Aurora B expression modulates paclitaxel response in non-small cell lung cancer

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ABSTRACT

Background

Taxanes are mitotic poisons widely used in the treatment of non-small cell lung cancer (NSCLC), however, little is known about potential molecular modulators of response to these compounds. Aurora B (AURKB) is a critical regulator of the mitotic spindle assembly, previously shown overexpressed in NSCLC. Here, we investigated the hypothesis that AURKB expression modulates the efficacy of taxanes in NSCLC cells.

Methods

AURKB mRNA expression was determined by qPCR in 132 frozen NSCLC tissues and 9 NSCLC cell lines. AURKB expression was knocked down in cell lines using multiple shRNA constructs. Barasertib was used to specifically inhibit Aurora B activity, determined by the level of H3S10 phosphorylation.

Results

Frequent AURKB mRNA upregulation was observed in NSCLC tissues ($p<0.0001$), being more prominent in squamous carcinomas ($p<0.0001$). AURKB expression in cell lines strongly correlated with sensitivity to both docetaxel ($p=0.004$) and paclitaxel ($p=0.007$). AURKB knock-down derivatives consistently showed a dose-dependent association between low AURKB expression and resistance to paclitaxel. Specific chemical inhibition of Aurora B activity also demonstrated a strong dose-dependent efficiency in triggering paclitaxel resistance.

Conclusion

41 Aurora B activity is an important modulator of taxane response in NSCLC cells. This may lead

42 to further insights into taxane sensitivity of NSCLC tumours.

INTRODUCTION

Lung cancer is currently responsible for almost a quarter of cancer-related deaths in the developed countries. Non-Small Cell Lung Carcinoma (NSCLC) is the most frequent type with adenocarcinoma (AdC) and Squamous Cell Carcinoma (SqCC) being the most common sybtypes (Travis *et al*). Despite the recent advances in targeted therapies for lung cancer, including agents targeting EGFR, VEGF and ALK, the therapeutic regimes currently used for NSCLC treatment are largely still based on the use of organometallics and anti-mitotics (Camidge *et al*, 2014).

Taxanes are microtubule targeting agents (MTAs), which are used to treat a variety of human cancers (Del Mastro *et al*, 2013), (Isonishi *et al*, 2013), (Van Veldhuizen *et al*, 2003) including lung carcinomas (Maemondo *et al*, 2014). Paclitaxel and Docetaxel represent the most prominent members of the taxane family and have manifested significant activity, mainly used as part of complex chemotherapeutic regimens (Tsao *et al*, 2011), (Oh *et al*, 2013) or less frequently as monotherapy (Herbst *et al*, 2010), (Chu *et al*, 2005). The cytotoxic action of these compounds is mediated primarily through their binding of β tubulin monomers, leading to microtubule stabilization, thus blocking their depolymerization and subsequently triggering cell cycle arrest at the G2/M phase (Monzo *et al*, 1999). However, many other pathways may also be involved in modulating their therapeutic effect. Thus far, the range of relevant interactions is not clear and the mechanisms related to taxane resistance acquisition are largely unexplored, in spite of their long clinical use in cancer therapeutics (Murray *et al*, 2012), (Che *et al*, 2013). Although an increasing number of targeted therapeutic agents have come into clinical use (Kris *et al*, 2014), it is estimated that taxanes will still be used in chemotherapy regimens for lung cancer treatment for the

foreseeable future (Cagle & Chirieac, 2012). Therefore, the identification of relevant response predictors will highly benefit clinical practice by stratifying patients into suitable currently available schemes (Cottini & Lautenschlaeger, 2013).

A number of genes have been previously implicated in taxane resistance in NSCLC. Functional p53 was shown to induce sensitivity to docetaxel (Jinturkar *et al*, 2012) and paclitaxel (Guntur *et al*, 2010) in NSCLC cell lines. A phase-III randomized trial showed that *KRAS* mutations decrease response of NSCLC to therapy involving paclitaxel, carboplatin and erlotinib (Eberhard *et al*, 2005). It is reasonable to hypothesize that the expression of genes involved in mitotic spindle formation may be predictors of sensitivity to taxanes. Tubulin βIII mutations have been associated with resistance to taxanes in NSCLC patients (Kaira *et al*, 2013) as well as cultured lung cancer cells (Kavallaris *et al*, 1999). However, the inclusion of *TUBB3* expression levels in the prediction model for docetaxel sensitivity shows no improvement while certain drug pump genes (MRP5 and MVP) and detoxification genes appear as better predictors (Glaysher, Yiannakis et al. 2009). The CHFR gene has also been suggested as a potential predictor of response of NSCLC patients to first-line therapy with carboplatin and paclitaxel (Pillai *et al*, 2013). Nevertheless, the available information on taxane efficacy predictors does not provide conclusive evidence. Considering the role of Aurora kinases in spindle formation and the reported extent of their deregulation in cancer, one may hypothesize that Aurora-dependent modulation of taxane efficiency is probable.

Aurora kinase B (AURKB) is a member of the Aurora kinase subfamily of conserved Serine/Threonine kinases, which also includes Aurora A and Aurora C. Deregulation of Aurora kinases leads to impairment of mitotic spindle checkpoints causing abnormal spindle assembly (Fu *et al*, 2007). Aurora B is encoded by the *AURKB* gene (17p13.1) and plays a key

role during mitosis by regulating chromosomal alignment, segregation and cytokinesis, as the catalytic protein of the Chromosomal Passenger Complex (CPC). Activation of Aurora B by INCENP is required for promoting transfer of the CPC sub-complex (INCENP–Survivin–Borealin subcomplex) to the spindle midzone during mitotic exit (Xu *et al*, 2009). Here, it is localized to the centromere and is required for kinetochore localization and chromosome attachment to the mitotic spindle (Hauf *et al*, 2003), as well as establishing the spindle assembly checkpoint (SAC) to correct anomalous chromosome-kinetochore microtubule attachment before the cell enters anaphase (Carmena *et al*, 2012).

AURKB is frequently overexpressed in NSCLC (Vischioni *et al*, 2006) and is associated with genetic instability (Smith *et al*, 2005), aneuploidy and poor patient prognosis (Takeshita *et al*, 2013). However, high Aurora B kinase expression has been associated with improved relapse-free survival (RFS) in ovarian cancer patients on taxane-based therapy (Beussel *et al*, 2012). Currently, there is very limited information on the potential impact of Aurora B on taxane response in cancer cells. In this study, we confirmed the extent of AURKB deregulation in NSCLC and utilized *in vitro* approaches to investigate the hypothesis that AURKB activity may modulate and thus be predictive of the sensitivity to taxanes in NSCLC.

METHODS

Primary NSCLC tumour samples: This study was undertaken in association with the Liverpool Lung Project. Appropriate Ethical approval has been acquired and all patients have provided written informed consent. 132 snap-frozen tumour samples were obtained at surgery and included in the study; 56 from AdC and 76 from SqCC. Pathological review of the sections used in this study confirmed inclusion of only specimens with >70% tumour cell content. In addition, 44 non-tumour paired surgical tissues (20 from AdC and 24 from SqCC

patients) were available for analysis, taken from the distant edge of the lung resection. The clinicopathological characteristics of this cohort are provided in Supplementary Table 1. The median age of those patients was 67 (45-82); 56 of the patients were females and 77 were males.

Cell lines & Growth conditions: All cell lines in this study were authenticated using the GenePrint® 10 System (Promega) and analysis on a 3130 Genetic Analyzer (Life Technologies) and mycoplasma tested utilising the e-MycoTM plus Mycoplasma PCR Detection Kit (Intron Biotechnology). Nine NSCLC cell lines (A549, Calu-3, CALU6, CRL5802, COR-L23, H358, LUDLU-1, SK-LU-1 & SK-MES1) were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's Nutrient Mixture F-12 (1:1) containing 5% Fetal Bovine Serum (Sigma-Aldrich). Non tumourigenic immortalised human bronchial epithelial cells (HBEC-3KT) along with their isogenic derivatives; p53 knockouts (HBEC-3KT-53), KRAS mutants (HBEC_3KT_R) and cells with both aberrations (HBEC-3KT-R53) were also employed in this study. HBECs were cultured in Keratinocyte-SFM medium supplemented with 50μg/mL Bovine Pituitary Extract (BPE) and 5 ng/mL human recombinant Epidermal Growth Factor (rEGF) (Life Technologies). All cell lines were maintained at 37^{o}C, 5% CO2.

Knock-down of *AURKB* by short hairpin RNA (shRNA): A549 and SK-MES1 cell lines were transfected with five different *AURKB*-targeting MISSION® shRNA constructs (Sigma-Aldrich, clone numbers: TRCN0000000777, TRCN0000000776, TRCN0000000778, TRCN0000000779 and TRCN0000010547) along with a scrambled control construct, using Attractene Transfection Reagent (Qiagen). Stable clones were selected by exposure to Puromycin (4 nM) and knockdown efficiency was confirmed by qPCR and western blotting.

AURKB overexpression

The AURKB-PCMV6-XL4 construct (Cat no. PCMV6XL4, SC117526 NM_004217 AURKB – OriGene, USA) was digested with PsiI (New England Biolabs, UK) and ligated with a Blasticidin cassete containing fragment which was derived by SmaI digestion of the pCMV/Bsd plasmid (Catalogue no. V510-20 - Life Technologies, UK). Standard blunt end ligation was performed using T4 DNA ligase (New England Biolabs, UK). One Shot TOP10 Chemically Competent E. coli cells (Invitrogen) were transformed and clones were selected for validation. The derived constructs were confirmed by dideoxyterminator sequencing (Life Technologies, UK).

CALU3 cell line was transfected with pCMV6-XL4-AURKB/Bsd recombinant plasmid using Attractene Transfection Reagent (Qiagen). Stable transfectant clones were selected in the presence of 5 µg/ml Blasticidin (SIGMA, UK). mRNA overexpression of AURKB was confirmed by qPCR.

Taxane and AZD1152 (Barasertib) exposure experiments. Depending on the growth rate of each cell line, 5×10^4 to 8×10^4 cells were seeded in each well of flat-bottomed 48-well plates in 6 replicates and cultured in 500 µl of medium. After overnight incubation, the medium was replaced with media containing a range of Paclitaxel (Sigma-Aldrich) or Docetaxel (FLUKA) concentrations (0-35nM) and/or Barasertib (Selleck Chemicals) (0-3.2 nM). Cells were incubated for 72 hours with replenishment of medium with drug at 36 hours. Toxicity was measured by MTT assay. Briefly, 200 µL of fresh medium containing 0.75 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) was added and incubated at 37^{o}C for three hours. Cells were lysed using 0.04M HCl in isopropanol and the optical density (O.D.) was measured at 590nm with 630nm as reference in a GENios plate reader (Tecan Austria GmBH).

DNA and RNA extraction, Reverse Transcription and qPCR: DNA was extracted from cell lines and primary lung tumours using the DNeasy® Blood and Tissue Kit (QIAGEN). Total RNA was extracted using Direct-zol™ RNA MiniPrep Kit (Zymo Research). Quality and quantity of DNA and RNA were determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). 200ng RNA was Reverse Transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). A predesigned FAM labelled Taqman expression assay (Hs00945858_g1 - Life Technologies) was employed to analyse *AURKB* mRNA expression, with a VIC-labelled *ACTB* Taqman assay (4326315E – Life Technologies) serving as endogenous control. Real-time PCR assays were performed in triplicate using a StepOnePlus™ Real-Time PCR System (Life Technologies). HBEC-3KT RNA was used as calibrator for calculating delta delta cycle thresholds (ΔΔCt) and relative quantification (RQ) values.

Western blotting: Cultured cells were lysed in SDS-based cell lysis buffer containing protease/phosphatase inhibitors and whole cell lysates were sonicated 10 times for 5-15 sec with 10 sec intervening pauses [AC 100V 50/60 HZ]. Protein concentrations were determined using the BCA assay (Thermo Scientific) on a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Ten to fifty µg of total protein was reduced by NuPAGE Reducing Agent (Life Technologies) and incubated at 70°C for 10 minutes. Samples were run in NuPAGE Novex Bis-Tris Gels utilising NuPAGE Antioxidant containing NuPAGE MES Running Buffer (Life Technologies). Electroblotting on PVDF membranes was undertaken by iBlot Dry Blotting System (Life Technologies). The iBind Western System (Life Technologies) was employed for application of blocking, primary and secondary antibodies (at dilution 1:1000) and washing steps. Mouse monoclonal primary antibodies to total Histone H3 (phospho S10) (ab14955) and β-actin (ab8226) (Abcam) were used, while IRDye 800CW Goat Anti-Mouse (LI 925-

32210) antibody (Odyssey®- LI-COR Biosciences) served as secondary. The immune complexes were detected using Odyssey® CLx Infrared Imaging System (Odyssey®- LI-COR Biosciences).

Statistical analysis:

All Statistical analyses were performed using IBM® SPSS® statistical software version 22.0 (Armonk, NY: IBM Corp) unless otherwise stated. Covariates were assessed for compliance to normal distribution graphically using histograms and by analytical methods using Kolmogorov-Smirnov/Shapiro-Wilk tests. In the absence of normality, Mann-Whitney, Wilcoxon rank or Kruskal-Wallis was employed to assess the difference in *AURKB* mRNA expression and response to taxanes. Kaplan-Meier method was employed for survival analysis and statistical differences between groups were examined with Log-rank test. The IC50 values were calculated using Prism 5 (GraphPad) in comparison to untreated cells at time 0.

RESULTS

AURKB mRNA is overexpressed in NSCLC tissues and cell lines.

mRNA expression profiling of *AURKB* in snap-frozen tissues demonstrated significant overexpression of the gene transcript in tumour tissue compared to the adjacent normal tissues (Mann-Whitney test, $p<0.0001$, Figure 1A). This overexpression was more profound in SqCC than AdCs (Mann-Whitney test, $p<0.0001$, Figure 1B). In addition, *AURKB* expression was more elevated in higher pathological T stages (T1 vs T2, $p=0.012$, Supplementary figure 1), however, this finding has to be treated with caution as the great majority of tumours in this sample set fall into the pT2 stage group (n=101) while pT1 and pT3/4 group comprised 19 and 12 patients respectively. This bias reflects the stage distribution among the operable NSCLC cases in the UK and therefore our access to the relevant tissue. *AURKB* upregulation did not demonstrate a significant impact on overall survival (OS) in this set, although a non-significant trend was demonstrated in adenocarcinoma patients ($p=0.079$, Supplementary Figure 2). Survival analysis using the top and bottom terciles instead of the median did not result in a significant relationship, most probably due to the significant reduction of numbers of patients in these groups. This weak correlation prompted us to check the available data in the public domain. In there (http://kmplot.com/analysis/index.php?p=service&cancer=lung) when selecting all NSCLC, AURKB expression significantly reduces survival ($p<10^{-16}$). However when selecting only the NSCLC group subjected to chemotherapy this correlation is not true ($p=0.3$). No further associations were found between *AURKB* mRNA expression and age, gender, clinical stage or nodal status in our patient set.

AURKB mRNA expression profiling was also undertaken in a panel of lung cancer cell lines, along with immortalized normal human bronchial epithelial cells (HBEC-3KT and its isogenic

p53 knockout and *KRAS* mutant derivatives). *AURKB* expression was variable among the lung cancer cell lines, however, it was markedly higher in comparison to that of non-tumourigenic HBECs (Figure 2). It is of note that among HBECs *AURKB* mRNA expression was higher in the p53 knockout derivatives (HBEC3KTp53 and HBEC3KTRp53) while a borderline reduction was seen in *KRAS* mutants (HBEC3KTR).

Modulation of NSCLC cells resistance to taxanes by AURKB expression.

The IC50 values for paclitaxel and docetaxel toxicity among the available lung cell lines were determined following treatment with a range of concentrations (1 - 35 nM) of the two drugs (Supplementary table 2). IC50s for docetaxel were consistently lower than those of paclitaxel with one exception (SKLU1 cells). Interestingly, mRNA expression of *AURKB* in NSCLC cell lines inversely correlated with resistance to both docetaxel (Spearman's test, rho=-0.850, p=0.004) and paclitaxel (rho= -0.817, p=0.007, Supplementary table 2). We explored the hypothesis this effect simply being a correlation between taxane resistance and doubling time (Supplementary table 3). However, our results did not demonstrate any correlation between doubling time with either AURKB expression (Spearman's test, rho=-533, p=0.139) or resistance to paclitaxel (rho=0.333, p=0.381) or docetaxel (rho=0.500, p=0.170, Supplementary table 2).

In order to further explore the possible modulation of taxane response by *AURKB*, we investigated the resistance of lung cancer cell lines to paclitaxel by (a) knocking down *AURKB* expression and (b) chemically inhibiting its activity. Successful *AURKB* knock down clones were derived from A549 and SK-MES1 cells using different shRNA constructs. Knock-down efficiency was assessed by qPCR and western blot (Figure 3). The clones, as expected, demonstrated variable knock down efficiency. When exposing these clones to paclitaxel it

was apparent that response to the drug inversely correlated to the level of *AURKB* mRNA expression in a dose-dependent manner. This was true for both clones derived from A549 and 4 clones derived from SK-MES1, while scrambled shRNA clones did not demonstrate altered response to paclitaxel when compared to the paternal cells (Figure 3).

Additional support to the knock down experiments came from overexpressing AURKB in Calu-3 cell line. A stable overexpressing clone derived from these cells demonstrated higher sensitivity to paclitaxel (Supplementary figure 6).

Selective inhibition of AURKB desensitizes NSCLC cells to paclitaxel.

In order to gain additional supporting evidence, we undertook selective inhibition of Aurora B using a highly specific Aurora B inhibitor (Barasertib). After experimentally determining the IC50s of Barasertib in A549 SK-MES1 and SKLU1 as 0.9 nM 1.2 nM and 2.3 nM respectively, we simultaneously exposed cells to paclitaxel and a range of Barasertib concentrations. AURKB inhibition was confirmed by measuring phosphorylation of histone 3 serine 10 (H3S10), which is on one of its prime substrates (Supplementary Figure 3). In addition, we confirmed that Barasertib exposure does not alter the mRNA expression of AURKB (Supplementary Figure 3). Barasertib-mediated AURKB inhibition clearly demonstrated a dose-dependent effect on paclitaxel efficiency; increased Barasertib concentrations resulted in increased resistance to paclitaxel in all three cell lines (Figure 4). We subsequently confirmed the Barasertib-mediated resistance to paclitaxel in the remaining NSCLC cell lines included in this study (LUDLU1, CRL5807, CRL5802, CORL23, CALU6 and CALU3) as well as all the AURKB knock-down derivatives from SKMES and A549 (supplementary Figure 4). It is of note that in the AURKB knockdown clones the Barasertib effect, while still visible, is reduced in comparison to the non-AURKB engineered NSCLC cell lines, as expected.

DISCUSSION

Aurora B kinase is an important contributor to the mitotic spindle assembly and its overexpression in human cancer has been frequently reported (Sorrentino *et al*, 2005), (Lin *et al*, 2010) (Pohl *et al*, 2011), therefore attracting significant interest in both cancer biology and therapeutics. In this study, we hypothesized that Aurora B activity may be implicated in modulating cellular response to taxanes, due to its role in mitotic spindle function, which is the target of these compounds. mRNA profiling of our NSCLC patient cohort confirmed the extensive overexpression of this gene, as previously reported (Takeshita *et al*, 2013), (Vischioni *et al*, 2006), (Smith *et al*, 2005). *AURKB* overexpression seems to be the main abnormality related with this gene in NSCLC. The relevant entries in COSMIC database (http://cancer.sanger.ac.uk/cosmic) report low number of point mutations (5/1985, 0.2%), copy number variations (4/843, 0.4%) and frequent overexpression (139/1008, 13.8%). In addition to the frequency difference in histological NSCLC types, AURKB overexpression appeared to be weakly associated with higher pathological stages. This observation may indicate that *AURKB* expression deregulation is a progressive phenomenon; however, one has to replicate this finding in additional cohorts. Nevertheless, investigation of the data available in TCGA study (http://www.cbioportal.org/index.do) do not show any pT stage related differences in lung adenocarcinoma or tumour dimensions for squamous lung carcinomas (pT stage was not available in the query in this histology).

The molecular triggers behind AURKB overexpression in NSCLC are not yet clear. We have investigated a possible relationship between AURKB and KRAS/ TP53 status in the studied cancer cell lines, however no such correlation was obtained. This is not surprising, as tumour cell lines are highly abnormal and diverse at the genetic and epigenetic levels, therefore

many confounding factors will influence AURKB expression. An interesting observation came from our HBEC isogenic lines (Figure 2), where the addition of mutant KRAS actually significantly reduces AURKB expression, either in the presence or absence of TP53. This is in contrast to a single report available (Dos Santos *et al*, 2016) which demonstrates upregulation of AURKB by KRAS in a different immortalised lung epithelial cell line. Therefore it is not yet clear whether KRAS has a positive or negative impact on AURKB expression. In contrast, TP53 knock-down is clearly associated with increased AURKB levels. While no evidence for direct transcriptional inhibition of AURKB by p53 exists, a possible mechanism is through c-myc. p53 represses, through induction of miR-145 c-myc (Sachdeva *et al*, 2009), which is known to induce expression of both AURKA and AURKB (den Hollander *et al*, 2010).

AURKB mRNA overexpression alone did not correlate with overall survival, in agreement with two previous studies (Smith *et al*, 2005) (Perumal, Singh et al. 2012), although the latter has suggested that *AURKB* expression can be part of a prognostic multi-gene signature. In contrast, immunohistochemical analysis of AURKB protein expression was reported to correlate with poor prognosis (Takeshita *et al*, 2013; Vischioni *et al*, 2006) suggesting that stability and/or correct localization of the protein may also be important.

AURKB mRNA expression was higher in all lung cancer cell lines tested in comparison to HBECs, as has been previously described (Hayama *et al*, 2007). The most significant observation of this study is the inverse correlation found in NSCLC cell lines between *AURKB* mRNA expression and sensitivity to both tested taxanes, with low levels of *AURKB* being associated with taxane resistance. The hypothesis that this change in resistance might be simply the reflection of a reduction in doubling times was disproved, as no such correlation was demonstrated. In order to verify the inverse correlation found between the endogenous

310 *AURKB* levels and taxane resistance, we knocked down *AURKB* mRNA expression in two lung
311 cancer cell lines. This knockdown consistently triggered a marked increased resistance to
312 paclitaxel. Moreover, it was observed that different knock down efficiency in each clone led
313 to a different level of resistance to paclitaxel, showing for the first time a quantitative effect
314 between expression of this gene and modulation of resistance to a taxane in human cancer
315 cells.

316 In order to exclude possible off-target effects of genetic manipulation, we used a highly
317 selective AURKB inhibitor (Barasertib or AZD1152), which was reported to have a 3700-fold
318 higher specificity for AURKB inhibition in comparison to AURKA (Walsby *et al*, 2008; Yang *et*
319 *al*). This experiment also demonstrated a clear dose-dependent increase of resistance to
320 paclitaxel with increasing concentrations of Barasertib in all cell lines tested. Barasertib
321 effect, as hypothesized, was moderated when tested in AURKB knockdown clones, due to
322 the already reduced availability of AURKB protein in these clones. This data is in contrast to a
323 previous report suggesting that Aurora B inhibition sensitizes cells to antimitotic agents
324 (Tanaka *et al*). However, that study utilized a generic kinase inhibitor AT9283, which inhibits
325 Aurora A and other kinases, such as Jak2, Jak3 and Abl kinase as well as Aurora B (Curry *et*
326 *al*). It is well known that Aurora A inhibition enhances chemosensitivity to paclitaxel in
327 pancreatic cancer cells (Lin *et al*), to docetaxel in human oesophageal squamous cell
328 carcinoma (Isham *et al*) and ovarian cancer cells (Scharer *et al*), therefore this sensitization
329 can be most probably attributed to AURKA inhibition. The association of high Aurora B
330 kinase expression sensitivity to taxane-based therapy has been also shown for ovarian
331 cancer (Beussel *et al*, 2012). In addition, miRNA-dependent knock-down of AURKB also
332 results in resistanse to taxanes in an in vivo breast cancer model (Winsel *et al*, 2014) .

Therefore the mechanism behind resistance to pacitaxel therapy due to AURKB mitotic checkpoint abrogation is not limited to NSCLC and appears to be getting well established.

Possible mechanisms to explain the effect of Aurora B on taxane efficiency may be drawn from a number of previous observations. Paclitaxel acts by perturbation of mitotic spindle assembly and activating the spindle assembly checkpoint (SAC) (Zachos *et al*), (Hung *et al*). This leads to a cell death response triggered through p53-independent apoptotic pathways that depend upon maintaining mitotic arrest (Huang *et al*). If mitotic slippage occurs, evasion of apoptosis is allowed, mainly due to the stabilization of anti-apoptotic proteins (Wertz *et al*). The use of Aurora B inhibitors (Hauf *et al*, 2003), (Ditchfield, 2003), (Vader *et al*), (Girdler *et al*), deactivation of Aurora B (Wang *et al*) or depletion of Aurora B activating proteins (Petsalaki *et al*), (Vader *et al*) have shown to relieve mitotic arrest caused by paclitaxel. Taken together, these studies provide further evidence for the central role of Aurora B function for spindle checkpoint activation and cell death in response to low-dose paclitaxel and further support our findings.

Concerning the evidence so far, AURKB expression presents with a potential paradox: Its overexpression reduces survival in the chemotherapy naïve patients but appears to have a beneficial effect in patients treated with taxane regimens. The latter may draw additional support from the loss of the very strong correlation between AURKB and NSCLC patient survival available online and mentioned in the results section, when limiting the question in the chemotherapy recipient group. Of course “chemotherapy” term in there is inclusive of every scheme used but nevertheless raises the question for investigating AURKB involvement in the resistance modulation of other common chemotherapeutics.

The present study clearly demonstrated a role for Aurora B in the response of NSCLC cells to paclitaxel and provided unique evidence for a dose-dependent association. Given the large extent of *AURKB* deregulation in NSCLC, these findings suggest that assessing the levels of AURKB protein in surgical samples and/or biopsy material could assist in the clinical decision tree for managing patients with NSCLC and have potential for development as a predictive biomarker. Unfortunately, chemotherapy data were not available for our tissue cohort, thus we could not investigate any correlation between *AURKB* expression and response to taxanes in this clinical set. Our findings, however, highlight the need for conducting such studies in large cohorts of NSCLC patients. Moreover, it may advise against the use of AURKB inhibitors in regimens involving taxanes and this should be further investigated in clinical studies.

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Figure Legends

Figure 1. Boxplots demonstrating *AURKB* mRNA expression in primary lung tumours compared to adjacent normal lung tissues (A) and in squamous cell carcinomas (SqCC) compared to adenocarcinomas (AdC) (B). P values are derived from Mann-Whitney tests. RQ was calculated using RNA from the non-tumourigenic cell line HBEC3KT as a calibrator. Larger circles represent outlier values (>1.5 x interquartile range); smaller circles represent extreme values (>3 x interquartile range).

Figure 2. *AURKB* mRNA expression in human bronchial epithelial cells (HBEC) and lung cancer cell lines. Bars represent mean values from 6 biological repeats and error bars represent standard error of the mean. All NSCLC cell lines show a higher expression than the non-tumourigenic immortalized bronchial cells (HBEC3KT). Also, an increased *AURKB* expression is shown in the isogenic p53 derivatives (3KT-53 and 3KT-R53) of the latter.

Figure 3. Paclitaxel response of lung cancer cell lines A549 and SK-MES1 and their *AURKB* knock down derivative clones in relation to *AURKB* mRNA and protein expression. Error bars in both the line graphs and the expression bar charts represent 95%CI. –PAR: parental, -SCR: scrambled, -Bx-y: knockdown clones where x is the shRNA construct and y is the clone number from this transfection. It is of note that the different knockdown efficiencies in the clones are associated with inverse impact on paclitaxel sensitivity.

Figure 4. Sensitivity of lung cancer cell lines to paclitaxel in the presence of the highly selective Aurora B inhibitor (Barasertib). Error bars represent 95%CI. A clear quantitative effect is demonstrated where increasing concentrations of Barasertib, and therefore inhibition of AURKB activity, is associated with increased resistance to paclitaxel.

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Figure 1

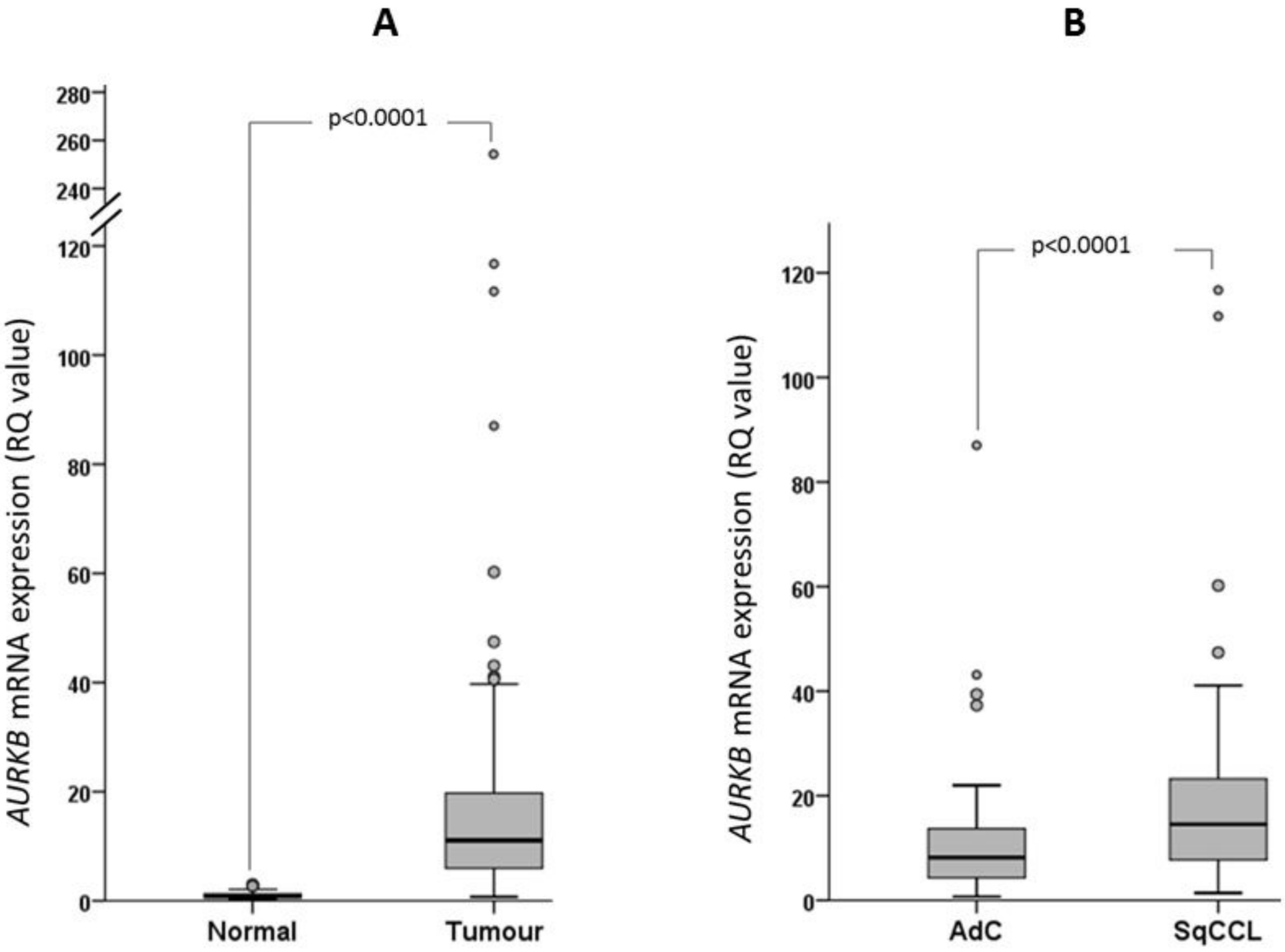

A
B
p<0.0001
p<0.0001
AURKB mRNA expression (RQ value)
AURKB mRNA expression (RQ value)
280
260
240
120
100
80
60
40
20
0
Normal
Tumour
AdC
SqCCL

Figure 2

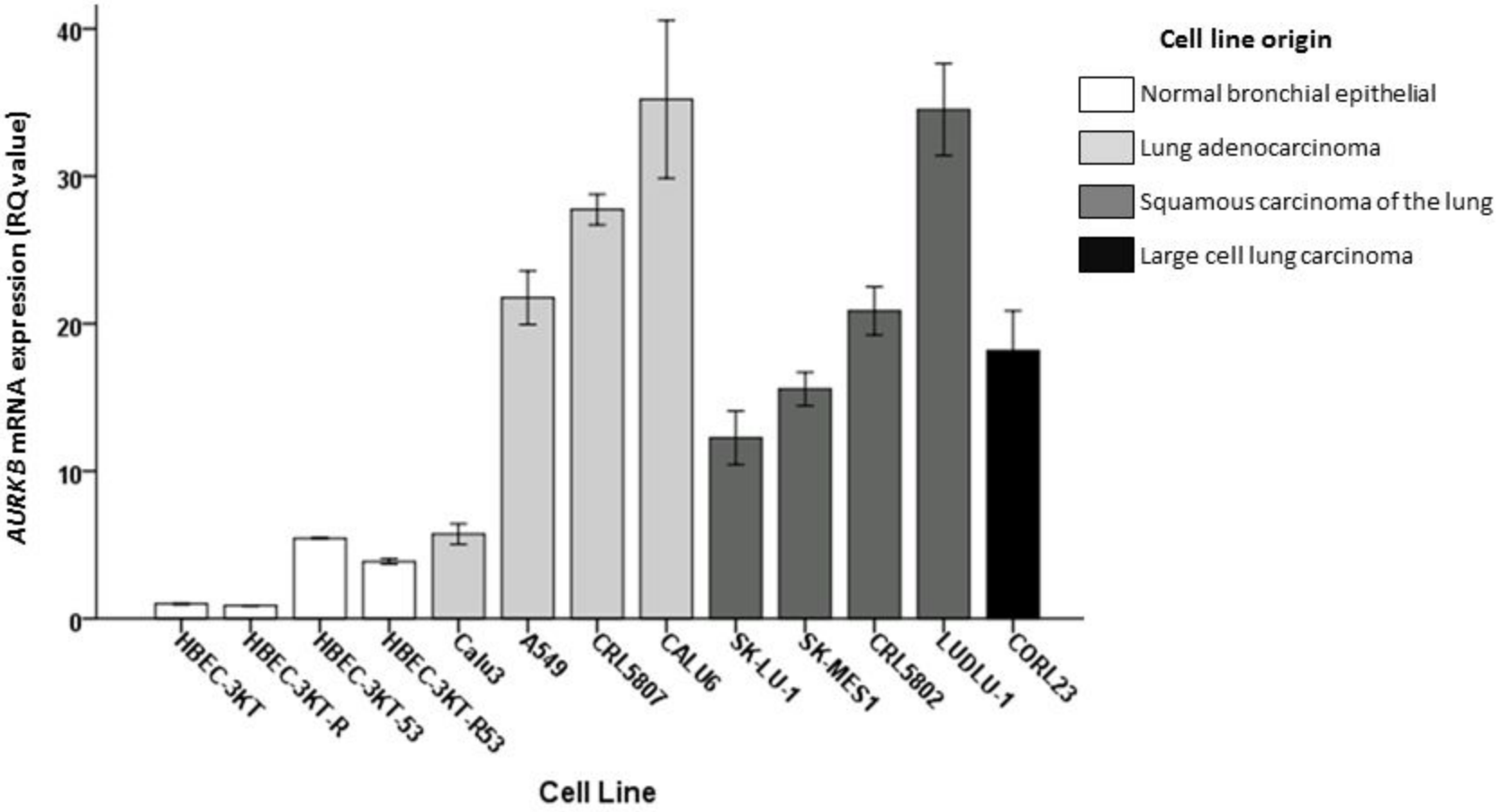

AURKB mRNA expression (RQ value)
40
30
20
10
0
HBEC-3KT
HBEC-3KT-R
HBEC-3KT-53
HBEC-3KT-R53
Calu3
A549
CRL5807
CALU6
SK-LU-1
SK-MES1
CRL5802
LUDLU-1
CORL23
Cell Line
Cell line origin
Normal bronchial epithelial
Lung adenocarcinoma
Squamous carcinoma of the lung
Large cell lung carcinoma

Figure 3

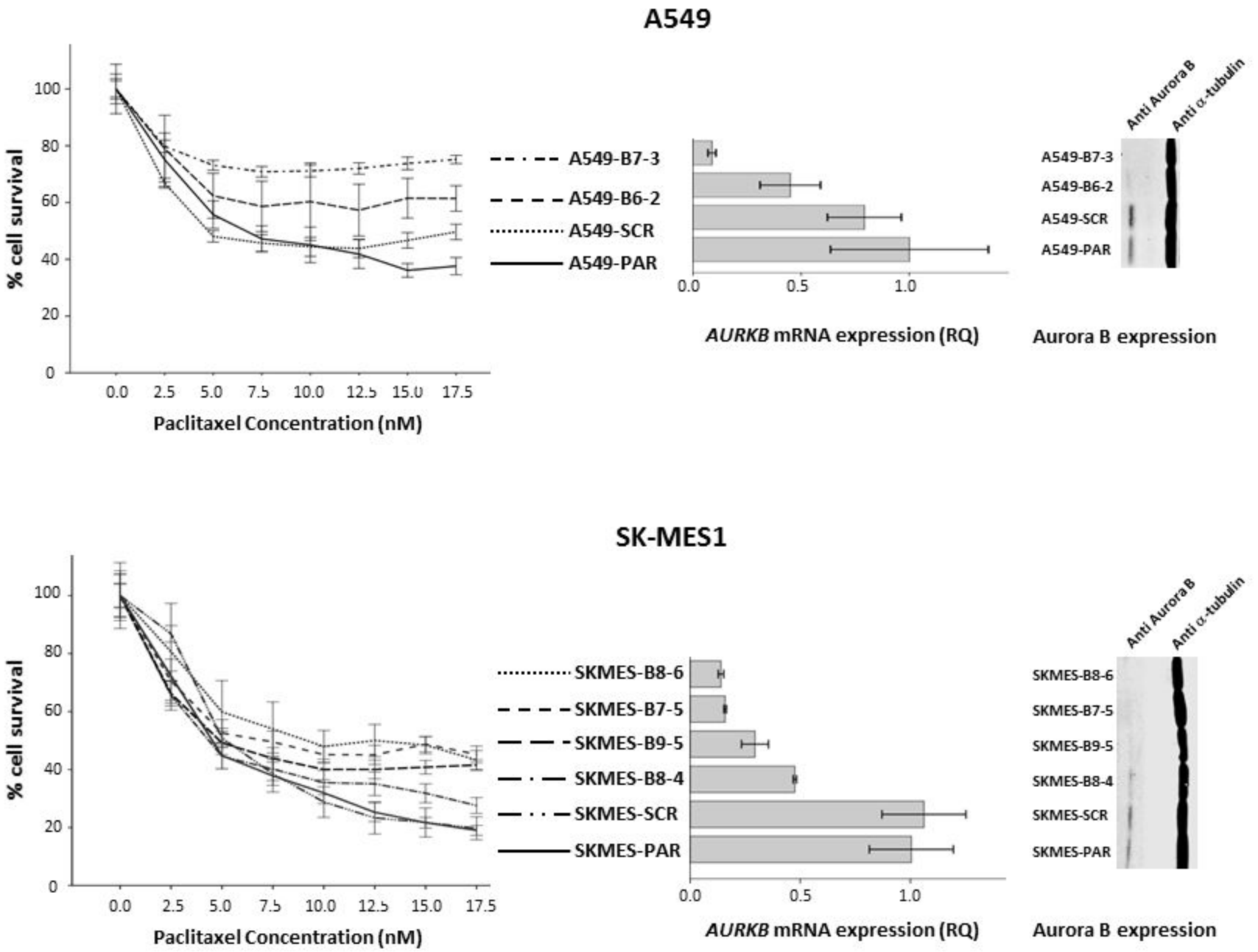
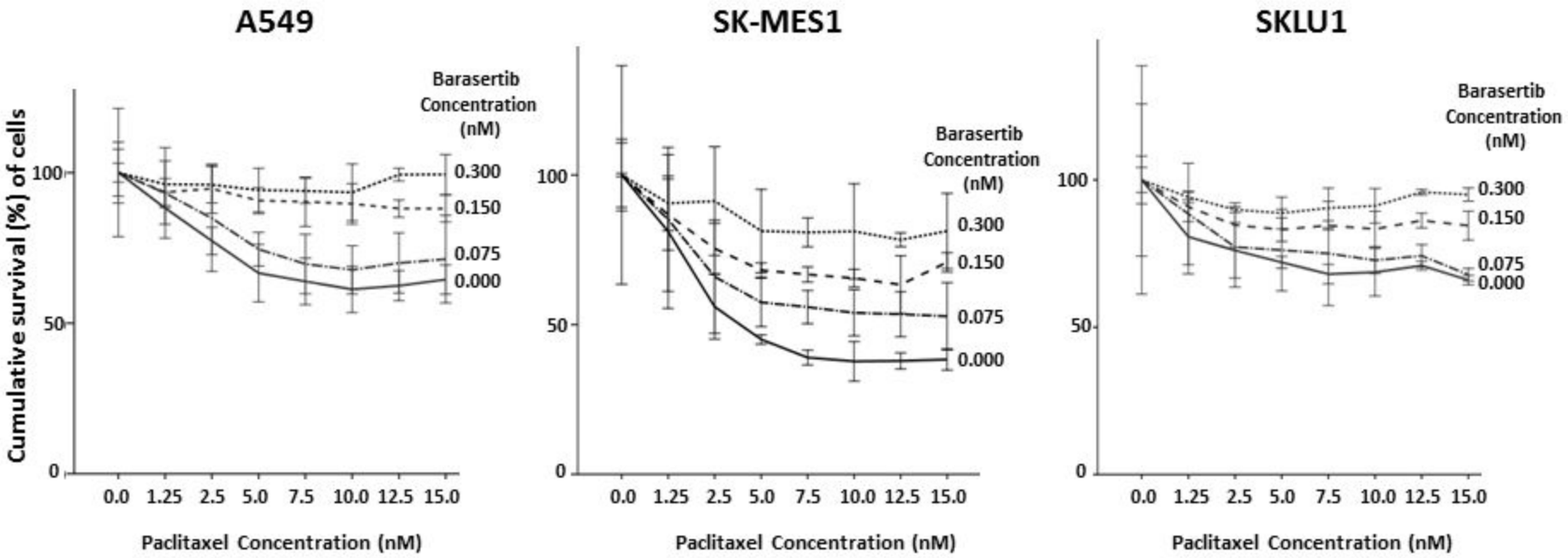

A549
% cell survival
100
80
60
40
20
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Paclitaxel Concentration (nM)
A549-B7-3
A549-B6-2
A549-SCR
A549-PAR
0.0
0.5
1.0
AURKB mRNA expression (RQ)
Anti Aurora B
Anti α-tubulin
A549-B7-3
A549-B6-2
A549-SCR
A549-PAR
Aurora B expression
SK-MES1
% cell survival
100
80
60
40
20
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Paclitaxel Concentration (nM)
SKMES-B8-6
SKMES-B7-5
SKMES-B9-5
SKMES-B8-4
SKMES-SCR
SKMES-PAR
0.0
0.5
1.0
AURKB mRNA expression (RQ)
Anti Aurora B
Anti α-tubulin
SKMES-B8-6
SKMES-B7-5
SKMES-B9-5
SKMES-B8-4
SKMES-SCR
SKMES-PAR
Aurora B expression

Figure 4


A549
SK-MES1
SKLU1
Cumulative survival (%) of cells
100
50
0
Barasertib Concentration (nM)
0.300
0.150
0.075
0.000
0.0 1.25 2.5 5.0 7.5 10.0 12.5 15.0
Paclitaxel Concentration (nM)