased expression of EGR-1 in prostate cancer promotes the growth and invasiveness of this cancer (35). Compared with other types of cancer, prostate cancer is not responsive to taxol (1, 2). The relative cytotoxic potency of the microtubule-stabilizing agents against various cell lines or tumors will be complex because it depends on at least three effects: (*i*) their potency as microtubule-stabilizing agents, (*ii*) their potency as cell-cycle inhibitors, and (*iii*) the net effects of gene induction on cell survival and apoptosis. A comprehensive understanding of the scope of gene induction will be a prelude to design and interpretation of such experiments.

As expected with taxol, induction of TNFα activates the NFκB transcriptional pathway (36). Induction of *chuk* was unexpected, but it is also self-consistent with NFκB activation. CHUK is a kinase that phosphorylates IκB, releasing NFκB from its cytosolic, inactive IκB:NFκB complex (37, 38). Thus, coordinate induction of both TNFα and CHUK facilitates NFκB transcription in cells treated with taxol. Our data substantiate that changes such as these in gene expression have pharmacological consequences *in vitro*. For instance, the induction of TNFα that occurs with taxol, but not taxotere, accentuated apoptosis in RAW 264.7 cells. Other investigators recently showed that TNFα, administered exogenously, can also augment the antineoplastic efficacy of taxol *in vivo* (39) and conditioned medium from taxol-treated macrophages induces p53-independent apoptosis of transformed mouse embryonic fibroblasts (40). We draw attention to the fact that many clinical protocols require pretreatment of patients with corticoids to neutralize the hypersensitivity reactions that accompany taxol administration. Because corticoids also block transcription of TNFα we speculate they may affect the efficacy as well as the adverse effects of taxol.

Our data suggest that taxol can influence transcriptional pathways in several ways. First, it can act indirectly, via induction of proteins like CHUK that govern a transcriptional pathway without changing the levels of the transcription factor (e.g., NFκB). Second, it can act directly, by increasing the expression of transcription factors (e.g., ATF-4 and Krox-24). Third, it can induce genes that activate latent nucleases in cells (e.g., 2′-5′-oligoadenylate synthetase). ATF-4 and 2′-5′-oligoadenylate synthetase often are part of a general "stress-response" mechanism of cells. However, their selective induction by taxol, but not taxotere, argues against their induction by, or in response to, stress. The induction of 2′-5′-oligoadenylate synthetase is particularly striking because of its

established role in mediating the pleiotropic effects of interferon, including its antineoplastic effects. Activation of latent nucleases is also consistent with degradation of genomic DNA during apoptosis.

We have identified several new members in a growing array of genes induced by taxol. This sample is large enough to establish that taxol induces different types of genes, not simply cytokines (14–18). However, there are other genes that we have not yet identified. The emerging availability of cDNA microarray technology will enable us to establish the full scope of this phenomenon. So far we have identified genes induced by taxol and verified that their induction is dissociated from microtubule stabilization or cell-cycle effects. We have not yet identified any genes induced by taxotere but not by taxol. Taxotere may lack the structural features required for gene induction, or its potency may be inadequate to induce changes under the conditions we used. Alternatively, its structural features may restrict its profile of gene induction compared with the profile displayed by taxol. A 12,13-isotaxane analog, PNU-105319, did induce certain genes as effectively as taxol, substantiating that taxol is not unique in this context.

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