Modulating microtubule stability enhances the cytotoxic response of cancer cells to paclitaxel

Ahmed Ashour Ahmed\textsuperscript{1,2,3,}, Xiaoyan Wang\textsuperscript{8}, Zhen Lu\textsuperscript{1}, Juliet Goldsmith\textsuperscript{2,3}, Xiaofeng Le\textsuperscript{1}, Geoffrey Grandjean\textsuperscript{1}, Geoffrey Bartholomeusz\textsuperscript{1}, Bradley Broom\textsuperscript{4}, Robert C. Bast Jr\textsuperscript{1,*}

1- Department of Experimental Therapeutics, M.D. Anderson Cancer Center, University of Texas, Houston, TX 77030, USA.
2- Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Oxford, UK OX3 9DU.
3- Department of Oncology, University of Oxford, Oxford, UK OX3 7DQ.
4- Bioinformatics and Computational Biology, M.D. Anderson Cancer Center, University of Texas, Houston, TX 77230-1410, USA.
*Corresponding authors.
\textsuperscript{8} Current address: Institute of Immunology, Medical School, Shandong University, Jinan, Shandong, 250012, China.

Running title:
Increasing microtubule stability enhances taxane response

Key words
Microtubule stability, paclitaxel, ovarian cancer, targeted therapy, antimitotic therapy.

Financial support
AAA was funded by Cancer Research UK, the University of Oxford and the Camilla Samuel Fund. AAA and all other authors were funded by the Zarrow Foundation, Mr. and Mrs. Stuart Zarrow, the Ovarian Cancer Research Fund Program Project Development Grant, the National Foundation for Cancer Research and the University of Texas M.D. Anderson Cancer Center Ovarian Cancer Specialized Program of Research Excellence (P50 CA083639).

Corresponding authors:
1- Dr Ahmed Ashour Ahmed. Email: ahmed.ahmed@obs-gyn.ox.ac.uk
Phone: +44 (0)1865 740382. Fax: +44 (0)1865 769141.
2- Dr Robert C Bast Jr. Email: rbast@mdanderson.org. Phone: (+1) 713 792 7743; Fax: (+1) 713 7452107.

Potential conflicts of interest: None declared
Abstract

The extracellular matrix protein TGFBI enhances the cytotoxic response of cancer cells to paclitaxel by affecting integrin signals that stabilize microtubules. Extending the implications of this knowledge, we tested the more general hypothesis that cancer cell signals which increase microtubule stability before exposure to paclitaxel may increase its ability to stabilize microtubules and thereby enhance its cytotoxicity. Toward this end, we performed an siRNA screen to evaluate how genetic depletion affected microtubule stabilization, cell viability and apoptosis. High content microscopical analysis was performed in the absence or presence of paclitaxel. Kinase knockdowns that stabilized microtubules strongly enhanced the effects of paclitaxel treatment. Conversely, kinase knockdowns that enhanced paclitaxel-mediated cytotoxicity sensitized cells to microtubule stabilization by paclitaxel. The siRNA screen identified several genes that have not been linked previously to in microtubule regulation or paclitaxel response. Gene shaving and Bayesian resampling used to classify these genes suggested three pathways of paclitaxel-induced cell death related to apoptosis and microtubule stability, apoptosis alone, or neither process. Our results offer a functional classification of the genetic basis for paclitaxel sensitivity and they support the hypothesis that stabilizing microtubules prior to therapy could enhance antitumor responses to paclitaxel treatment.
Introduction

Taxanes (1, 2) are microtubule stabilizing drugs used in the standard management of two of the most common solid tumors, breast and ovarian cancer, in addition to several other cancers (3, 4). In each of these cancers, only a fraction of patients will benefit. Understanding mechanisms of response to primary taxane therapy is an essential step in developing novel combination therapies that will improve response rates. Improving response rates to an established therapy is likely to improve survival outcomes for patients.

The taxane, paclitaxel, binds to specific pockets within $\beta$ tubulin in $\alpha/\beta$ tubulin dimers (5), that are incorporated within preformed microtubule polymers (6, 7). Paclitaxel binding increases microtubule polymerization and stability, and induces mitotic arrest of cycling cancer cells followed by apoptosis. Increasing the fraction of unstable microtubules in the cell by mutations outside the paclitaxel-binding sites (8, 9), overexpression of $\beta3$ tubulin (10, 11) or overexpression of depolymerizing microtubule associated proteins (12) results in a disproportionate increase in free tubulin dimers and a consequent decrease in paclitaxel binding sites, leading to profound paclitaxel resistance.

We have previously shown that loss of the extracellular matrix protein TGFBI results in decreased Focal Adhesion Kinase (FAK) activation, decreased microtubule stability and an increase in unstable microtubules resulting in significant paclitaxel resistance (13). We have also shown that ovarian cancers which lack TGFBI are intrinsically resistant to single agent paclitaxel therapy. Based on these findings we proposed a model whereby the proportion of baseline stable microtubules determines the availability of binding sites for paclitaxel and consequently the magnitude of paclitaxel-induced microtubule stabilization. In this report, we tested the hypothesis that modulating baseline microtubule stability by perturbing signaling pathways would alter paclitaxel response and could be utilized therapeutically.
Materials and Methods

Cell culture and siRNA transfection

Cell culture and transfections

All cell lines were obtained from the American Tissue Type Culture Collection. Cells were maintained in RPMI-140 medium supplemented with 10% fetal bovine serum and incubated at 37°C and 5% CO₂. Human skin fibroblasts were a kind gift from Dr Karl Morten. A549 and A549-T12 lung carcinoma cells were a kind gift from Dr Susan Horwitz. Cell lines were authenticated using short tandem repeat profiling.

Transient knockdown experiments for the screen were performed by transfection using a pool of 4 individual siRNA duplexes targeting individual genes (siGenome Smart pool, Dhharmacon), or individual duplexes, using Dhharmafect 4 (Dharmacon) according to the manufacturers' instructions. In summary, 0.1 μl of 20 μM siRNA was mixed with 0.1 μl of Dhharmafect 4 and 10 μl of Optimem medium (Invitrogen) for 20 min in a well of 384-well plate and overlaid with 40 μl of cell suspension (1750 cells) in antibiotic-free cell line-specific media. This gave a final siRNA concentration of 40 nM per well. The catalogue numbers and sequences of the siRNAs used are listed in Supplementary table S1. siControl non-targeting siRNA number 4 (Dharmacon) was used as a transfection control in all experiments. Pools of 4 siRNAs targeting Tau, MAP2 and Stathmin were obtained from Dhharmacon (siGenome library).

For the purpose of the screen, the first three columns of every plate were reserved for transfection controls as follows; siControl, siTOX (Dharmacon) as control for transfection efficiency and cells only.

Immune-staining microscopy techniques, image acquisition and image analysis
SKOv3 ovarian cancer cells were transfected using siRNAs in individual wells of black 384-well plates (Greiner, 1750 cells per well) for 48 hrs then either treated with paclitaxel (Sigma) 8 nM or diluent only then fixed using 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 3 to 5 min. The cells were washed to remove PFA and were incubated in absolute ethanol at -20°C for at least 1 hr before being washed with Tris-buffered saline, 0.04% SDS (Sigma), and 0.2% Triton X-100 (Sigma). Blocking was performed using 1.5% bovine serum albumin in Tris-buffered saline. Nuclear DNA was stained using Hoechst 33258 dye (Invitrogen) and stable microtubules were revealed by rabbit anti-Glu tubulin antibody (Millipore) and counterstained using anti-rabbit tagged with alexa-594 (Invitrogen). Image acquisition was obtained as previously described using the In Cell Analyzer 1000 (GE Healthcare) (14). For quantification of α tubulin intensity, mouse anti-α tubulin antibody (Sigma) was counterstained with anti-mouse antibody counterstained with alexa-488.

**Statistical analysis:**
Analysis of data for the screen from individual cells (n~4 million) was automated using the R programming language and statistical computing environment. In brief, each cell analyzed was annotated to individual wells representing a gene knockdown. Background subtracted mean pixel intensity values were calculated for each cell and the mean of all cells per well was used as an estimate of the magnitude of microtubule stability for each well. The median of triplicate values was obtained for each gene with and without paclitaxel and expressed as a percentage of the mean value for microtubule stability following transfection with non-targeting siRNAs. The values in each plate were centered on 100 using median scaling. The same method was used for analyzing caspase and viability data.

Robust siRNA clusters were obtained using bagged gene shaving (15). We used 256 bootstrap resamples and obtained each resample by first ranking the individual cells in each sample, and then applying a weighted ranking across
each siRNA using weights obtained from the Bayesian bootstrap distribution. Aggregated clusters obtained from the adjacency matrix were required to contain siRNA with at least 50% similarity within the cluster.

**Cytotoxicity assays**

For measuring apoptosis, Caspase 3/7 activity were estimated 48 hrs following treatment by adding equal volume of the Caspase-Glo 3/7 Assay reagent (Promega). Luminescence was read following 1 hr of incubation using a luminescence plate reader. Cell viability was estimated by adding 5 μl of the CellTiter blue kit reagent (Promega) according to the manufacturer's instructions. Fluorescence was measured 4 hours later using a plate reader.

For colony forming assays, SKOv3 cells (2×10^5 cells/well) or Hey cells (9×10^4 cells/well) were seeded in the six-well plates. Twenty-four hours later, cells were transfected using either non-targeting siRNA or siRNAs targeting ILK or RAPGEF4 for 48 hrs, then the cells were reseeded in another six-well plates at the density of 250 cells/well for SKOv3 or 200 cells/well for Hey. After two days, the cells were treated with paclitaxel 1nM for 48 hrs and cells were maintained in the corresponding non-antibiotic medium for either 7 days (SKOv3 cells) or 4 days (Hey cells) then fixed and stained using Coomassie Blue and individual colonies were counted.

For measuring cell kinetics, 5-Bromo-2’-deoxy-uridine (BRDU) incorporation was measured using an ELISA based assay (Roche Applied Sciences) following the manufacturer’s instructions as a measure of the extent of cell cycling.

**Reagents**

Paclitaxel and nocodazole were obtained from Sigma. Antibodies for Tau, MAP2 and Stathmin were obtained from Millipore and for p-STAT3 was from Cell Signaling. All chemicals were obtained from Sigma unless otherwise indicated.
Results
A hypothesis-driven screen to investigate the influence of baseline microtubule stability on paclitaxel cytotoxicity

To test the hypothesis that modulating signaling-induced microtubule stabilization can directly influence paclitaxel-induced microtubule stabilization and cytotoxicity, we conducted an siRNA screen to identify candidate genes which upon knockdown enhance paclitaxel-induced microtubule stability, apoptosis and cell death. We chose the dharmacon kinome library, as microtubule associated proteins are strongly regulated by phosphorylation (16-21). SKOv3 ovarian cancer cells were transfected with either non-targeting control siRNA or a pool of 4 siRNAs targeting each of the 779 genes included in this library.

To measure microtubule stability in a high-throughput manner we performed triplicate immunofluorescence experiments in 384-well plates using an antidetyrosinated alpha tubulin (also called Glu-tubulin) antibody, a specific marker for stable microtubules (22, 23), that was counterstained with an alexa-594 -tagged antibody. Automated image capture was performed followed by automated image analysis to extract background-subtracted mean pixel intensity values from the cytoplasm of individual cells as a measure of microtubule stability. When microtubule stability was measured following exposure to increasing concentrations of paclitaxel (Supplementary Fig.S1) the Glu-tubulin-based assay yielded a wide (4.3 fold) dynamic range compared to the narrower range (2.5 fold) of alpha-tubulin-based assays. This difference may relate to the relatively small fraction of stable microtubules (glu tubulin) in cancer cells compared with total microtubules (alpha tubulin) prior to paclitaxel treatment (Supplementary Fig.S1). Use of automated microscopy and image analysis for individual cells yielded a consistent and reproducible linear relationship between paclitaxel dose and microtubule stability (Supplementary Fig.S1) in contrast to
the use of other high-throughput methods based on measuring total fluorescence from individual wells or measuring well luminescence in assays that utilize peroxidase-conjugated antibodies (data not shown). To verify the fidelity of the technique in identifying changes in microtubule stability, we tested the effect of depletion of the microtubule destabilizing protein Stathmin (12) and the microtubule polymerizing protein MAP2 (24). As expected, depletion of Stathmin resulted in a significant increase in baseline microtubule stability and paclitaxel-induced microtubule stabilization while depletion of MAP2 resulted in the opposite effect. In addition, depletion of Tau, which has previously been shown to compete with paclitaxel for the binding site on β tubulin (24-26), resulted in a marked increase in paclitaxel-induced microtubule stabilization (Supplementary Fig.S2). In addition, the above results were also reproduced by measuring total microtubule polymer formation by using an antibody for alpha tubulin (Supplementary Fig.S2).

Microtubule stability was measured following one hour of paclitaxel treatment as this time point highly correlated with cytotoxicity after 48 hrs of treatment (Pearson correlation coefficient = 0.97, p<0.001) (Supplementary Fig.S3). Later estimation of microtubule stability was less reproducible because of easier detachment of cells following arrest in mitosis (not shown). In addition to measuring microtubule stability, we also measured caspase activity and cell viability with or without paclitaxel treatment. To avoid the bias introduced by lifting off of mitotic cells when measuring apoptosis or viability, we utilized assays that did not require washing of media prior to estimation of cytotoxicity (see methods). All three outcome measures were optimized to maximize their dynamic range. Our assay development experiments showed that decreasing the proportion of cycling SKOv3 cells significantly decreases paclitaxel-induced apoptosis and this may decrease the dynamic range of the cytotoxicity tests and increase the false negative rate in siRNA experiments (Supplementary Fig.S4). As transfection itself can decrease proliferation rate and increase the proportion of non-cycling cells, we conducted detailed assay development experiments to optimize our
transfection conditions to avoid the bias introduced by shifting cell kinetics. We tested multiple starting cell densities (1000, 1250, 1500, 1750 and 2500 cells per well), 8 transfection reagents, 9 non-targeting siRNAs and 4 siRNAs that were known to induce cell death and measured cell viability using CellTiter blue kit reagent (Promega) following 48 hrs of transfection (Supplementary Fig.S5). These experiments showed that using 1750 cells, and Dharmafect 4 transfection reagent minimized the inhibitory effect of transfection on cell proliferation when compared to non-transfected cells and resulted in the least variability across replicates (Supplementary Fig.S5). Apoptosis was measured following 48 hrs as this gave a detection range of 9 fold (Supplementary Fig.S6) and viability was measured after 72 hrs as this resulted in a 2.6 fold detection range (Supplementary Fig.S7A) compared to only 1.6 fold detection range after 48 hrs (Supplementary Fig.S7B). A dose of 8 nM of paclitaxel was chosen to maximize the chance of detecting sensitizing siRNAs by providing at least an 80% window for detecting enhanced taxane effects. Using 8 nM of paclitaxel resulted in only 2-fold increase in microtubule stability, a 2-fold increase in apoptosis and a 15% decrease in cell viability. This dose was also chosen because cytotoxicity measurements tended to plateau at higher concentrations (Supplementary Fig.S7).

Thus, three kinome screens were conducted in parallel to measure the effect of siRNA transfections on microtubule stability, cell viability and apoptosis with or without paclitaxel (Fig.1A). Each of the three screens was conducted in triplicate. To estimate data precision, we measured the mean correlation coefficients among the three replicates for microtubule stability, cell viability and apoptosis and found that the average Pearson correlation coefficients were 0.77, 0.86 and 0.96, respectively (Fig.1B). This high reproducibility across replicates is a strong indicator of the high precision of data (27) obtained in this screen.

**Baseline microtubule stability strongly correlates with paclitaxel-induced microtubule stabilization**
To test the hypothesis that increasing baseline microtubule stability results in enhanced paclitaxel-induced microtubule stabilization we calculated the median microtubule stability values of three replicates prior to treatment and correlated this with the median value following treatment and found a highly statistically significant positive correlation (Pearson $r^2 = 0.66$, $p<0.001$). This indicated that on a global scale, baseline microtubule stability predicted taxane induced microtubule stabilization (Fig.2).

Enhanced microtubule stabilization is a significant mechanism for enhanced paclitaxel-mediated cytotoxicity

We next sought to identify a group of kinases that regulate sensitivity of cancer cells to paclitaxel. We selected siRNAs that 1) induced a two fold enhancement of cell growth inhibition when combined with paclitaxel compared to the cytotoxic effect of siRNA alone (normalized paclitaxel and siRNA treated value divided by normalized siRNA treated value of more than 2 fold) or 2) increased paclitaxel-induced cytotoxicity following siRNA by 2.5 fold when compared to paclitaxel-induced cytotoxicity following transfection with non-targeting siRNA. To identify enhancers of paclitaxel-mediated microtubule stability we selected siRNA pools that were in the upper quartile of all siRNAs tested (microtubule stability > 116.1% compared to paclitaxel treated cells transfected with non-targeting siRNAs). Using these criteria, we identified 61 siRNA pools that enhanced paclitaxel induced apoptosis and 50 siRNA pools that enhanced paclitaxel induced cell death (Supplementary tables S2 and S3). Importantly, 34.4 % of the genes that sensitized cells to paclitaxel induced apoptosis (Fig.3A and Supplementary table S2) and 46% of the genes that sensitized cells to paclitaxel-mediated cell death (Fig.3B and Supplementary table S3) also enhanced paclitaxel-induced microtubule stability. The probability of these proportions occurring by chance alone is 0.03 and <0.001, respectively, based on 100,000 random samplings without replacement. This analysis confirmed that enhancing
microtubule stability following paclitaxel treatment is strongly associated with enhanced paclitaxel-induced cell death. Interestingly several genes that enhanced apoptosis and microtubule stability such as CHUK (also known as inhibitor of kappa B kinase A [IKKalpha]), or enhanced cytotoxicity such as CARD14, are known to activate the Nuclear Factor Kappa B (NFkB) pathway (28, 29). This pathway is constitutively active in SKOv3 cells (30) and its inhibition has been previously reported to enhance paclitaxel-mediated cell death (31-33).

**Measuring microtubule stability identifies three distinct mechanistic classes for modulating paclitaxel-induced cytotoxicity**

We next sought to classify siRNA pools targeting individual genes according to their similarities in the mechanism of modulating paclitaxel response. To do this we first used gene shaving to obtain classes of genes that behave similarly in modulating cell viability and apoptosis with or without paclitaxel and paclitaxel-induced microtubule stability (15). We next used Bayesian resampling to identify classes that were statistically robust to permutation analysis. This method identified predominantly three robust classes of genes (Fig.4A, Supplementary tables S4 – S6) in which paclitaxel induced microtubule stability either; 1) significantly correlated with paclitaxel-induced apoptosis (Pearson $r^2 = 0.5$, p<0.001, n=208 siRNA pools, Fig.4B) and to a lesser extent with paclitaxel-induced loss of viability (Pearson $r^2 = -0.2$, p<0.001, n=208, Fig.4B), 2) significantly correlated with both paclitaxel-induced apoptosis (Pearson $r^2 = 0.5$, p<0.001, n=132 siRNA pools, Fig.4B) and loss of cell viability (Pearson $r^2 = -0.7$, p<0.001, n=132 siRNA pools, Fig.4B) or 3) paradoxically, inversely correlated with paclitaxel-induced apoptosis (Pearson $r^2 = -0.6$, p<0.001, n=105 siRNA pools, Fig.4B) and loss of cell viability (Pearson $r^2 = 0.6$, p<0.001, n=105 siRNA pools, Fig.4B). Interestingly, the latter class was also characterized by a poor correlation between paclitaxel-induced apoptosis and paclitaxel-induced loss of cell viability (Pearson $r^2 = -0.3$, Fig.4B). To explain the paradoxical inverse correlation between microtubule stability and apoptosis in the third gene cluster,
we tested the hypothesis that depletion of genes that result in inhibition of apoptosis may result in resistance to paclitaxel regardless of the effect on microtubule stability. To test this possibility, we identified all siRNA pools that resulted in increased paclitaxel-induced microtubule stabilization and decreased paclitaxel-induced apoptosis (n=34). Examination of the known function of these genes revealed that several of them were either genes with known critical function in inducing apoptosis or genes that played a role in recognition of cell damage. For example, RIPK2 (Receptor Interacting Serine Threonine Kinase 2, also known as CARD3) is a known apoptosis inducer (34). Depletion of RIPK2 resulted in decreased apoptosis irrespective of paclitaxel administration. Also, loss of the cell cycle checkpoint protein ATM (35), resulted in enhanced microtubule stability but decreased paclitaxel-induced apoptosis and improved cell survival following paclitaxel treatment. We, therefore, reasoned that loss of these genes may result in a significant decrease in apoptosis that is independent of paclitaxel effect. Indeed, the mean normalized apoptosis induction following transfection with these siRNAs (n=34) was less than half the mean of the rest (n=745) of the genes (65% vs 140%, respectively, p<0.001, t test, Fig.4C).

Secondary validation identifies novel mechanisms of modulating microtubule stability and paclitaxel cytotoxicity

To investigate genetic specificity of siRNA pools we used 4 individual siRNAs to knockdown each of 35 genes that enhanced paclitaxel effect on microtubules and also enhanced cytotoxicity, as detected by measuring cell viability or apoptosis. We measured the effect of KD on microtubule stability and viability with or without paclitaxel treatment in 384-well plates in triplicates. 34 out of the 35 genes tested had at least one siRNA that resulted in a significant increase in microtubule stability. 29 genes were validated using at least 2 independent siRNAs (Fig.5A and B, table 1). Importantly, there was a strong positive correlation between baseline microtubule stability and paclitaxel-induced microtubule stability.
following siRNA transfections (correlation coefficient = 0.87, Fig.5C). We next examined the correlation between paclitaxel-induced microtubule stabilization and paclitaxel-induced cell death and found a highly significant correlation (p<0.001, Fig.5D). Taken together, these results further validate the notion that baseline microtubule stability is a strong determinant of paclitaxel-mediated microtubule stabilization and identify previously unrecognized roles of a group of genes in modulating microtubule stability.

**Modulating microtubule stability directly influences microtubule mass and cytotoxicity in ovarian cancer cell lines**

Paclitaxel induces a significant increase in microtubule stability and in total microtubule mass resulting in the induction of the characteristic feature of paclitaxel-induced bundles (36) which perturbs the ability of the cell to form an organized mitotic spindle. To investigate whether enhanced paclitaxel-induced microtubule stability also resulted in an enhanced paclitaxel-induced microtubule polymerization we knocked down 7 genes (ILK, FER, RAPGEF4, AATK, CHEK1, FLT4, SGK2) using siRNA pools and measured the effect of knockdown on microtubule stability and total microtubule mass following paclitaxel treatment at 10 nM and 100 nM using both alpha and glu-tubulin staining. As expected, knockdown of each of the 7 genes enhanced glu-tubulin formation following paclitaxel treatment at each of the two concentrations used (Fig.6A). In addition, gene depletion also resulted in an increase in microtubule mass for at least one of the concentrations tested for each of the 7 genes (Fig.6B). Depletion of these genes resulted in enhanced paclitaxel-induced loss of cell viability in short-term assays and a significant decrease in the ability of cancer cells to form colonies in long-term assays in SKOv3 and Hey ovarian cancer cell lines (Fig.6C and D). Similar results were obtained following depletion of RAPGEF4 in the paclitaxel-resistant A549-T12 lung carcinoma cells that are known to have microtubule-level of resistance to paclitaxel (12). We first confirmed that paclitaxel-induced microtubule stabilization was significantly reduced in A549-T12 cells compared to
the parent paclitaxel-sensitive A549 cells (Supplementary Fig.S8). RAPGEF4-specific siRNA treatment of the A549-T12 cells resulted in a significant increase in paclitaxel-microtubule stabilization and partially restored paclitaxel sensitivity (Supplementary Figure S8). These results confirm the strong mechanistic effect of modulating microtubule stability on microtubule polymer mass and paclitaxel-mediated cytotoxicity.

Discussion

Pharmacological approaches for overcoming resistance to chemotherapy in general and to antimitotic agents in particular have often focused on enhancing general mechanisms of cytotoxicity such as apoptosis (37-39), rather than on modulating drug-specific targets. Methods used to enhance cytotoxicity often lack specificity and such approaches, while important, are likely to induce unwanted off-target effects. In-spite of the detailed knowledge of the mechanism of action of paclitaxel, identifying mechanism-specific methods for modulating its cytotoxic effect via modulating microtubules or mitosis has remained elusive. This work confirms and generalizes the concept that modulating microtubule stability in cancer cells is an effective method for enhancing paclitaxel cytotoxicity. Our current study has also discovered several targets that could be exploited for therapeutic benefit.

In contrast to several previously conducted siRNA screens that have focused on measuring a single general endpoint in multiple cell lines, we have conducted a comprehensive analysis of the effect of siRNA depletion on multiple related but independent outcomes, focusing on the paclitaxel-specific end-point of microtubule stability as well as the more general parameters of cell viability and apoptosis. This approach revealed a previously underestimated level of complexity in regulating cell fate following paclitaxel treatment. Discordant results in the three assays after knockdown of certain groups of kinases indicate that promoting cell death through one mechanism does not necessarily predict
outcomes for other seemingly parallel mechanisms of cell death. This may, in part, explain the inconsistency in the results obtained between different studies when evaluating a novel target for therapy.

Our work identified several gene clusters that influence paclitaxel response in strikingly different ways. More than a third of the genes that sensitized cells to paclitaxel induced apoptosis and nearly half of the genes that sensitized cells to paclitaxel-mediated cell death also enhanced paclitaxel-induced microtubule stability ($P=0.03$ and $<0.001$, respectively). This analysis confirmed that enhancing microtubule stability following paclitaxel treatment is strongly associated with enhanced paclitaxel-induced cell death. While, it is intuitive to hypothesize that enhancing microtubule stability following paclitaxel treatment should be followed by higher apoptosis and loss of cell viability, not all siRNAs that increased microtubule stability enhanced sensitivity to paclitaxel in this screen. An important limitation of short-term assays as those conducted in this screen, is the undue sensitivity of the assay to cell population kinetics. As paclitaxel-induced apoptosis is dependent on passage through mitosis, decreasing cell population doubling time due to suboptimal transfection conditions may result in false negative results. We have, therefore, extensively optimized assay development conditions prior to the screen to avoid this potential pitfall. However, the total elimination of this possibility is difficult to confirm.

How microtubule stabilization results in enhanced paclitaxel-induced cytotoxicity is likely to be multifactorial. However, our findings suggest that induction of apoptosis following increased mitotic arrest is likely to be the predominant mechanism. Consistent with previous findings (40), our work reveals the importance of the effect of modulating apoptosis on cell fate such that a negative effect on apoptosis may negate a therapeutic advantage of enhancing microtubule stability. In contrast to targeting the intrinsically promiscuous kinases that share and phosphorylate many substrates, targeting microtubule-specific
molecular targets may result in a tighter concordance between the magnitude of microtubule stability and cytotoxicity.

Another possibility for the effect of enhancing microtubule stability on increasing paclitaxel-induced cell death is interfering with vesicular transport of key survival and transcription factors to the nucleus. To investigate this hypothesis we tested whether microtubule stabilization or depolymerization decreased the nuclear transport of a constitutively activated transcription factor in SKOV3 cells, STAT3 (41, 42). This revealed that in contrast to microtubule depolymerization using Nocodazole which resulted in a significant decrease in nuclear accumulation of phospho-STAT3, paclitaxel used at concentrations up to 100 nM did not (Supplementary Fig.S9). Whether depletion of motor proteins involved in nuclear transport of transcription factors may result in sensitization to paclitaxel by targeting several transcription factors at the same time will remain to be tested.

The identification of molecular predictive markers is required for personalization of therapy (43). A significant obstacle to identifying clinically relevant predictive markers for taxane response is that, in clinical practice, most tumors are treated by a combination of paclitaxel and other chemotherapies (3, 4). Therefore, using standard end points for measuring response, such as radiological monitoring of tumor size or measuring the level of tumor markers, cannot distinguish the effect of individual drugs. Similarly, it is not possible to use early indicators of cytotoxicity, such as apoptosis markers, as endpoints in such studies. In this work we showed that baseline microtubule stability is an important determinant of paclitaxel-induced microtubule stabilization and cytotoxicity. This presents a testable hypothesis that microtubule stability could be measured either as a predictive marker of paclitaxel response or as a surrogate endpoint for paclitaxel response that could be utilized to identify clinically relevant predictive molecular biomarkers. Measuring the magnitude of microtubule stability at presentation is feasible by measuring Glu-tubulin levels in tumors (44). In addition to measuring base line microtubule stability, it is possible to hypothesize that a certain
threshold of microtubule stability is required for paclitaxel to exert its cytotoxic effect in patients. Measuring paclitaxel-induced microtubule stability in mouse models and in specifically designed early phase clinical trials will help in testing the relevance of this method in predicting paclitaxel response.

Mechanisms regulating microtubule dynamics are most probably shared between cancer and non-cancer cells. For example, depletion of the microtubule associated protein Tau, which is thought to compete with paclitaxel for binding sites in β tubulin, in human skin fibroblasts grown in culture results in a significant increase in paclitaxel-induced microtubule stabilization (Supplementary Fig.S10). However, we have shown that paclitaxel-induced apoptosis is dependent on cell cycling (Supplementary Figure S4). As many non-tumour cell types are non-cycling, the concept of specific targeting of microtubules to enhance paclitaxel-induced stabilization ensures the minimization of toxicity. Importantly, our study identified enhancers of microtubule stabilization following the administration of substoichiometric concentrations of paclitaxel (8 nM) to mimic the concentrations that would be expected in the vicinity of ovarian cancers in patients. In contrast to poorly vascularized cancer tissue, Non-tumour cycling cells such as those in bone marrow are probably exposed to much higher concentrations of paclitaxel (above 50 nM) that result in near stoichiometry and full microtubule stabilization (45, 46). Therefore, we hypothesize that enhancers of paclitaxel-induced microtubule stability may only increase cell death in cancer cells and not normal bone marrow cells as the former is exposed to much lower doses of paclitaxel. Future experiments in animal models of cancer will address this hypothesis. In addition, prioritization of targets for testing in preclinical and clinical models will depend on the pattern of expression and activity of the identified targets in ovarian and other cancers and the availability of inhibitors for the identified targets. In those instances where kinases are overexpressed or activated, selective enhancement of microtubule stability might be achieved within cancer cells, increasing rates of response. In contrast to many earlier studies, the goal
would be to enhance primary sensitivity, rather than reverse mechanisms of acquired resistance.

In summary, our work has shown that modulation of baseline microtubule stability is a key determinant for paclitaxel-induced microtubule stabilization and cytotoxicity. This finding has important predictive and therapeutic implications that could be tested in preclinical and clinical models of paclitaxel therapy.

Acknowledgements
We would like to thank Dr Gordon Mills and Catherine Nguyen for technical help.
References


Table 1: Shown is the fold change in paclitaxel-induced microtubule stability following siRNA depletion of the indicated genes by 4 individual siRNA compared to paclitaxel-induced microtubule stability following non-targeting siRNA transfection. The p value is the result of one-sided t-test comparison of triplicate values of paclitaxel-induced microtubule stability following siRNA depletion of an individual gene compared with triplicate values of paclitaxel-induced microtubule stability following transfection using non-targeting control siRNA.
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>siRNA number 1</th>
<th>siRNA number 2</th>
<th>siRNA number 3</th>
<th>siRNA number 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>AATK</td>
<td>apoptosis-associated tyrosine kinase</td>
<td>1.65 0.025</td>
<td>1.09 0.025</td>
<td>0.92 0.973</td>
<td>1.42 0.008</td>
</tr>
<tr>
<td>BMP2K</td>
<td>BMP2 inducible kinase</td>
<td>1.53 0.030</td>
<td>1.01 0.356</td>
<td>1.07 0.173</td>
<td>1.45 0.021</td>
</tr>
<tr>
<td>CDK5RAP3</td>
<td>CDK5 regulatory subunit associated protein 3</td>
<td>1.12 0.231</td>
<td>1.30 0.012</td>
<td>0.93 0.953</td>
<td>0.97 0.813</td>
</tr>
<tr>
<td>CHEK1</td>
<td>CHK1 checkpoint homolog (S. pombe)</td>
<td>1.19 0.142</td>
<td>1.86 &lt;0.001</td>
<td>1.45 0.006</td>
<td>1.60 0.018</td>
</tr>
<tr>
<td>CHEK2</td>
<td>CHK2 checkpoint homolog (S. pombe)</td>
<td>1.58 0.054</td>
<td>1.21 0.104</td>
<td>0.97 0.819</td>
<td>1.15 0.066</td>
</tr>
<tr>
<td>CHUK</td>
<td>conserved helix-loop-helix ubiquitous kinase</td>
<td>1.77 0.013</td>
<td>1.10 0.145</td>
<td>1.18 0.053</td>
<td>1.59 0.003</td>
</tr>
<tr>
<td>COPB2</td>
<td>coatomer protein complex, subunit beta 2 (beta prime)</td>
<td>1.81 0.001</td>
<td>1.74 0.013</td>
<td>1.64 0.011</td>
<td>1.76 &lt;0.001</td>
</tr>
<tr>
<td>CSF1R</td>
<td>colony stimulating factor 1 receptor</td>
<td>1.67 0.023</td>
<td>1.34 0.033</td>
<td>1.17 0.080</td>
<td>1.12 0.068</td>
</tr>
<tr>
<td>DAPK1</td>
<td>death-associated protein kinase 1</td>
<td>1.19 0.102</td>
<td>2.13 0.004</td>
<td>1.54 0.020</td>
<td>1.17 0.074</td>
</tr>
<tr>
<td>EDN2</td>
<td>endothelin 2</td>
<td>0.86 0.986</td>
<td>1.58 0.005</td>
<td>1.15 0.006</td>
<td>1.21 0.122</td>
</tr>
<tr>
<td>ERBB4</td>
<td>v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)</td>
<td>1.72 0.016</td>
<td>1.31 0.018</td>
<td>1.95 0.035</td>
<td>1.12 0.132</td>
</tr>
<tr>
<td>ERN1</td>
<td>endoplasmic reticulum to nucleus signaling 1</td>
<td>1.04 0.264</td>
<td>1.12 0.112</td>
<td>1.96 0.018</td>
<td>1.35 0.014</td>
</tr>
<tr>
<td>FER</td>
<td>fer (fps/fes related) tyrosine kinase</td>
<td>1.28 0.008</td>
<td>1.52 0.027</td>
<td>1.32 0.001</td>
<td>0.96 0.693</td>
</tr>
<tr>
<td>FLT4</td>
<td>fms-related tyrosine kinase 4</td>
<td>1.49 0.017</td>
<td>0.99 0.548</td>
<td>1.26 0.045</td>
<td>1.58 0.001</td>
</tr>
<tr>
<td>GALK2</td>
<td>galactokinase 2</td>
<td>1.14 0.017</td>
<td>1.22 0.078</td>
<td>2.01 0.008</td>
<td>2.43 0.008</td>
</tr>
<tr>
<td>GRK4</td>
<td>G protein-coupled receptor kinase 4</td>
<td>1.66 0.012</td>
<td>2.00 0.026</td>
<td>1.27 0.028</td>
<td>1.19 0.095</td>
</tr>
<tr>
<td>IKKB</td>
<td>inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta</td>
<td>2.01 0.023</td>
<td>1.28 0.025</td>
<td>1.17 0.021</td>
<td>1.30 0.010</td>
</tr>
<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
<td>1.58 0.005</td>
<td>1.11 0.148</td>
<td>1.80 0.001</td>
<td>1.38 0.001</td>
</tr>
<tr>
<td>PANK4</td>
<td>pantothenate kinase 4</td>
<td>1.02 0.363</td>
<td>1.02 0.399</td>
<td>1.52 0.004</td>
<td>2.42 0.001</td>
</tr>
<tr>
<td>PDGFRB</td>
<td>platelet-derived growth factor receptor, beta polypeptide</td>
<td>0.92 0.760</td>
<td>2.44 0.012</td>
<td>1.16 0.016</td>
<td>1.30 0.067</td>
</tr>
<tr>
<td>PI4KB</td>
<td>phosphatidylinositol 4-kinase type 2 beta</td>
<td>1.18 0.076</td>
<td>1.49 0.002</td>
<td>1.33 &lt;0.001</td>
<td>1.25 0.001</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>phosphoinositide-3-kinase, class 2, alpha polypeptide</td>
<td>1.42 0.042</td>
<td>1.79 0.008</td>
<td>1.83 0.011</td>
<td>1.99 0.005</td>
</tr>
<tr>
<td>PIK3R1</td>
<td>phosphoinositide-3-kinase, regulatory subunit 1 (alpha)</td>
<td>1.07 0.264</td>
<td>1.12 0.151</td>
<td>1.55 0.016</td>
<td>1.07 0.208</td>
</tr>
<tr>
<td>PKM2</td>
<td>pyruvate kinase, muscle</td>
<td>1.09 0.190</td>
<td>1.35 0.001</td>
<td>1.25 0.073</td>
<td>1.27 0.051</td>
</tr>
<tr>
<td>RAPGEF3</td>
<td>Rap guanine nucleotide exchange factor (GEF) 3</td>
<td>1.16 0.065</td>
<td>1.53 0.036</td>
<td>1.64 0.006</td>
<td>1.00 0.526</td>
</tr>
<tr>
<td>RAPGEF4</td>
<td>Rap guanine nucleotide exchange factor (GEF) 4</td>
<td>0.98 0.653</td>
<td>1.03 0.310</td>
<td>1.11 0.013</td>
<td>1.22 0.001</td>
</tr>
<tr>
<td>RFP</td>
<td>ring finger protein. Also known as tripartite motif containing 27 (TRIM27)</td>
<td>0.87 0.979</td>
<td>1.23 0.073</td>
<td>1.51 0.002</td>
<td>1.66 0.008</td>
</tr>
<tr>
<td>SGK2</td>
<td>serine/threonine kinase 2</td>
<td>1.47 0.025</td>
<td>1.02 0.305</td>
<td>1.50 0.013</td>
<td>1.14 0.038</td>
</tr>
<tr>
<td>SNF1LK</td>
<td>SNF1-like kinase</td>
<td>1.30 0.001</td>
<td>1.04 0.271</td>
<td>1.08 0.114</td>
<td>1.00 0.524</td>
</tr>
<tr>
<td>STK22B</td>
<td>serine/threonine kinase 22B. Also known as testis specific serine kinase 2 (TSSK2)</td>
<td>1.04 0.245</td>
<td>1.54 0.001</td>
<td>1.05 0.363</td>
<td>1.37 0.010</td>
</tr>
<tr>
<td>STK24</td>
<td>serine/threonine kinase 24</td>
<td>1.44 0.002</td>
<td>1.08 0.216</td>
<td>1.25 0.031</td>
<td>0.98 0.755</td>
</tr>
</tbody>
</table>
**Figure Legends:**

**Figure 1**

**A hypothesis-driven screen to investigate the influence of baseline microtubule stability of paclitaxel cytotoxicity.**

A) An outline of the siRNA screens conducted. The 779 siRNA pools used in each screen were divided into three 384-well plates (320 pools for plates 1 and 2 and 139 pools in plate 3) and three replicates were used for each plate with and without paclitaxel giving a total of 54 384-well plates for the entire experiment. B) Shown in the lower diagonal of each plot are dot plots representing the correlation between the measures obtained for each pair of the three replicates used in the screen following paclitaxel treatment. The corresponding Pearson correlation coefficient for each pair is shown in the upper diagonal of each plot.

**Figure 2**

**Baseline microtubule stability strongly correlates with paclitaxel-induced microtubule stabilization.**

779 siRNA pools targeting individual genes were used to transfect SKOv3 cells followed by immunofluorescence as described in methods. The mean cytoplasmic pixel intensity values for each cell were extracted and background subtracted using the In-Cell Investigator (GE Healthcare) software from a total of 64,800 images including a total of ~ 4 million cells. The mean value of all cells in a well, that represent a single gene, was used to calculate the percentage microtubule stability following knockdown of a gene compared to the mean value obtained for non-targeting siRNA transfected cells. In this plot each circle represents the median of triplicate percentage values obtained following siRNA treatment alone, representing baseline microtubule stability, or siRNA and paclitaxel treatment.

<table>
<thead>
<tr>
<th>TAF1L</th>
<th>TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 210kDa-like</th>
<th>0.96</th>
<th>0.91</th>
<th>0.91</th>
<th>0.750</th>
<th>1.88</th>
<th>0.039</th>
<th>1.20</th>
<th>0.141</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBK1</td>
<td>TANK-binding kinase 1</td>
<td>1.75</td>
<td>0.001</td>
<td>1.57</td>
<td>&lt;0.001</td>
<td>1.44</td>
<td>0.003</td>
<td>1.28</td>
<td>0.023</td>
</tr>
<tr>
<td>TESK2</td>
<td>testis-specific kinase 2</td>
<td>1.20</td>
<td>0.093</td>
<td>1.32</td>
<td>0.021</td>
<td>1.38</td>
<td>0.002</td>
<td>0.87</td>
<td>0.994</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
<td>1.05</td>
<td>0.184</td>
<td>2.61</td>
<td>0.008</td>
<td>1.46</td>
<td>0.013</td>
<td>1.24</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Figure 3
Enhanced microtubule stabilization is a significant mechanism for enhanced paclitaxel-mediated cytotoxicity.

SKOv3 cells were transfected, and treated as described in figure 1 and methods. After this, the following parameters were measured; 1) the fold change in apoptosis or cytotoxicity following siRNA pool transfection alone compared to non-targeting siRNA (fold no drug), 2) the fold change in apoptosis or cytotoxicity following siRNA pool transfection and paclitaxel compared to non-targeting siRNA transfection and paclitaxel (fold drug treated) and 3) the ratio of 2 over 1. siRNA pools were identified that either increased the fold drug treated over 2.5 or increased the ratio over 2. In the figure, each circle represents the median of triplicate values of the indicated ratio. Data for apoptosis and cell viability is presented in (A) and (B), respectively. The blue lines indicate the cut-offs used in the screen for identifying hits. The green circles indicate genes that also enhanced paclitaxel-induced microtubule stability.

Figure 4
Measuring microtubule stability identifies three distinct mechanistic classes for modulating paclitaxel-induced cytotoxicity.

A cluster co-occurrence matrix showing the results of gene clustering (from 1 to 779) following permutation analysis based on the similarity between genes in modulating paclitaxel-induced cytotoxicity, apoptosis and microtubule stabilization. In this figure, red signifies high similarity and dark blue signifies no similarity. This analysis identified three main gene clusters. In B, the pair-wise correlation between the fold change in microtubule stability, apoptosis and cell viability following siRNA and paclitaxel treatments. Note the good correlation between microtubule stability and apoptosis and viability for cluster 2, the good correlation between microtubule stability and apoptosis for cluster 1 and the paradoxical inverse correlation between microtubule stability and cell viability and apoptosis for cluster 3. In cluster 3 circles in magenta represent genes that upon depletion increase microtubule stability but paradoxically decrease apoptosis and
improve cell viability following paclitaxel treatment. In C, a box plot is presented to compare the fold change in apoptosis following siRNA-mediated depletion of genes shown in magenta in cluster 3 (n=34) with the rest of the genes (n = 745). Note that for this cluster of 34 genes, siRNA depletion results in a significant decrease in apoptosis irrespective of paclitaxel treatment.

**Figure 5**

Secondary validation identifies novel mechanisms of modulating microtubule stability and paclitaxel cytotoxicity

A) SKOv3 cells were transfected as described in methods followed by treatment using diluent or paclitaxel as in the primary screen followed by immunofluorescence to measure microtubule stability. The bar plot shows the number of siRNAs per gene that significantly (p<0.05) enhanced paclitaxel-induced microtubule stability. In B, an example of increased % of paclitaxel-induced microtubule stability following depletion of PIK3C2A using 4 individual siRNAs compared to non-targeting siRNA-transfected (control), paclitaxel-treated cells. Shown is the mean + s.d. of triplicate measurements for each siRNA used.

C) Same experiment as in A. Shown is the correlation between baseline microtubule stability following siRNA transfection alone and microtubule stability following siRNA transfection and paclitaxel treatment. D) In parallel to the secondary validation described in A, an additional secondary validation was performed to measure cell viability 72 hrs following paclitaxel treatment in a similar way to that used in the primary screen. Shown is the correlation between the percentage of microtubule stability and the percentage of cell viability following siRNA transfection and paclitaxel treatment.
Figure 6
Modulating microtubule stability directly influences microtubule mass and cytotoxicity in ovarian cancer cell lines
A-B) SKOv3 cells were transfected using the indicated siRNAs for 48 hrs then treated with paclitaxel for one hour using the indicated concentrations then fixed and probed using anti-Glu tubulin counterstained with alexa-594 (A) or anti-α tubulin counterstained with alexa-488 (B). Images were collected and segmented as previously described. Shown in the mean background- (BG-) subtracted intensity +/- s.e.m. for each stain.  C-D) Cells were transfected using either non-targeting siRNA or siRNAs targeting ILK (C) or RAPGEF4 (D) for 48 hrs then treated with paclitaxel 1nM for 48 hrs and cells were maintained for either 4 days (Hey cells) or 7 days (SKOv3 cells) then fixed and stained using Coomassie Blue and individual colonies were counted. Shown is the mean number of colonies +/- s.d. of counts from two independent experiments.
Figure 1

A

Knockdown of 779 genes using siRNAs for 48 hr

Treatment with diluent or paclitaxel

1 hr

Immuno-fluorescence and high content microscopy

48 hr

Caspase activity

72 hr

Cell viability

B

Microtubule stability

Cell viability

Cell apoptosis
Figure 2

% mt stability compared to non-targeting siRNA (siRNA pools + paclitaxel)

% mt stability compared to non-targeting siRNA (siRNA pools only)
Figure 4

A. Cluster co-occurrence matrix

B. 

Cluster 1

\[ \text{Fold paclitaxel caspase activity} \]
\[ \text{Fold paclitaxel microtubule stability} \]
\[ r^2 = 0.5 \]

Cluster 2

\[ \text{Fold paclitaxel caspase activity} \]
\[ \text{Fold paclitaxel microtubule stability} \]
\[ r^2 = 0.5 \]

Cluster 3

\[ \text{Fold paclitaxel caspase activity} \]
\[ \text{Fold paclitaxel microtubule stability} \]
\[ r^2 = 0.6 \]

C. 

Fold caspase siRNA only

Cluster 3 siRNAs
- high stability - low apoptosis
Other siRNAs
n=745
Modulating microtubule stability enhances the cytotoxic response of cancer cells to paclitaxel

Ahmed Ashour Ahmed, Xiaoyan Wang, Zhen Lu, et al.

Cancer Res  Published OnlineFirst July 20, 2011.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-0025

Supplementary Material  Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/07/20/0008-5472.CAN-11-0025.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.