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Gene expression of peripheral blood cells reveals pathways downstream of glucocorticoid receptor antagonism and nab-paclitaxel treatment

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Abstract

Objectives—While paclitaxel treatment is associated with leukopenia, the mechanisms that underlie this effect are not well-characterized. Additionally, despite the importance of glucocorticoid signaling in cancer treatment, the genomic effects of glucocorticoid receptor (GR) antagonism by mifepristone treatment in primary human cells have never been described.

Methods—As part of a randomized Phase 1 clinical trial, we used microarrays to profile gene expression in peripheral blood cells sampled from each of 4 patients at baseline, after placebo/nab-paclitaxel treatment (cycle 1), and after mifepristone/nab-paclitaxel treatment (cycle 2).

Results—We found that 63 genes were differentially-expressed following treatment with nab-paclitaxel, including multiple genes in the tubulin pathway. We also found 606 genes that were differentially expressed in response to mifepristone; genes down-regulated by mifepristone overlapped significantly with those previously identified as being up-regulated by dexamethasone.

Conclusions—These results provide insights into the mechanisms of paclitaxel and GR inhibition in peripheral blood cells.

Keywords

pharmacology; transcriptome; chemotherapy; mifepristone

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Conflict of interest: SDC and the University of Chicago have been issued a patent that describes the discovery that mifepristone and chemotherapy could be an effective treatment for estrogen receptor negative breast cancer.

Introduction

Taxanes are a class of drugs commonly used as chemotherapeutics for various types of cancer, including breast cancer. Taxanes inhibit mitosis by stabilizing microtubules and, as a consequence, inhibit mitotic spindle formation (1, 2). Paclitaxel, originally extracted from the Pacific yew tree, was the first compound in this class to be used pharmacologically. Numerous synthetic and semi-synthetic analogs have subsequently been developed (3). In 2005, nanoparticle albumin-bound paclitaxel (nab-paclitaxel) was approved by the FDA for breast cancer patients with disease that is refractory to alternative treatments (4). Among other benefits, this new formulation allows paclitaxel to be administered without Cremophor, which is associated with hypersensitivity reactions; therefore, nab-paclitaxel generally does not require pre-treatment with glucocorticoids.

The need for pre-treatment with glucocorticoids for previous formulations of paclitaxel could be especially problematic because corticosteroids can initiate GR-mediated pro-survival pathways in tumor epithelial cells (reviewed in (5)). Additionally, there is evidence that endogenous glucocorticoid (cortisol) production is deregulated in metastatic breast cancer patients (6), and that this may negatively influence clinical outcomes (7, 8). These observations have raised the intriguing possibility that mifepristone, a potent antagonist of the glucocorticoid (and progesterone) receptors (9), could be used to improve breast cancer treatment outcome.

Taxanes, including nab-paclitaxel, also have effects on non-tumor cells, leading to a number of dose-limiting toxicities that require careful monitoring. Many of these effects, such as myelosuppression and neutropenia are well known, but profound lymphopenia has also been described (10, 11). However, the mechanisms of taxane effects on leukocytes are not well-characterized. Similarly, mifepristone effects on GR target gene signaling in patients have not been described. To characterize the *in vivo* transcriptional effects of these treatments on peripheral blood cells, we used expression microarrays to profile gene expression in peripheral blood collected from four patients before treatment (baseline), after a dose of nab-paclitaxel, and then 24 hours after combined mifepristone and nab-paclitaxel treatment. Comparing expression levels of each gene between these treatment conditions, we found that both nab-paclitaxel and mifepristone alter transcriptional regulation in peripheral blood cells. These results provide important insights into the biological effects of these treatments, revealing that nab-paclitaxel affects expression of genes in the tubulin pathway and that mifepristone indeed modulates expression of genes that are responsive to GC treatment.

Methods

Patients

Peripheral blood samples were taken from patients with chemotherapy-refractory breast cancer enrolled in a randomized Phase 1 clinical trial examining the safety and efficacy of placebo/nab-paclitaxel versus a combination of mifepristone with nab-paclitaxel. Four patients were included in this study. Each of these patients was treated with a cycle of placebo/nab-paclitaxel and then subsequently treated with a cycle of nab-paclitaxel/mifepristone. Blood samples were drawn from each patient (between 8–10 am) at baseline

(i.e. before any therapy was administered), 24h after initial treatment with placebo/nab-paclitaxel, and then 24h after initial treatment with mifepristone/nab-paclitaxel. This study design allowed us to compare baseline expression to expression after each treatment within the same patient (see Figure 1A). This study was approved by the Institutional Review Board at the University of Chicago.

Gene expression measurements

Blood was drawn into PaxGene RNA tubes and extracted using the PaxGene RNA isolation protocol. Total RNA was reverse transcribed into cDNA, labeled, hybridized to Illumina HumanHT-12 v4 Expression BeadChips and scanned at the Functional Genomics Facility at the University of Chicago. Summary data were obtained using the BeadStudio software (Illumina).

Low-level analysis of expression data

Low-level microarray analysis was performed using the Bioconductor software package LUMI (12) in R (<http://www.r-project.org>). We applied variance stabilizing transformation (13) to all arrays. We also removed probes with intensities indistinguishable from background fluorescence levels in all samples. Finally, we performed quantile normalization across all arrays. We annotated each probe by mapping probe sequences to the RNA sequences from RefSeq using BLAT. We removed probes that mapped to multiple genes to avoid ambiguity in the source of a signal due to cross-hybridization of similar RNA species. We also removed probes that contained one or more HapMap SNPs.

Principal component analysis to model and correct for latent covariates

We found that gene expression measurements were generally consistent across RNA samples, with stronger correlations between RNA samples from the same patients versus between different patients (Supplementary Figure 1, Supplemental Digital Content 1, <http://links.lww.com/FPC/A749>). To summarize patterns of shared variation across genes, and to explore factors that may contribute to variation within and between patients, we performed principal component analysis using the ‘pccomp’ function in the R statistical package. We found that the first principal component of the expression data matrix was significantly correlated ($P=4\times 10^{-7}$) with the date of sample collection. We then corrected for date of collection, and found that the first principal component of the corrected expression levels was significantly associated ($P=5\times 10^{-7}$) with patient (i.e. gene expression was much more similar between different collections from the same patients versus comparisons between patients after any particular treatment). After correcting for both date of collection and patient, we found that the first principal component of the corrected expression levels was significantly correlated ($P=0.011$) with the RNA quality as determined by an Agilent Bioanalyzer and summarized as an RNA integrity number (RIN). While this observation indicates that overall RNA quality has a measurable effect on expression level estimates, we found that RNA quality was generally high (RIN range = 8.2–9.5, Supplementary Table 1, Supplemental Digital Content 2, <http://links.lww.com/FPC/A750>). Furthermore, correcting for RIN did not qualitatively change the results of subsequent analyses (Supplementary Figure 2, Supplemental Digital Content 3, <http://links.lww.com/FPC/A751>). We corrected the expression levels for date of collection, patient, and RNA quality, we found that the first

principal component was significantly associated ($P=0.0013$) with treatment (i.e. baseline, placebo/nab-paclitaxel, and mifepristone/nab-paclitaxel). Technical factors that influence measurements of gene expression can add noise or act as confounders in analyses of expression microarray data. To avoid these issues, linear regression can be applied at each gene to estimate and remove the effects of confounders in expression microarray studies (as described in (14)). Consequently, we used linear regression to correct expression levels at each gene for data of collection, patient, and RNA quality. Corrected values were used in subsequent analyses.

Identification of differentially expressed genes and overlap with dexamethasone response

We used the Bioconductor package LIMMA (15), in R, to identify differentially-expressed genes. Specifically, we used this software to perform linear regression (in the framework of a hierarchical model) to test for treatment effects and to estimate $\log_2$ fold changes in expression at each gene. We then applied the *q*value function in R to estimate and control the false discovery rates (FDR) (16).

We used permutations to assess the statistical significance of the overlap between differentially expressed genes in response to the synthetic glucocorticoid dexamethasone (1 μ M) in a previous study (17). Specifically, we generated 10,000 permutations of the gene expression data with sample labels randomly swapped. This allowed us to preserve the correlation structure (between genes across samples) in the expression data and account for it in our estimates of the distribution of overlapping genes under the null distribution. [This issue is discussed in greater depth in (18)]. We identified differentially expressed genes in each permutation using the same framework as described for our primary analysis. We then calculated the number of 'differentially-expressed' genes from each permutation that were also differentially-expressed following glucocorticoids treatment. Finally, we used this as a null distribution of the number of overlapping genes expected by chance.

Gene ontology analyses

We performed pathway analyses using the online tool DAVID (19, 20). This allowed us to query multiple different pathway and Gene Ontology (GO) databases. We included GO biological processes, GO molecular function, BBID pathways, BIOCARTA pathways, EC_NUMBER pathways, KEGG pathways, PANTHER pathways, PANTHER molecular function, PANTHER biological processes, and REACTOME pathways. We used the list of all genes expressed in peripheral blood cells (based on microarray data from this experiment) as a background.

Results

Nab-paclitaxel treatment results in tubulin-related pathway gene expression

To characterize the effects of nab-paclitaxel treatment on gene regulation in peripheral blood cells, we used linear regression (in a hierarchical model implemented in the software LIMMA) to compare gene expression in samples taken before treatment (baseline) to expression in samples taken 24 hours after individuals were treated with either nab-paclitaxel alone or in combination with mifepristone (see Figure 1B). While this test could

also identify genes that are strongly responsive to mifepristone, we chose to include mifepristone samples to increase the sample size and improve our sensitivity. We found that the results of this analysis were strongly correlated with results where mifepristone-treated samples are excluded (see Supplementary Figure 3, Supplemental Digital Content 4, <http://links.lww.com/FPC/A752>).

We found 63 significantly genes differentially-expressed following nab-paclitaxel treatment ($FDR < 0.05$, Figure 2A). Most of these genes (42 of 63) were down-regulated, which was a significant departure from random expectations (i.e. 50:50) ($P = 0.01$). We then used pathway analysis to shed light on the biological implications of these changes in expression. Consistent with the established effects of paclitaxel on microtubule formation (1, 2), we found that the most significantly enriched pathway for up-regulated genes was the PANTHER molecular function category ‘tubulin’ ($P = 1 \times 10^{-7}$, $FDR = 4 \times 10^{-6}$). Indeed, we found that the most significantly differentially expressed gene, *TUBB*, encoded the tubulin beta chain protein ($\log_2$ fold change = 0.5, $P = 6 \times 10^{-8}$, Figure 2B). The enrichment signal for the PANTHER ‘tubulin’ pathway reflected the significant up-regulation of five genes that encode sub-units of tubulin (*TUBB*, *TUBA1B*, *TUBA1A*, *TUBA4A*, and *TUBB4B*). Pathway analyses also revealed that differentially expressed genes were enriched for a number of other pathways and gene categories related to microtubule formation (Supplementary Table 2, Supplemental Digital Content 5, <http://links.lww.com/FPC/A753>). Enrichment for these pathways and categories of genes also reflected differential expression of these five tubulin genes, although some pathways related to GTP also involved an additional gene, the guanylate-binding protein *GBP4*. No pathways or categories were significantly enriched among the down-regulated genes after multiple test correction.

Mifepristone has widespread effects on gene expression in peripheral blood cells

We then investigated the effects of mifepristone on gene expression. To do so, we used linear regression to compare gene expression levels in cells taken after individuals were treated with mifepristone and nab-paclitaxel to gene expression in blood samples taken before individuals were treated with mifepristone (i.e. both at baseline and after nab-paclitaxel monotherapy was administered, see Figure 1B). This analysis revealed 606 genes that were differentially-expressed following treatment with mifepristone ($FDR < 0.05$). Unexpectedly, we found that most of these genes (412 out of 606) were down-regulated following mifepristone treatment (Figure 3). This was a significant deviation from random expectations ($P = 5 \times 10^{-19}$).

Although no pathways or categories were significant after multiple test correction, we found that the most significantly enriched pathway for down-regulated genes was ‘Signaling by Rho GTPases’ ($P = 7 \times 10^{-3}$, $FDR = 0.3$). The top ten pathways also included several pathways related to immune function (Supplementary Table 3, Supplemental Digital Content 6, <http://links.lww.com/FPC/A754>). For example, the second most significantly enriched category was the GO biological process ‘regulation of immune system processes’ ($P = 7 \times 10^{-4}$, $FDR = 0.4$). The most significantly enriched pathway for up-regulated genes was the PANTHER molecular function ‘Extracellular matrix structural protein’ ($P = 5 \times 10^{-4}$, $FDR = 0.05$). The top ten pathways for genes up-regulated following mifepristone treatment

are listed in Supplementary Table 4(Supplemental Digital Content 7, <http://links.lww.com/FPC/A755>).

Mifepristone and glucocorticoid signaling in peripheral blood mononuclear cells

To explore the relationship between mifepristone effects (i.e. GR antagonist effects) on gene expression in peripheral blood and glucocorticoid (pure GR agonist) signaling, we compared our data to a previous *in vitro* study of glucocorticoid (1 μ M dexamethasone) treatment on gene expression in peripheral blood mononuclear cells (17). Genes down-regulated following mifepristone treatment *in vivo* were indeed enriched for glucocorticoid-induced genes, and this enrichment was higher for genes with greater evidence of differential expression (Figure 4). For example, we found that 20 of the 412 genes down-regulated by mifepristone also showed very strong evidence of being differentially-expressed following dexamethasone treatment (threshold of $FDR < 10^{-35}$). This represented a significantly greater overlap than expected by chance ($P=0.03$). In contrast, only 4 of the 194 genes up-regulated following mifepristone treatment were also differentially-expressed following dexamethasone treatment (threshold of $FDR < 10^{-35}$). The majority of these genes (19 of 20) were up-regulated following glucocorticoid treatment. This included the well-characterized glucocorticoid receptor target *TSC22D3*, which was strongly up-regulated by dexamethasone ($P=3 \times 10^{-47}$, $FDR=1 \times 10^{-44}$) and down-regulated following mifepristone in patients' peripheral blood cells ($P=6 \times 10^{-5}$, $FDR=0.007$, Figure 4). In contrast, we found that genes up-regulated following mifepristone treatment were not enriched for genes differentially-expressed following glucocorticoid treatment *in vitro* (Figure 4).

We then used pathway analysis to explore the biological function of the genes that were differentially-expressed following mifepristone treatment but not altered by dexamethasone treatment (threshold of $P > 0.05$). We found that these 164 genes were significantly enriched for the REACTOME pathway "Diabetes pathways" ($P=4 \times 10^{-4}$, $FDR=0.01$). We also used pathway analyses to explore the biology of gene expression changes following dexamethasone treatment ($FDR < 0.05$) but not following mifepristone treatment ($P > 0.05$). We found that these genes were significantly enriched for the Gene Ontology Biological Process "ncRNA metabolic processes" ($P=1 \times 10^{-6}$, $FDR=5 \times 10^{-3}$).

Discussion

We profiled expression across the genome before and after treatment with nab-paclitaxel and mifepristone. In so doing, we show that both these drugs have *in vivo* effects on gene regulation in peripheral blood cells. Through analyses of differentially expressed genes, we describe patterns that provide insights into the molecular basis of systemic effects of these compounds.

Genes differentially-expressed following nab-paclitaxel treatment were enriched for the tubulin pathway. This is also consistent with the mechanism of action of paclitaxel in tumor cells. Paclitaxel can directly bind the β -subunit of tubulin to stabilize microtubules and prevent mitosis (21). We found that the most responsive gene to nab-paclitaxel treatment was *TUBB*, which encodes the β -subunit of tubulin. We found that nab-paclitaxel treatment results in the up-regulation of expression of *TUBB* and of four other genes encoding sub-

units of tubulin. These results indicate that in addition to direct interactions, nab-paclitaxel signaling leads to an increase in the expression of genes encoding target proteins. This mechanism could enhance the effects of direct paclitaxel binding to tubulin proteins by leading to higher concentrations of target proteins. This phenomenon could cause non-continuous pharmacodynamics, where the same concentration of drug has a greater effect at a later time point as compared to an earlier time point. These results also suggest that nab-paclitaxel targets similar pathways in tumor cells and leukocytes. This observation could support the use of peripheral blood cells, which are much more accessible than epithelial cancer cells, as a model system for characterizing inter-individual variation in taxane response.

The transcriptional response to nab-paclitaxel treatment was not limited to tubulin-related genes and included effects on genes such as *FOLR3*, which encodes folate receptor 3, and the RAS oncogene family member *RAB6A*. These results are consistent with previous studies that have shown transcriptional effects of taxanes independently of microtubule formation (22, 23). For example, we found that nab-paclitaxel treatment results in increased expression of *IL1 β* , which encodes a pro-inflammatory cytokine. This gene has been previously shown to be induced following *in vitro* treatment with paclitaxel in murine macrophages (22) and human monocytes (24). Indeed, paclitaxel has very similar pro-inflammatory effects to lipopolysaccharide, independently of its effects on microtubules and potentially due to interaction with heat shock proteins (25). These results suggest that nab-paclitaxel has important effects besides stabilization of microtubules (26); however, the extent and clinical implications of these additional effects remain poorly understood.

Compared to nab-paclitaxel, treatment with mifepristone had more widespread effects on gene regulation. Most of these changes reflected down-regulation of gene expression, and were enriched for effects on GR-regulated genes. This was particularly true for genes up-regulated by glucocorticoids. It is likely that this reflects mifepristone antagonizing endogenous glucocorticoid receptor signaling in these individuals. For example, endogenous cortisol signaling may have induced expression of a number of genes in the peripheral blood cells of patients at the time of the ‘baseline’ collection, when they are likely to experience an increased stress response. We hypothesize that subsequent treatment with mifepristone antagonizes GR-mediated gene expression, leading to relatively decreased expression (compared to baseline) of these genes after treatment with mifepristone. Interestingly, we found that this overlap was not complete, in that some genes differentially-expressed in response to mifepristone showed little evidence of differential expression due to glucocorticoid treatment. It is likely that mifepristone regulates a set of genes that endogenous cortisol or dexamethasone do not regulate. While the incomplete overlap between genes differentially expressed by mifepristone and GC treatment could reflect incomplete power to identify response to glucocorticoids, this explanation is unlikely because the glucocorticoid study was performed in a large sample (88 individuals) and had high power to detect significant differentially-expressed genes. These effects could also represent a mifepristone mechanism that is independent of its role as a GR ligand (i.e. off target effects).

The patterns we observed in this study have important clinical implications. We found that nab-paclitaxel targeted tubulin pathway genes in peripheral blood cells. This observation suggests that similar mechanisms may mediate the effects of nab-paclitaxel on epithelial cancer cells and non-cancerous blood cells. Future studies are needed to follow-up on this hypothesis. For example, comparative analysis of the gene expression response to nab-paclitaxel in cancer versus normal blood cells could reveal the overlap in underlying pathways, which could help inform the development of novel therapies with reduced hematological effects. Our results also suggest that an individual's hematological sensitivity to nab-paclitaxel could be measured using gene expression profiling, potentially forming the basis of an assay that might predict individualized risk of adverse events (e.g. neutropenia).

We found that, compared to nab-paclitaxel, mifepristone had much broader effects on gene regulation in peripheral blood cells. This includes modulation of expression of known GR target genes. These results suggest that mifepristone may counteract the effects of cortisol signaling in blood cells. Cortisol can cause apoptosis and prevent proliferation of leukocytes (27). Mifepristone may counteract these effects, potentially leading to a protective effect on white blood cells. We speculate that mifepristone may, therefore, act to reduce overall risk of leukopenia when included with nab-paclitaxel therapy, and future studies are warranted to explore this possibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Study design for treatment and expression profiling is depicted. A) Schedule of blood draws relative to treatment is shown. Peripheral blood samples were taken from each patient before any therapy was initiated (baseline), 24 hours after nab-paclitaxel/placebo was administered, and 24 hours after mifepristone/nab-paclitaxel was administered. Microarrays were used to profile expression across all genes in each blood sample from each patient. B) Comparisons made to identify nab-paclitaxel (top box) and mifepristone (bottom box) effects on gene expression levels are depicted relative to the treatment and sampling scheme shown in A.

Figure 2.

Transcriptional response of peripheral blood cells from patients 24 hrs following treatment with nab-paclitaxel. A) Volcano plot shows log2 fold change in expression in samples treated with nab-paclitaxel alone, or with mifepristone, versus baseline plotted against the significance of differential expression (as $-\log_{10}$ of P-value). B) Boxplots show expression by treatment for the five tubulin sub-unit genes up-regulated following nab-paclitaxel treatment. Relative expression levels (y-axis) represent mean-centered expression estimates corrected for covariates as described in methods.

Figure 3.

Transcriptional response of peripheral blood cells 24 hours following mifepristone treatment. A) Volcano plot shows log2 fold change in expression in samples from patients treated with mifepristone (with nab-paclitaxel) versus baseline, or versus monotherapy with nab-paclitaxel treatment, plotted against the significance of differential expression (as $-\log_{10}$ of P-value). B) As an example of gene expression changes following mifepristone, the boxplot shows expression by treatment for the gene with strongest evidence of response to mifepristone (*ESYT1*). Relative expression levels (y-axis) represent mean-centered expression estimates corrected for covariates as described in methods.

Figure 4.

Overlap between mifepristone and glucocorticoid-mediated gene expression. A) The relative enrichment of overlapping genes (differentially-expressed by both mifepristone and dexamethasone) is shown for genes up-regulated (black dots) and down-regulated (red dots) following mifepristone treatment. Enrichment represents the ratio of the observed number of overlapping genes relative to the number of overlapping genes expected by chance. B) Boxplot shows expression by treatment for the glucocorticoid-induced leucine zipper (*GILZ*), a well-established target of GR activation that is down-regulated following mifepristone treatment *in vivo*. Relative expression levels (y-axis) represent mean-centered expression estimates corrected for covariates as described in methods.